An RNAi screening of clinically relevant transcription factors regulating human adipogenesis and adipocyte metabolism

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ABSTRACT

Objective Healthy hyperplasic (many but smaller fat cells) white adipose tissue (WAT) expansion is mediated by recruitment, proliferation and/or differentiation of new fat cells. This process (adipogenesis) is controlled by transcriptional programs mostly identified in rodents. A systemic investigation of adipogenic human transcription factors (TFs) that are relevant for metabolic conditions has not been revealed previously.

Methods TFs regulated in WAT by obesity, adipose morphology, cancer cachexia and insulin resistance were selected from microarrays. Their role in differentiation of human adipose tissue-derived stem cells (hASC) was investigated by RNA interference (RNAi) screen. Lipid accumulation, cell number and lipolysis were measured for all screened factors (148 TFs). RNA (RNAseq), protein (western blot) expression, insulin and catecholamine responsiveness were examined in hASC following siRNA treatment of selected target TFs.

Results Analysis of TFs regulated by metabolic conditions in human WAT revealed that many of them belong to adipogenesis-regulating pathways. The RNAi screen identified 39 genes that affected fat cell differentiation in vitro, where 11 genes were novel. Of the latter JARID2 stood out as being necessary for formation of healthy fat cell metabolic phenotype by regulating expression of multiple fat-cell phenotype-specific genes.

Conclusions This comprehensive RNAi screening in hASC suggests that a large proportion of WAT TFs that are impacted by metabolic conditions might be important for hyperplastic adipose tissue expansion. The screen also identified JARID2 as a novel TF essential for the development of functional adipocytes.

Keywords: obesity, insulin resistance, RNAi, human adipose-derived stem cells, adipogenesis, RNAseq, JARID2
1. INTRODUCTION

White adipose tissue (WAT) can expand by increase in size of existing adipocytes (hypertrophy) or by number of adipocytes (hyperplasia) (1). Fat tissue morphology resulting from the balance between hypertrophic and hyperplastic expansion within an individual has a profound impact on metabolic health (1). Expansion through hyperplasia associates with a benign metabolic phenotype, while expansion through hypertrophy promotes obesity-associated metabolic complications (2). Hyperplastic adipose tissue expansion is mediated by several adipogenic events including recruitment, proliferation and/or differentiation of new fat cells, whereas hypertrophic expansion mainly is governed by size increase of already present adipocytes (1).

Adipogenesis is controlled by a large network of TFs that regulate phenotypic transition of adipose tissue mesenchymal stem cells to mature adipocytes (3). The differentiation cascade can be divided into at least two waves of TFs that drive the adipogenesis. The first wave activates several early adipogenic factors including C/EBP β/δ, KLFs, CREB, and SREBP-1c (4-10). Such TFs, in turn, induce expression/recruitment of the second wave of TFs, of which PPARγ and C/EBPα are the key players that lead towards the mature adipocyte phenotype (11-13). Apart from these TFs which promote adipogenesis, there are secreted factors and TFs—WNT10b, GATA factors 2 and 3, KLFs 2 and 7—which inhibit fat cell differentiation and guide mesenchymal stem cells to different lineages (14, 15). Therefore, a balance between positive and negative regulation is necessary to have a controlled effect on adipogenesis and adipocyte metabolism (15).

It has been shown that 10% of adipocytes are renewed each year throughout adulthood (16) suggesting that adipogenesis continues throughout life. Adipocyte differentiation is known to be affected by WAT metabolic conditions such as obesity, insulin resistance (IR) and morphology (17-
21), which is often also associated with the changes in transcriptional program (22-29). For example, PPARγ is downregulated while NFAT5 and HIF-1α are upregulated during obesity and IR (22-24); cancer cachexia, characterized by high lipid mobilization and loss of both adipose tissue and muscle mass, is associated with reduction in expression of C/EBPα, C/EBPβ, PPARγ and SREBP-1c in WAT (25); furthermore, reduced EBF1 levels cause adipose hypertrophy (30). Taken together, adverse WAT metabolic conditions can result or be dependent on impairment not only in the lipid and glucose metabolism, but also in altered transcriptional program regulating adipogenesis.

Although adipogenesis is a well-studied process especially in murine in vitro systems, systemic investigations of human adipocyte TFs impacted by metabolic conditions have not yet been revealed. Our hypothesis was that altered fat cell function in such conditions was dependent on fluctuations in the expression of TFs regulating adipogenesis. RNAi screening is a powerful tool for identification of functional genes. To our knowledge, only two previous studies used RNAi screens to identify factors that regulate human adipogenesis (31, 32). In the first study, the focus was to find druggable protein targets affecting adipogenesis and the complete list of screen hits was not published. In the second study, the osteosarcoma cell line U2OS was employed to identify the small set of genes that work via PPARG. In this current study, we used RNAi screening in human primary adipose-derived stem cells (hASCs) to identify TFs that are regulated by metabolic conditions in human WAT (hWAT) and that would affect fat cell differentiation. The role of a novel hit gene, JARID2, (Jumonji And AT-Rich Interaction Domain Containing 2), in adipogenesis was validated in some detail.
2. MATERIALS & METHODS

2.1. Human cohorts

We re-investigated published global gene expression arrays in subcutaneous WAT (scWAT) from four different cohorts. The regional ethics board approved all studies and the donors gave their written informed consent. The first cohort consists of 30 obese (BMI > 30 kg/m²) and 26 non-obese (BMI < 30 kg/m²) metabolically well characterized women described previously (33). All women were of Caucasian origin, pre-menopausal and free from any medication. Global gene expression from scWAT was used to select genes that were regulated by obesity (obese/non-obese) defined by FDR<5%. Data from the 26 non-obese women from the same cohort was used for picking genes regulated by morphology (hyperplasia/hypertrophy) as described in (30).

The second cohort comprises 27 gastrointestinal cancer patients with (n=13, 10 men and three women) or without (n=14, nine men and five women) cachexia (34). Cancer cachexia was defined as gastrointestinal cancer with self-reported unintentional weight loss of >5% of the habitual weight during last three months or >10% unintentional weight loss during last six months. Results from global gene expression profiles of abdominal scWAT were used to define genes regulated by cancer cachexia (cachexia/control) as a proxy of weight loss using FDR<5% as a cut-off (34).

The third cohort, which consists of 21 insulin-sensitive obese and 18 insulin-resistant obese women was used to find genes regulated by IR as described in (35). To define genes regulated by IR (insulin resistant/insulin sensitive) data from global gene expression profiles was used with ±20% fold change and p<0.05 as selection criteria.

The fourth cohort included 15 non-obese and 61 obese women, where purified subcutaneous adipocytes were available (36). These samples were used to examine JARID2 expression and correlate it to WAT morphology value.
2.2. Cell culture

hASC were isolated from subcutaneous adipose tissue of a male donor (16 years old, BMI 24 kg/m²), expanded and differentiated in vitro into adipocytes as previously described (37).

2.3. RNAi screening procedure

Confluent hASC were trypsinized and reverse transfection was performed using an automated setup (Multidrop dispenser (Thermo Fisher Scientific, Waltham, MA USA) and BioMek plate aspirator/washer (Beckman Coulter, Indianapolis, IN, USA) in 96-well plates. 0.35μl Dharmafect3 transfection reagent (GE Healthcare Dharmacon Inc, Lafayette, CO, USA) was mixed with a specific siRNA pool (ON-Target plus, GE Healthcare Dharmacon Inc) reaching a final concentration in cell culture media of 50nM. After 20 minutes of incubation, 10,000 cells in proliferation medium were added to each well in a 96-well plate. A full list of siRNAs containing the order numbers and sequences of the 148 selected transcriptional regulators, non-targeting siRNA (Negative Control) pool and siRNA targeting the known adipogenic TFs like PPARG and CEBPA are given in Supplemental table 1 (38). Each siRNA pool was run in triplicate. PPARG (n=3) and non-targeting control (n=6) were included on each test plate (in total nine plates were utilized for one screen). 24-hours after transfection, medium was changed to differentiation medium and the cells differentiated until day 9 as previously described (37). In addition, to 148 siRNAs used in the screen, siRNA targeting glucocorticoid receptor (GR) was used for optimization experiments (Supplemental table 1) (38).
2.4. Neutral lipid and nuclei staining

At day 9 of differentiation, cells were washed, fixed using 4% PFA and stained for neutral lipids (Bodipy 493/503, Molecular Probes, Thermo Fisher Scientific), cell nuclei (Hoechst 33342, Molecular Probes, Thermo Fisher scientific) and quantified as previously described (39).

2.5. Data normalization and hit identification in RNAi screens

Data were normalized within each plate based on cell number and lipid accumulation in six negative control wells as previously described (40). For hit identification, the normalized values from each screen were used to calculate the Strictly Standardized Mean Difference (SSMD) = \( \frac{\mu_i - \mu_N}{s_i + s_N} \), where \( \mu_i \) and \( s_i \) are sample mean and variance of each siRNA and \( \mu_N \) and \( s_N \) are sample mean and variance of the negative control per plate (41). The values and SSMD from each screen were put together to produce a mean outcome from both screens. To interpret the size of siRNA effects the following SSMD-based classification was used: \( \geq 5 \) extremely strong, \( 5 > \) to \( \geq 3 \) very strong, \( 3 > \) to \( \geq 2 \) strong, \( 2 > \) to \( \geq 1.645 \) fairly strong, \( 1.645 > \) to \( \geq 1.28 \) moderate, \( 1.28 > \) to \( \geq 1 \) fairly moderate, \( 1 > \) to \( \geq 0.75 \) fairly weak, \( 0.75 > \) to \( > 0.5 \) weak, \( 0.5 \geq \) to \( > 0.25 \) very weak and \( 0.25 \geq \) to \( > 0 \) extremely weak.

2.6. siRNA transfections with electroporation

Selected hits were verified using NEON electroporation system (Invitrogen, Thermo Fisher Scientific) as transfection method. 1600V/20ms pulse and 10\( \mu \)l or 100\( \mu \)l electroporation tips were used to transfect 100,000 or 10\(^6\) cells, respectively. Final concentration of 4nM siRNA was used for 10\( \mu \)l and 20nM for 100\( \mu \)l tips. After electroporation, cells were seeded in a proliferation media without FGF2 in a density of 40,000 cells/cm\(^2\). Differentiation was induced 24-hours post-transfection and cells were differentiated as previously described (37). To verify specificity of targeting in the RNAi screens,
1 or 2 different siRNAs from Ambion (Silencer select, Thermo Fisher Scientific) were used for validation experiments (Supplemental table 2) (38).

2.7. RNA isolation and RT-qPCR

Total RNA from hASC was extracted either with RNAeasy Micro Kit (Qiagen, Hilden, Germany) (96-well plates) or nucleospin RNA Macherey Nagel kit (Macherey Nagel, Duren, Germany) (24-well plates) according to manufacturer’s recommendation. cDNA from total 50ng of RNA was prepared using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative qPCR was run on CFX96 Touch™ detection system (Bio-Rad). Taqman probes (Thermo Fisher Scientific) were used for quantification of CEBPA (Hs00269972.s1), NR3C1 (Hs00353740_m1) and JARID2 (Hs00192089_m1) (in differentiation kinetics experiment), whereas Sybr primers were utilized for the rest of the genes (sequences are listed in Supplemental table 3) (38). Expression of mRNA was normalized to 18S ribosomal RNA using the ΔΔCt –method (42).

2.8. Nuclear protein extraction

Nuclei were first isolated from the cells and then nuclear proteins were extracted using RIPA buffer (Thermo Fischer Scientific), as described previously in (43, 44). The protein concentrations were measured using Pierce™ BCA Protein Assay Kit (Pierce, Thermo Fischer Scientific) according to the manufacturer’s instructions.
2.9. Western blot

For protein detection, 15μg of nuclear protein was separated on a 12% SDS-PAGE gel (Bio-Rad) and transferred to a polyvinyl difluoride membrane (GE Healthcare). The membrane was blocked in 3% ECL Advance blocking agent (GE Healthcare) and incubated with primary antibodies against JARID2 (D6M9X, 1:500 dilution, Cell Signaling Technology Danvers, MA, USA Cat# 13594, RRID:AB_2798269 (45)). Secondary anti-rabbit HRP antibody was used at a 1:10,000 (Sigma-Aldrich, St.Louis, MO, USA, Cat# A0545, RRID:AB_257896 (46)). The proteins were visualized by chemiluminescence using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare), ChemiDoc XRS+ system and Quantity One Software (Bio-Rad). Protein quantification was performed by Image lab software (Bio-Rad). JARID2 values were normalized to total nuclear proteins per lane (Supplemental figure 1)(38).

2.10 RNAseq

Total RNA from in vitro differentiated hASC was subjected to quality control with Agilent Tapestation (Agilent Technologies Inc. Santa Clara, CA, USA) according to the manufacturer’s instructions. Libraries were constructed using Illumina TruSeq stranded mRNA sample preparation protocol according to manufacturers’ instructions (Illumina, San Diego, CA, USA). The yield and quality of the amplified libraries were analyzed using Qubit (Thermo Fisher Scientific) and the Agilent Tapestation (Agilent). The indexed cDNA libraries were normalized, combined and the pools were sequenced on the Illumina Nextseq 550 (Illumina) for a 75-cycle v2 sequencing run generating 75bp single-end reads. Sample demultiplexing was performed using bcl2fastq (v2.20.0). Sample quality was assessed using FastQC (v0.11.8) and MultiQC (v1.7). Reads were aligned to a reference built from Ensembl GRCh38 genome sequences using STAR (v2.6.1d). Counts for each gene were obtained by featureCounts (v1.5.1) using the GRCh38.99 GTF file from Ensembl. Bioconductor package DESeq2 was used for count normalization and sample group comparisons, generating log2 fold changes,
Wald test p-values and p-values adjusted for multiple testing (Benjamini-Hochberg method). RNAseq data are accessible on GEO with the accession number GSE164413 (reviewer token ctonmgksrjqpdwz).

2.11 Glycerol release and stimulated lipolysis

Cell culture medium (48-hours conditional media) was collected on day 9 of hASC differentiation and glycerol release as a measure of basal lipolysis was assessed and normalized as previously described (39). Stimulated lipolysis (also measured by glycerol release) was determined in control and isoprenaline-treated (1 µM) samples on day 13 of differentiation as described previously (39, 47, 48), except that insulin was removed from cell culture media 24-hours before the experiment. During lipolysis fat (mainly triglycerides) in the lipid droplet of fat cells are broken down to glycerol and fatty acids, which are released from the cells. Glycerol is a more accurate lipolysis measure than fatty acids as it is not re-utilized to an important extent by the fat cells.

2.12. Total glucose uptake

Insulin (100nM)-stimulated and basal glucose uptake in hASC was assessed on day 13 of differentiation as previously described (49). Glucose uptake was normalized to protein amount in each cell culture well measured by BCA assay.

2.14. Pathway analysis

Ingenuity pathway analysis (IPA) software (Ingenuity® Systems, QIAGEN, http://www.ingenuity.com) was used to determine the upstream regulators in RNAseq experiments and for Canonical pathway analysis in the screening.
2.15. Statistical analysis

The data was tested for normal distribution with the Shapiro-Wilk test. If the criteria for normal distribution were fulfilled, one-way analysis of variance (ANOVA) with subsequent Bonferroni correction post-hoc test was utilized. If only two groups were compared, student’s t-test was used. When the criteria for normal distribution were not achieved, the non-parametric Kruskal-Wallis and Mann-Whitney pair-wise comparison tests were applied. Pearson test was used to correlate normally distributed measures (JARID2 expression with adipose tissue morphology, glycerol release with number of differentiated cells) and Spearman test was used to correlated the results from two screens. The level of statistical significance was set as 0.05 with *p < 0.05, **p < 0.01 and ***p < 0.001. IBM SPSS Statistics 22 software (SPSS, Chicago, IL, USA) or GraphPad Prism version 7 and 9 (GraphPad Software, La Jolla, California, USA) were used for statistical analysis. As regards power calculation we focused on the published microarrays and used the method by Liu and Hwang (50) to determine the appropriate sample size for such experiments. Assuming that significant differences in gene expression between the two studied groups were defined with a false discovery rate (FDR) of 5%, the proportion of non-differentially expressed genes between groups was 0.95. Assuming a standard deviation for effect size of 1, a population consisting of two groups composed of 15 subjects each yields a power of approximately 90% to detect differentially expressed genes. This is close to the actual number of 13 cachectic and 14 weight stable patients used in the smallest of the examined groups (Cohort 2).

3. RESULTS

3.1. Performing RNAi screen for adipogenesis-regulating TFs

To screen for adipogenesis-regulating TFs affected by metabolic conditions, we selected genes present in Dharmacon siRNA TF library (n=1530) and influenced by adipose tissue metabolic status in our previously described cohorts. We found that 115 TFs were regulated by obesity (33), 16 by cancer cachexia (34), 24 by IR (35) and 20 by adipose tissue morphology (30). Thereafter we utilized
previously published FANTOM5 CAGE data (51) to verify if selected TFs are expressed at some time point during in vitro differentiation of hASC (Figure 1A). This resulted in 148 genes (Figure 1A, B). Several belonged to more than one category of metabolic regulation (the TFs regulated by several categories are in bold). To identify the possible functions of these selected genes, canonical pathway analysis was performed using IPA software. The analysis revealed adipogenesis to be the most enriched pathway (Figure 1C) and suggested that large part of selected genes may be involved in regulation of fat cell differentiation. To evaluate the role of all 148 genes on adipogenesis, we designed RNAi screening approach in in vitro differentiated hASC in 96-well format. The knockdown was performed one day before induction of differentiation (day -1) and lipid accumulation was quantified on day 9 (Figure 2A). The knockdown efficiency was relatively stable until day 9 of adipogenesis and differences between negative siRNA and siPPARG-treated cells were large (Figure 2B, C). Two automated RNAi screens were performed using six wells of negative (non-targeting siRNA) and three wells of positive (PPARG and/or CEBPA) controls in each screening plate (Figure 2C). Wells on the edges showed edge effects during optimization experiments (data not shown), therefore the edges were excluded and filled with the buffer. Number of differentiated cells was adjusted by total number of cells in each well and the average Z’ factor for each screen was calculated. The Z’ factor for the first screen was 0.44 and 0.15 for the second, which suggested they are of good quality. Screens with Z’ of zero or greater are successful in identifying validated hits (40). In addition, visualization of ratio of lipid-containing cells per total cells showed a good reproducibility of control samples throughout all the plates and identification of several well-known adipogenesis regulators such as EBF1 (52, 53), KLF15 (8, 54) and FBXW7 (55) (Figure 2D). Furthermore, there was a strong correlation between the effect of specific TFs on lipid accumulation in the first and second screens (p<0.001, r=0.8191) (Figure 2E) and lipid accumulation correlated well with glycerol release (Figure 2F), which in our experiment setup was another measure of adipogenesis. In summary, the analysis of screening results strongly suggested that adipogenesis-regulating TFs could be identified by data analysis.
3.2. Thirty-nine TFs are identified as adipogenesis regulating factors

To classify and select the hit genes, we first set lipid accumulation of SSMD ≥ ±1 as a cut-off threshold, which gave us 79 primary hits as shown in Figure 3A. Lipid accumulation was used as an indirect measure of differentiation. Thereafter, to exclude the siRNAs with consistent but weak effects, the biologically more favorable measure namely mean fold change (±10%) was used, which excluded 29 previous hits. As a third step, to exclude variations in cell seeding during transfection, lipid accumulation was adjusted to the total number of cells (nuclear staining) using a threshold of SSMD ≥ ±1, which resulted in 39 hits (Figure 3B). 25 hit TFs were regulated by obesity (21.7% of all obesity-regulated screened genes), 11 by IR (45.8% of selected IR-regulated genes), five by morphology (25% of selected genes), and three by cancer cachexia (18% of selected genes) (Figure 3C). Among those, there were genes regulated by several conditions – two by obesity and morphology, one by obesity and IR, one by cachexia and morphology and one by cachexia and IR. Canonical pathway analysis of these 39 selected hits, showed that adipogenesis was among top six significantly regulated pathways. In addition, two cell cycle checkpoints were among most highly enriched pathways suggesting that cell-cycle regulating TFs might have been affecting lipid accumulation in our screening setup (Figure 3D). However, only four TFs among 39 hits were affecting cell numbers (with SSMD ≥ ±1 and 10% effect compared to non-targeting siRNA); GTF2F2 and ANKRD30A were decreasing cell number while HMGA1 and FBXW7 were increasing. The list of significantly enriched canonical pathways included also several other adipogenesis-affecting pathways - PPARG, PDGF, Wnt/β-catenin, TGF-β and FGF pathways (Supplemental table 4) (38). Literature analysis (Pubmatrix (56)) of these 39 hits revealed that many of the genes have been reported to regulate adipogenesis by earlier studies, but 11 genes were novel with no reports on their function in adipogenic regulation (Table 1).
3.3 Twenty TFs are affecting hASC cell number

Enrichment of canonical pathways for adipogenesis-affecting TFs described above suggested that cell cycle regulation can be important for human adipocyte differentiation. In addition, previous reports indicated that ASCs need to re-enter cell cycle before releasing differentiation program (101), therefore we decided to investigate whether the screened TFs affect adipocyte cell number in vitro. In the analysis described above, quantity of differentiated cells was normalized for total cell numbers, therefore TFs that affect cell number, but not differentiation/lipid accumulation efficiency have been hidden the normalization. To reveal TFs possibly affecting cell proliferation, we have performed the analysis of total cell numbers for each screened TF using the same cut-off as for quantification of differentiation (SSMD ≥ ±1 and 10% effect compared to non-targeting siRNA). This analysis identified 20 TFs that affected cell number in the RNAi screen (Figure 3E and Table 2). Largest number of these TFs (15) were regulated by obesity, four by morphology, three by cancer cachexia and four by IR (Figure 3F). Only for eight out of 20 TFs, the role in adipogenesis regulation has been reported by previous studies, while almost all of them (18) had a documented role in cell proliferation (Table 2). Interestingly, adipogenesis pathway was the most significantly enriched pathway in IPA canonical pathway analysis while cell cycle regulation being a third one (Figure 3G, Supplemental table 5) (38). This strongly suggested that novel adipogenic regulators might be identified even by this analysis.

3.4 Validation of the novel hit TFs

To find novel hit TFs, regulating adipogenesis, we used a strict definition criteria, selecting TFs that (1) affected the percentage of differentiated cells in the screen and (2) which function in adipogenesis had not been demonstrated in publications previously. Eleven such TFs were detected (Table 1, genes in bold). Among these genes, two genes (ANKRD30A and GTF2F2) had no significant effect on glycerol release and therefore were removed from following analysis. Most of these
selected genes were regulated by obesity (five), two by IR, one by morphology, and one by cancer cachexia and morphology (Figure 4A). Using siRNA from a different manufacturer (Ambion) and a different transfection method (electroporation), we have knocked down these nine genes as well as PPARG to validate the effect on adipogenesis obtained by the RNAi screen. The knockdown was efficient and stable throughout differentiation (Supplemental figure 2A showing JARID2 suppression (38)) for all TFs except one (NFKBIZ) (Figure 4B). The attenuation of TGIF1, SOX4, NFKBIZ, CSRNP1 and JARID2 expression resulted in significant reduction of lipid accumulation. Strikingly, a knockdown of CSRNP1 using Ambion’s siRNA caused an opposite effect than siRNA from Dharmacon, suggesting possible off-target effects. Inhibition of JARID2 caused most consistent and significant effect on lipid accumulation. JARID2 is a member of the Jumonji C (JmjC) and ARID domain protein family (113), which is essential for early embryonic development (114, 115), but its role in adipogenesis and fat cell function was unknown. Therefore, this gene was further validated.

3.5 JARID2 expression is regulated by adipogenesis

JARID2 expression (RNA and protein) was examined during in vitro differentiation of human adipocytes. mRNA expression of JARID2 increased between day 2 and 4 of adipogenesis and reached its maximum on day 8 (Figure 5A). Western blot analysis detected two isoforms of JARID2 as described previously (116). Lower molecular weight isoform around 80kD was expressed from initial phase of adipogenesis (day 2), whereas the higher molecular weight protein (around 140kD) appeared only at later stage (day 6 and 13) (Figure 5B). Image quantification showed that only 140kD isoform of JARID2 was downregulated by siRNA treatment (Figure 5B) although four different oligonucleotides covering different regions of JARID2 sequence were used for the knockdown. Furthermore, only one transcription start site for JARID2 was found in hASC by CAGE analysis (Supplemental figure 2B) (38). Therefore, the observed 80kD band could represent unspecific binding of the antibody and that the 140kD isoform is likely to be functional in adipocyte differentiation.
3.6 JARID2 regulates lipid accumulation and fat cell metabolism

Attenuation of JARID2 expression with siRNA decreased the lipid accumulation and basal glycerol release (Figure 5C, D). In addition, isoprenaline-stimulated lipolysis as well as insulin-stimulated glucose uptake were decreased in siJARID2-treated cells compared to control (Fig. 5E, F). These effects were not dependent on induced toxicity or lower cell number (Supplemental figure 3A, B). Furthermore, JARID2 siRNA was not affecting hASC proliferation or induction of proliferation marker (Supplemental figure 3C, D) (38).

3.7 JARID2 regulates the adipogenic program

We further defined JARID2-regulated genes by performing RNAseq in siJARID2 and control siRNA-treated hASC on day 6 and day 13 of differentiation (due to JARID2 expression pattern). In addition, knockdowns of JARID2 on day 2 of differentiation gave similar results as day -1 siRNA treatments (Supplemental figure 4) (38). These experiments suggested that JARID2 affects adipocyte phenotype during later stages of in vitro differentiation. Principal component (PC) analysis of RNAseq data showed that samples from different days of differentiation were separated by PC1 and siJARID2 samples were separated from controls by PC2 (Figure 6A). JARID2 knockdown affected gene expression stronger on day 13 than on day 6 (Figure 6B, Supplemental table 6) (38). Probably this reflects stronger expression of JARID2 on day 13. Analysis of pathways and networks (IPA software) suggested that pro-adipogenic factors (PPARG, CEBPA, SREBF2) were downregulated on day 6, while factors leading to disglycemia and inflammation (TNF, IFNG) were upregulated (Figure 6C). On day 13, pathways characterizing undifferentiated preadipocytes (TGFB1-3, TGFBR2, FGF2, SMAD3) and inflammation (TNF, IL1B) were upregulated (Figure 6D). Twenty-six genes were commonly regulated between day 6 and day 13 (Figure 6E). Several of these genes were previously reported to be involved in adipogenesis (MESD, PCSK9, INSIG1, PTGIS, KCNB1, PLIN1, SLC2A4...
Furthermore, IPA analysis of upstream regulators for the genes affected by siJARID2 on day 6 and day 13 revealed that genes that were under control of pro-adipogenic factors, such as PPARγ, insulin, SREBF1/2, PPARGC1A were gradually downregulated during differentiation in siJARID2-treated cells (Figure 6F and the supplemental table 7 (38, 124-131), while genes controlled by negative regulators like TGFB, TNF and lipopolysaccharide (132-134) were upregulated. These results indicated that JARID2 affects adipogenesis modulating the balance between stimulators and suppressors of this event.

3.8 JARID2 expression in adipocytes is influenced by obesity and hyperplasia

Large adipocytes (WAT hypertrophy) are indicative of lower adipogenic capacity of the tissue as discussed [121]. Therefore, we investigated whether there is any correlation between JARID2 expression and WAT morphology. In human adipose tissue, JARID2 is highly expressed in macrophages (data not shown), which affects correlation outcome if JARID2 expression is examined in intact tissue. Therefore, expression of this TF was examined in isolated adipocytes from scWAT (cohort 4). JARID2 expression was increased in lean subjects (Figure 6G) and correlated negatively with morphology delta value (Figure 6H). This value is the difference in fat cell volume between measured value and expected value obtained from the curve-linear relationship between fat cell volume and fat mass (135). The findings suggest that high JARID2 levels associates with hyperplastic WAT which, in turn, is linked to rapid rates of adipogenesis (135).

4. DISCUSSION

In this study, we have performed RNAi screening to detect TFs that are influenced by metabolic conditions in WAT and also affect adipogenesis. A large number of the presently identified hit TFs were previously known to influence fat cell differentiation. However, the study also defined novel TFs regulating lipid accumulation and cell number during human fat cell differentiation in vitro.
Herein, we provide literature sources for the known factors and validation of the newly detected hits. To further elucidate the usefulness of the screen one of the latter TFs, namely JARID2, was studied in some detail and defined as a TF required for efficient adipogenic program.

It is well known that genetically limited lipid accumulation/adipogenic capacity in WAT is causing lipodystrophies with ectopic fat storage resulting in IR (136, 137), while restoring adipogenic capacity in WAT is resolving IR (138). Therefore, few earlier attempts have been made to discover novel adipogenesis-regulating factors. A former RNAi screen for druggable hits (31) identified 459 genes. As the list was not published, it is impossible to determine if those hits overlap with the present study. However, none of our hits included in Table 1 and 2 are listed among its validated genes (31).

Interestingly, in the current screen 72% of significant hit TFs were found to be positive regulators of adipogenesis and only 28% were negative regulators, whereas in the previous screen only 22% of significant hits were positive regulators. This could reflect gene selection strategy for the screen, but also methodological differences; in the current study hASC were not proliferated, whereas in the previous screen hASC were proliferated for 3 days after siRNA transfection (31). In addition, usage of full differentiation media can also cause bias towards the detection of pro-adipogenic factors.

Another previous screen had a very limited scope. It was designed to find the novel regulators of PPARG and was based on PPARG reporter activity (32). In the latter study, only few siRNAs were tested as proof of principle for the reported methodology. Therefore, when comparing with the two previous studies, the present study provides unique and novel findings as well as a high throughput methodology for the in vitro study of adipogenesis-regulating genes. It is also important to note that the timing and stability of siRNA treatment in the current screen provided possibility to target genes that are both upregulated and downregulated during adipocyte differentiation.
Current screening results suggest that large part (26.4%; 39 genes out of 148) of metabolic condition-regulated TFs affect lipid accumulation reflecting differentiation and additional 13.5% TFs affect cell number, which mirrors proliferation. This suggests that adipogenic capacity of adipose tissue is affected in metabolic conditions and results in altered morphology of WAT (fat cell size versus number). As many as 45.8% of all IR-affected TFs were found to regulate adipogenesis. The high number goes in line with previously published data, namely that IR is associated with larger fat cells and impaired fat cell differentiation (138, 139).

Expectedly, many of significant hits were already known regulators of fat cell differentiation (EBF1, LXRα, MYC, KLF15) (references within Table 1). This can be interpreted as a good quality of the current screen. On the other hand, many genes that were found to affect fat cell number (Table 2) were not defined as adipogenic factors before but are well-known proliferation regulators mostly in cancer cells. For example, GLIS2 and ETV6 have been reported to regulate proliferation in leukemia (140), HMGAI in gastric cancer (141), FBXW7 in breast cancer (142). In addition, LRRFIP1 and ZNRD1 have been shown to regulate proliferation via Wnt/β-catenin pathway in mesenchymal stem cells and in tumour cells respectively (143, 144). Therefore, these genes might have a function in adipocyte progenitor expansion, which needs further studies.

It is important to acknowledge that the TF selection for the screen was done using gene expression profiling in the intact WAT, therefore there is a risk that metabolic conditions were targeting TFs expressed in different cell type than adipocyte or adipocyte progenitor. Furthermore, not all the hits could be validated by using siRNA obtained from a different vendor. Such a drawback is a known phenomenon and can be due to off-target effects (145). However, the reproducibility of siRNAs effects between the triplicate samples as well as between two screens was very high and a mixture of four different siRNAs were used to knock each gene in RNAi screen, which suggests that the screen produced highly reliable results.
It should also be noted that our screen was performed on clinically relevant TFs from scWAT. In theory, important additional TFs might have been discovered if other WAT regions such as the visceral also were included. Unfortunately, the visceral depot cannot be investigated during the presently used ambulatory conditions. In addition, one needs to point out that the hASC used for in vitro studies are from a male individual while our clinical cohorts are from females except cachexia cohort that includes both genders. Unfortunately, larger male clinical cohorts with defined global gene expression in WAT were not available to us while hASC used for in vitro studies is the best characterized primary cell model for such type of study. In addition, the screening identified a large number of known adipogenesis-related TFs that have been studied both in vitro and in vivo murine models (mostly in male mice). Although we cannot exclude that some sex-specific TFs were not examined in our study, we think that it is not likely that screening results are largely affected by this gender difference.

Nine TFs were detected having a hitherto unknown role in adipogenesis. As mentioned, we selected JARID2 for further investigations. Its expression was upregulated during adipocyte differentiation and correlated with hWAT hyperplasia. Knockdown of JARID2 affected cell metabolism and the adipogenic transcriptional program. Jarid2 belongs to a family of Jumonji histone demethylases but lacks an enzymatic activity. Instead, it functions as a regulator of developmental processes in embryonic cells (146, 147). JARID2 depletion is embryonically lethal (115) in mice and human mutations are associated with congenital defects (148). A short form of JARID2 (80kDa) is present in lineage-committed cells (116). It is predominant in keratinocytes, where the gene influences cell cycle and epidermal differentiation (116). IPA analysis of siJARID2-affected genes revealed that TGF-β signaling could be a central affected pathway in JARID2-attenuated cells. TGFβ inhibits adipocyte differentiation by mediating Smad3 interaction with C/EBPa (132). Furthermore, TGF-β signaling is inducing adipocyte progenitor cell proliferation (149), which if upregulated late during differentiation, can possibly switch cell fate towards undifferentiated state. In addition, pro-
inflammatory signals, induced by JARID2 knockdown are also driving hASC towards undifferentiated state (134), which further supports a role of JARID2 for hASC differentiation to mature adipocyte.

Although, the major focus of the study was the screening of clinically relevant TFs and JARID2 was studied to further prove a validity of this screen, we made an attempt to further investigate the mechanism of JARID2 function by ChIP-seq analysis. JARID2 has been shown to recruit Polycomb Repressor Complex 2 (PRC2), which methylates histone H3 on lysine 27 (i.e. H3K27me3) transcriptionally silencing the chromatin (146). Therefore, ChIP-seq analysis with the antibodies binding to PRC2 component EZH2 was also performed in control and siJARID2-treated cells. EZH2 was chosen due to its direct interaction to JARID2, which has been reported perviously (150). Unfortunately, none of three different anti-JARID2 antibodies showed sufficient JARID2 affinity (no binding) or specificity (the same number of peaks in control and siJARID2-treated cells) in ChIP-seq experiments. Number of EZH2 peaks was not affected by JARID2 siRNA (data not shown). Although these results suggest that JARID2 might not act via recruitment of PRC2 complex in differentiating fat cells, further mechanisms were not explored due to a different focus of the study.

To conclude, herein we report on a comprehensive screen for human fat cell TFs that are of relevance for important metabolic conditions and also can be implicated in adipogenesis. We provide methodological tools for an efficient RNAi screen in human in vitro differentiated adipocytes, lists of all metabolic condition-regulated TFs expressed in adipocytes and overview of all adipogenesis-regulating TFs detected by the current screen. It is apparent that a large number of metabolic condition-regulated TFs also impact adipogenesis. Most are known to possess this feature from previous studies. However, nine novel ones are described and for one, JARID2 we made some further analyses suggesting that it can de-repress early progenitor cell pathways and inhibit metabolism of mature adipocytes.
Author statement:

Conceptualization (CB, JL, PA); Data curation (CB, NS, JL, JPL, JA, BT, ABE); Formal analysis (CB, NS, JL, JPL, ABE); Funding acquisition (JL, PA); Investigation (CB, NS, JPL, BT, JA); Methodology (CB, NS, JPL, ABE); Project administration (CB, NS, JL); Validation; Visualization (CB, NS, JL); Roles/Writing - original draft (CB, NS, JL); Writing - review & editing (all co-authors).

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Data availability:

The RNAseq data are published on GEO with accession number GSE164413 (reviewer token ctonmgksrqpdz) and will be made publicly available upon publication of the study. Some or all data generated or analysed during this study are included in this published article or in the data repositories listed in References.
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FIGURE LEGENDS:

**Figure 1. Selection of TFs for RNAi screen.** **A.** Selection of the TFs for the screening. **B.** A list of selected TFs and their assignment to different categories of metabolic conditions. Genes belonging to several different categories are shown in bold. **C.** Ingenuity Pathway Analysis (IPA) for the 148 selected genes showing top five most significantly enriched canonical pathways.

**Figure 2. RNAi screening.** **A.** Schematic picture of siRNA treatment and differentiation of hASC. **B.** Efficiency of siRNA knockdown during differentiation for PPARG, GR (NR3C1) and CEBPA (RT-qPCR, n=3, 2-way ANOVA, *p<0.05; **p<0.01; ***p<0.001). **C.** A representative setup of a screening plate and representative images of non-targeting siRNA (Neg C) and siPPARG-treated cells stained for neutral lipids (Bodipy) and DNA (Hoechst). **D.** TF screening result showing amount of lipid droplets/cell in each well. Samples are represented by black symbols of different shapes, negative control (non-targeting siRNA) by yellow circles, positive control - siPPARγ (blue circles) and siCEBPA (purple circles) are also indicated. As a quality control, known adipogenic stimulators EBF1 and KLF15 and adipogenesis inhibitor FBXW7 are labeled. **E.** Correlation of a number of differentiated cells (bodipy objects) in first and second screen for each tested TF (Spearman correlation), samples of negative control are represented by yellow, siPPARG by blue and siCEBPA by purple circles. **F.** Correlation between the number of differentiated cells and glycerol release for each tested TF (Pearson correlation).

**Figure 3. Selection of adipogenesis-regulating TFs.** **A.** Workflow for TF selection for further analysis. **B.** Fold change of lipid accumulation per cell (bodipy objects per Hoechst objects normalized to control in each screening plate) for 39 significant TFs. **C.** Distribution of TFs significantly regulating
lipid accumulation in categories of metabolic conditions (Venn diagram). D. Six most significantly enriched pathways of IPA analysis for 39 TFs shown in B.

E. Fold change of cell number (Hoechst objects normalized to control in each screening plate) 20 significant TFs. F. Distribution of TFs significantly regulating cell amount in categories of metabolic conditions (Venn diagram). G. Six most significantly enriched pathways of IPA analysis for 20 TFs shown in E.

Figure 4. Validation of selected hit TFs. A. Venn diagram showing metabolic group distribution of nine novel adipogenesis-regulating TFs and effect direction of their siRNA on lipid accumulation in RNAi screen. B. siRNA knockdown efficiency using siRNA from Ambion for nine selected TFs and PPARG as a positive control. Fold change of expression in siRNA-treated sample compared to non-targeting control is shown for each TF (RT-qPCR, one-way ANOVA, with Bonferroni’s multiple comparisons n=3). C. Effect of siRNA (Ambion) on cell differentiation (bodipy objects per Hoechst objects compared to non-targeting control, n=3, *p<0.05; **p<0.01; ***p<0.001, one-way ANOVA with Bonferroni’s multiple comparisons).

Figure 5. JARID2 expression is regulated by adipogenesis and JARID2 knockdown affects fat cell phenotype in vitro. A. Expression of JARID2 mRNA in hASC during differentiation (RT-qPCR, n=3). B. Western blot analysis of JARID2 expression during adipogenesis. Both described isoforms of 140 and 80kD are indicated. Quantification of the expression of JARID2 isoforms during differentiation (80kD - C and 140kD - D, n=3, paired t-test). C. Representative picture of control and siJARID2-treated cells on day 9 of differentiation. D. Fold change of glycerol concentration in conditional media normalized to cell number (Hoechst) and compared to control on day 9 of differentiation (n=3, Kruskal-Wallis test). E. Basal and insulin-stimulated glucose uptake on day 13 of differentiation (n=3, 2-way
ANOVA). F. Basal and isoprenaline-stimulated lipolysis measured on day 13 of differentiation (n=4, 2-way ANOVA), *p < 0.05, **p < 0.01 and ***p < 0.001.

Figure 6. JARID2 regulates genes involved in adipogenesis process and fat cell phenotype. A. Principal component analysis of RNAseq results. B. RNAseq data represented by Volcano plots for RNA expression on day 6 and day 13. Significantly regulated genes are represented by red circles, top 6 regulated genes are labeled (n=3). C. IPA graphical summary of pathways and regulators for JARID2-regulated RNAs on day 6 of differentiation (blue indicates downregulation and orange–upregulation). D. IPA graphical summary of pathways and regulators for JARID2-regulated RNAs on day 13 of differentiation (blue indicates downregulation and orange–upregulation). E. Normalized heatmap of 26 genes common among JARID2-regulated genes on day 6 and 13 of differentiation. F. IPA analysis of upstream regulators based on significantly regulated genes by siJARID2. Top 15 most significantly enriched upstream regulators with Z-scores are shown. G. JARID2 expression in adipocytes obtained from scWAT of lean (n=9) and obese (n=60) patients (RT-qPCR) (t-test, *p<0.05). H. Pearson’s correlation of JARID2 mRNA expression and cell morphology index in non-obese patients (n=15).
Table legends.

Table 1. Genes regulating lipid accumulation normalized per cell. For the genes with known function in adipogenesis, up to three most relevant publications are cited. Fold change is calculated from non-targeting control siRNA.

Table 2. Genes regulating glycerol release independently of differentiation (glycerol release per differentiated cell. genes that overlap with table 1 excluded). For the genes with known function in adipogenesis, up to three most relevant publications are cited. Fold change is calculated from non-targeting control siRNA.
Table 1. Genes regulating lipid accumulation normalized per cell. 11 novel regulators of adipogenesis that have not been studied previously are shown in bold.

| Gene ID (alternative name) | Cells with lipids/total cells | Glycerol release/total cells | PubMed hits adipogenesis | Selected references |
|---------------------------|------------------------------|-----------------------------|--------------------------|---------------------|
|                           | SSMD Fold change              | SSMD Fold change            |                          |                     |
| EBF1                      | -4.92 0.58                   | -2.88 0.59                  | 17                       | (52, 57)            |
| DNM2                      | -3.99 0.72                   | -3.83 0.42                  | 0                        |                     |
| HMGA1                     | -3.93 0.63                   | -3.19 0.48                  | 12                       | (58-60)            |
| TGIF1                     | **-3.81** 0.67               | -3.99 0.57                  | 0                        |                     |
| LMO4                      | -2.73 0.72                   | -2.54 0.55                  | 2                        | (61, 62)           |
| SOX4                      | -2.69 0.73                   | -1.39 0.71                  | 2                        |                     |
| FOS (C-FOS)               | -2.62 0.82                   | -1.56 0.69                  | 30                       | (63)               |
| KLF15                     | -2.41 0.78                   | -1.75 0.60                  | 33                       | (8, 64)            |
| ID2                       | -2.4 0.74                    | -2.42 0.52                  | 10                       | (65-67)            |
| TFDP1 (DP1)               | -2.02 0.83                   | -2.86 0.70                  | 2                        | (68, 69)           |
| ARID5A                    | -2.01 0.88                   | -7.83 0.70                  | 1                        | (70)               |
| BCL3                      | -1.99 0.88                   | -4.52 0.60                  | 2                        |                     |
| IFI16                     | -1.85 0.78                   | -1.73 0.62                  | 1                        | (71)               |
| NR2F1 (COUP-TF)           | -1.83 0.83                   | -2.19 0.61                  | 1                        | (72)               |
| FOSB                      | -1.79 0.88                   | -0.68 0.85                  | 7                        | (73, 74)           |
| SQSTM1                    | -1.67 0.87                   | -0.42 0.92                  | 14                       | (75, 76)           |
| NR1H3 (LXRalpha)          | -1.57 0.85                   | -1.54 0.71                  | 28                       | (77-79)            |
| HIPK1                     | -1.44 0.86                   | -0.77 0.83                  | 0                        |                     |
| MYC                       | -1.39 0.84                   | -1.73 0.65                  | 72                       | (80-82)            |
| GATA6                     | -1.35 0.93                   | -1.02 0.71                  | 5                        | (83)               |
| Gene       | Log2 Ratio | Fold Change | p-Value | q-Value | P-Value | q-Value |
|------------|------------|-------------|---------|---------|---------|---------|
| USF1       | -1.35      | 0.87        | -1.30   | 0.81    | 4       | (84)    |
| JARID2     | -1.32      | 0.87        | -1.65   | 0.67    | 0       |         |
| NFKBIZ     | -1.32      | 0.88        | -1.39   | 0.81    | 0       |         |
| LMCD1      | -1.28      | 0.87        | -1.28   | 0.70    | 1       | (85)    |
| YWHAH      | -1.28      | 0.85        | -1.62   | 0.80    | 1       | (86)    |
| GAS7       | -1.27      | 0.9         | -0.91   | 0.78    | 0       |         |
| DDIT3 (CHOP)| -1.13    | 0.9         | -2.33   | 0.68    | 52      | (87-89) |
| KLF16      | -1.13      | 0.87        | -0.83   | 0.80    | 1       | (90)    |
| GTF2F2     | 1.36       | 1.15        | 0.91    | 0.75    | 0       |         |
| FOSL2 (FRA-2)| 1.52   | 1.13        | 0.95    | 1.28    | 2       | (91, 92)|
| TCF12      | 1.54       | 1.14        | 2.02    | 1.22    | 1       |         |
| EGR2       | 1.62       | 1.23        | 2.43    | 1.42    | 16      | (93-95) |
| STAT2      | 1.68       | 1.12        | 2.14    | 1.46    | 3       | (96)    |
| GCDH       | 1.69       | 1.22        | 1.63    | 1.21    | 0       |         |
| FBXW7      | 2.09       | 1.12        | 1.18    | 1.64    | 2       | (55)    |
| ABL1 (c-Abl)| 2.1      | 1.24        | 1.27    | 1.22    | 5       | (97, 98)|
| CDKN2A     | 3.15       | 1.21        | 0.95    | 1.17    | 18      | (99, 100)|
| ANKRD30A   | 3.68       | 1.24        | 0.85    | 1.09    | 0       |         |
| CSRNP1     | 4          | 1.29        | 2.76    | 1.56    | 0       |         |
**Table 2.** Genes regulating glycerol release independently of differentiation (glycerol release per differentiated cell, genes that overlap with table 1 excluded). Novel potential regulators of adipogenesis that have not been studied previously are shown in bold.

| Gene ID     | SSMD | Fold change | Pubmed hits Adipogenesis | Pubmed hits Proliferation | Selected references for adipogenesis |
|-------------|------|-------------|---------------------------|---------------------------|-------------------------------------|
| GTF2F2      | -3.1 | 0.79        | 0                         | 3                         |                                     |
| ETV1        | -1.64| 0.87        | 1                         | 63                        | (83)                                |
| ANKRD30A    | -1.53| 0.89        | 0                         | 2                         |                                     |
| ZNF219      | -1.51| 0.82        | 0                         | 0                         |                                     |
| ATM (Tel1)  | -1.47| 0.89        | 0                         | 13                        |                                     |
| DNMT1       | -1.45| 0.87        | 15                        | 837                       | (102, 103)                          |
| ELF1        | -1.29| 0.86        | 0                         | 34                        |                                     |
| SNAPC5      | -1.28| 0.87        | 0                         | 0                         |                                     |
| GLIS2       | -1.26| 0.85        | 0                         | 9                         |                                     |
| NOTCH3      | -1.26| 0.87        | 9                         | 375                       | (104, 105)                          |
| ETV6        | -1.19| 0.90        | 1                         | 94                        |                                     |
| ZNRD1       | -1.19| 0.90        | 0                         | 10                        |                                     |
| RNF14 (ARA54)| -1.18| 0.87        | 0                         | 7                         |                                     |
| SAP30       | -1.01| 0.90        | 0                         | 7                         |                                     |
| KLF5        | 1     | 1.10        | 21                        | 279                       | (7, 106)                            |
| HMGA1       | 1.06 | 1.15        | 13                        | 194                       | (15, 107, 108)                      |
| MAX         | 1.09 | 1.11        | 1                         | 126                       | (109)                               |
| LRRFIP1     | 1.18 | 1.11        | 0                         | 9                         |                                     |
| SPI1 (PU.1) | 1.72 | 1.13        | 9                         | 339                       | (110-112)                           |
| FBXW7       | 2.1  | 1.19        | 2                         | 269                       | (55)                                |
**Figure 1**

**A**

Dharmacon TF RNAi library (n=1530)

- **Genes regulated by obesity (n=115)**
- **Genes regulated by adipocyte morphology (n=20)**
- **Genes regulated by cancer cachexia (n=16)**
- **Genes regulated by insulin resistance (n=24)**

148 unique TFs expressed during adipogenesis in *in vitro* differentiated primary adipocytes

**B**

| Metabolic condition | Regulated TFs |
|---------------------|---------------|
| **Obesity**         | AHR, ATF6, BACH1, CALR, CDKN2B, CDKN2C, CREB3, DNMT1, ETV6, IFI16, LRRFIP1, MAGED1, MSC, MYC, MYCBP, NOTCH3, NR4A1, PDLIM1, PRRX1, TLE4, YWHAH, ZNF219, ABL1, AEBP1, AES, ARNTL, ATF3, ATM, BCL3, BHLHE40, BTBD11, CAMK4, CBL, CDKN2A, CTNNB1, DNM2, E2F7, ELF4, ELK3, ESR1, ETV1, ETV5, FBXW7, FOS, FOSL2, FOXC2, GAS7, GATAD2A, GLIS3, GTF2F2, HES1, HIF1A, HIX1, HMGA1, HOXC5, HTATIP2, ID2, JARID2, KLF16, KLF6, LMCD1, LM04, MAFF, MAX, MDM2, MEF2C, MLXIPL, MYBBP1A, NFKB1, NRP1, OSTF1, PIR, PPARD, PREB, PTP1, RAI14, RNP14, RUNX1, RUNX2, RYR1, SAP30, SERTAD2, SIX2, SMAD1, SMARCA4, SMARCC1, SNAPC3, SND1, SOX4, SQSTM1, SRA1, SREBF2, STAT2, STAT3, STAT5, TAF10, TAF13, TAX1BP3, TCF12, TDP3, TFE3, TGF1, TP53, TRIM25, TRPV2, TWIST1, UBP1, USF1, ZBTB7A, ZDHHC13, ZEB2, ZNF154, ZNF354A, ZNDR1 |
| **Morphology**      | AHR, ATF6, BACH1, CALR, CDKN2C, ETV6, GCDH, IFI16, LRRFIP1, MSC, PDLIM1, PRRX1, TLE4, YWHAH, EBFI, ELF1, GTF2H1, GTF2IRD1, HIPK1, RFXANK |
| **Cancer Cachexia** | CDKN2B, CREB3, DNMT1, GCDH, KLF15, LRRFIP1, MAGED1, MSC, MYCBP, NOTCH3, ZNF219, HOXC8, IRF2BP2, KLF10, NR1H3, YAP1 |
| **Insulin resistance** | CDKN2C, KLF15, MYC, NR4A1, ANKRD30A, ARID5A, CSRN1, Ddit3, EGR2, EGR3, FOSB, GATA6, GLIS2, IRF1, KLF5, MAFF, MLX, NFKB1, NR2F1, RARB, RBPU, SERTAD1, SOX6, SPI1 |

**C**

- Adipogenesis pathway
- Aryl Hydrocarbon Receptor Signaling
- Cell Cycle: G1/S Checkpoint Regulation
- Molecular mechanisms of cancer
- Small Cell Lung Cancer Signaling

![](https://academic.oup.com/endo/advance-article-doi/10.1210/endocr/bqab096/6272286) by guest on 08 May 2021
Figure 2

A
Adipose-derived stem cells, hASC → Differentiation day
   1 2 3 4 5 6 7 8 9 → Adipocytes

B
siRNA reverse transfection with cell seeding
Quantification of lipid droplets and cell nuclei

C
Negative Control
siPPARG
BODIPY Hoechst Merged

D
Adipogenesis screening

E
Average bodipy objects

F
Normalized glycerol release
Normalized bodipy objects
Figure 3

A

1) Lipid accumulation comp to NegC; SSMD ≥ ±1 (n=79)
2) Lipid accumulation ±≥10% of NegC (n=50)
3) Lipid accumulation adj. for cell number comp to NegC; SSMD ≥ ±1 (n=39)

B

Diffentiated cells/total cells

Fold change

1.5
1.0
0.5
0.0

C

Morphology Cachexia
Obesity

Cachexia

In

rphology

Cell Cycle: G2/M DNA Damage
Checkpoint Regulation
Adipogenesis pathway
Chronic Myeloid Leukemia Signaling
Bladder Cancer Signaling

D

-log(p-value)

Aryl Hydrocarbon Receptor Signaling
Cell Cycle: G1/S Checkpoint Regulation

E

Cell number

Fold change

1.5
1.0
0.5
0.0

F

Morphology Cachexia
Obesity

Insulin resistance

G

-log(p-value)

Adipogenesis pathway
DNA Methylation and Transcriptional Repression Signaling
Cell Cycle: G1/S Checkpoint Regulation
Th1 and Th2 Activation Pathway
DNA Double-Strand Break Repair by Homologous Recombination
Figure 4

A

B

C

[Diagram showing the relationships between obesity, insulin resistance, cancer cachexia, and morphology, with knockdown efficiency and lipid droplet count/cell graphs.]

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Figure 5

A

Relative JARID2 mRNA expression

B

KD Control siJARID2

80 kD

day 2 day 6 day 13

C

Glycerol release

D

Relative glycerol release

E

Glucose uptake

F

Lipolysis

Normalized 3H glucose uptake

Normalized glycerol release
Figure 6

A

PC scores

log2 (fold change)

-log10 (adjusted p)

Day 6

ATAD1
MESD
JARID2
KRT79
PLIN1

Day 13

TGFBI
ABI3BP
TIMP3
LTBP1

B

C

D

E

F

G

H

JARID2 mRNA expression

Lean
Obese

Morphology (delta value)

JARID2 mRNA

r = -0.6283
p = 0.0215