Research Article

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Epigenomics

The histone methyltransferase SMYD1 is induced by thermogenic stimuli in adipose tissue

Annunziata G Cicatiello[‡,1], Annarita Nappi[‡,1], Fabiana Franchini[‡,1], Immacolata C Nettore[1], Maddalena Raia[2], Carmine Rocca[3], Tommaso Angelone[3,4], Monica Dentice[1,2], Paola Ungaro*[,§,5] & Paolo E Macchia[§,1]

[1]Department of Clinical Medicine & Surgery, University of Naples 'Federico II', 80131, Naples, Italy
[2]CEINGE, Biotecnologie Avanzate S.c.a.r.l., 80131, Naples, Italy
[3]Laboratory of Cellular & Molecular Cardiovascular Pathophysiology, Department of Biology, Ecology & Earth Sciences (DiBEST), University of Calabria, Arcavacata di Rende, 87036, Cosenza, Italy
[4]National Institute of Cardiovascular Research (I.N.R.C.), 40126, Bologna, Italy
[5]National Research Council – Institute for Experimental Endocrinology & Oncology 'Gaetano Salvatore', 80131, Naples, Italy
*Author for correspondence: pungaro@ieos.cnr.it
[‡]Authors contributed equally; co-first authors
[§]Co-last authors

Aim: To study the expression of histone methyltransferase SMYD1 in white adipose tissue (WAT) and brown adipose tissue and during differentiation of preadipocytes to white and beige phenotypes. **Methods:** C57BL/6J mice fed a high-fat diet (and exposed to cold) and 3T3-L1 cells stimulated to differentiate into white and beige adipocytes were used. **Results:** SMYD1 expression increased in WAT of high-fat diet fed mice and in WAT and brown adipose tissue of cold-exposed mice, suggesting its role in thermogenesis. SMYD1 expression was higher in beige adipocytes than in white adipocytes, and its silencing leads to a decrease in mitochondrial content and in *Pgc-1α* expression. **Conclusion:** These data suggest a novel role for SMYD1 as a positive regulator of energy control in adipose tissue.

Plain language summary: In this study, a protein called SMYD1 was examined in the adipose tissue of mice to understand its role in the development of different types of fat cells. The authors used mice fed a high-fat diet or mice exposed to a cold environment. The experiments were also performed on cultured cells that were stimulated to form specific types of fat cells (white adipocytes, which store energy; or beige adipocytes, which are responsible for releasing energy in the form of heat). The study found that SMYD1 increased in white adipose tissue particularly in response to cold exposure and high-fat diet, suggesting involvement in body temperature regulation. SMYD1 was higher in beige adipocytes than in white fat cells, and when SMYD1 was reduced, there was a decrease in certain factors related to energy control. Overall, these results suggest that SMYD1 plays a novel role in energy regulation in adipose tissues.

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According to the WHO, obesity is a metabolic disease characterized by excessive accumulation of fat stored in the form of triglycerides in adipocytes [1]. It occurs as a result of the enlargement (hypertrophy) and proliferation (hyperplasia) of adipocytes [2]. Because of its role in the balance between energy storage and consumption, adipose tissue is an important regulator of metabolic homeostasis in mammals [3].

Mammalian adipose tissue is generally divided into white adipose tissue (WAT) and brown adipose tissue (BAT) [4,5]. WAT, which expands during obesity, stores energy as large lipid droplets that are hydrolyzed during increased metabolic demand [6,7]. In contrast, BAT releases energy in the form of heat through a process called non-shivering or adaptive thermogenesis, which promotes the maintenance of a lean, healthy phenotype [8]. More

recently, a third type of adipocyte, called brite/beige adipocytes, has been found mainly in the subcutaneous WAT (scWAT) after cold exposure or stimulation by β3-adrenergic agonists. These cells exhibit an intermediate morphology between white and brown adipocytes and have increased numbers and activity of mitochondria [2,9]. The exact origin of beige adipocytes is not fully understood; they arise postnatally from WAT through a process called 'browning' of white fat [10]. WAT browning can be triggered by certain environmental stimuli such as chronic cold exposure, diet, exercise, thyroid hormones, natriuretic peptides and drugs [11–15]. During the browning process, the number of mitochondria, energy expenditure and multilocularization of fat increased in WAT and the expression of several thermogenic genes, such as *PPAR-α* and *PPAR-γ*, *PRDM16*, *PGC1α*, *CIDEA* and *UCP1* were also increased [16–18].

The idea of converting adipocytes from an energy storage type to a disposal type has long intrigued researchers as a promising strategy for preventing obesity and its associated metabolic disorders [19,20]. For this reason, a better understanding of the key players in the regulation of browning could lead to an alternative approach in modulating energy balance by increasing energy expenditure.

Thermogenic gene activation in brown and beige adipocytes is influenced by interactions between genes and environmental stimuli, including temperature or diet [21,22], through epigenetic events. Some epigenetic modifiers responsible for post-translational histone modifications, such as the histone methyltransferases MLL3/4 and EHMT1 and demethylase LSD1, are involved in regulating the thermogenic gene program by altering the chromatin landscape. In addition, they play an important role in maintaining the chromatin architecture, which is required for brown/beige adipocyte fate [23].

The histone lysine N-methyltransferase SMYD1 is a component of the Su(Var)3–9, Enhancer-of-zeste and Trithorax (SET) and Myeloid, Nervy and DEAF-1 (MYND) domain-containing (SMYD) proteins. They catalyze the methylation of histone 3 lysine 4 (H3K4), suggesting a function as transcriptional activators [24]. However, other studies suggest that SMYD family members can either activate or repress transcription by targeting unique histone residues [25]. SMYD1 plays a critical role in cardiac and skeletal muscle morphogenesis through its interaction with the tissue-specific transcriptional activator skNAC [26]. Targeted deletion of *Smyd1* or *skNAC* in mice results in right ventricular hypoplasia in both cases [26,27], and this defect is even more severe in mice with *Smyd1* knockout, which die *in utero* due to cardiac defects [28]. Interestingly, *Smyd1* ablation in the adult mouse heart leads to a global downregulation of oxidative phosphorylation (OXPHOS) proteins and a decrease in H3K4me3 levels at the promoter of the *Pgc1α* gene, an important mitochondrial energy regulator, resulting in a decrease in its expression. These observations led the authors to conclude that SMYD1 regulates cardiac energetics through transcriptional control of the *Pgc1α* gene [29].

The role of SMYD1 has never been described in the context of adipose tissue. To investigate whether SMYD1 is involved in the activation of mitochondrial function of adipose cells and tissues under conditions that require activation of adaptive thermogenesis, we used two complementary *in vivo* approaches: first, exposure of mice to cold and second, exposure of mice to a high-fat diet (HFD). Both conditions are known physiological situations that require activation of mitochondria to generate heat (in the case of cold exposure) or to burn excess calories (in a state of positive energy balance, due to 'diet-induced thermogenesis'). Our studies have shown that SMYD1 is activated in WAT and BAT under both conditions, together with the classical markers of thermogenesis, such as PGC1A and UCP1. We also performed *in vitro* experiments to differentiate adipocytes into the white and brown phenotype and observed overexpression of *Smyd1*. Furthermore, knockdown of *SMYD1* led to a decrease in H3K4me3 at the *Pgc1α* locus in adipocytes and a decrease in mitochondrial activity. Overall, these data may indicate an additional role of SMYD1 in the metabolic adaptation of WAT.

Materials & methods

Animal care & treatment

The 4-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Lecco, Italy) and housed at the common facility of the Federico II University of Naples. After an acclimatization period of 3 weeks, mice were fed a standard diet (StD) or HFD as previously described [30]. At 8 weeks, mice were sacrificed, and brown adipose tissue (BAT) and epididymal WAT (eWAT), subcutaneous white adipose tissue (scWAT) and inguinal WAT (ingWAT) were collected. All samples were stored at -80°C until further analysis. For the cold exposure experiments, 12-week-old male C57BL/6J mice were housed individually in cages at the animal facility of CEINGE, Biotecnologie Avanzate S.c.a.r.l., Naples, and kept at 4°C for 16 h as previously described [31]. As control, male C57BL/6J mice

at room temperature (23–25°C) were used. BAT and eWAT, scWAT and ingWAT were collected and stored at -80°C for further analysis.

Animals were handled according to national and European Community guidelines, and protocols were approved by the Animal Research Committee of the University of Naples 'Federico II' (MIUR, approval code: 354/2019-PR).

Cell cultures & treatments

Mouse 3T3-L1 (CL-173) and NIH/3T3 (CRL-1658) fibroblasts were purchased from the American Type Culture Collection (VA, USA). 3T3-L1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum, 2% L-glutamine and 1% penicillin/streptomycin solution (proliferation medium) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 (media, sera and antibiotics for cell culture were purchased from Lonza (Basel, Switzerland). Fibroblasts were subcultured every 3–4 days at approximately 90% confluence. For the induction of adipogenesis, mouse 3T3-L1 cells were grown in proliferation medium for 48 h and then induced to differentiate into white or beige adipocytes as previously described [32,33]. Briefly, white adipogenesis was induced in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin, 0.25 µM dexa, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 µg/ml insulin. For brown adipogenesis, 1 µM rosiglitazone and 50 nM T3 were also added to the medium. After 48 h, the dexa was removed from the medium and the cells were harvested after 6 and 12 days. Media, cell sera, antibiotics, IBMX, dexa and T3 were provided by Sigma-Aldrich (MO, USA), rosiglitazone by Enso Life Sciences (NY, USA) and insulin by Life Technologies (MA, USA). Mouse NIH/3T3 fibroblasts were grown in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

3T3-L1 transient transfection

Approximately 80,000 cells were plated out on six-well plates and transfected with 25.0 nM chemically synthesized siRNA against *Smyd1* (siSMYD1) and scrambled (control) siRNA (siCTR) using TransIT-X2® after reaching 80–90% confluence, and then induced to differentiate into mature adipocytes. Transfection was performed every 48 h until day 12. The efficiency of *Smyd1* silencing was confirmed by RT-PCR and western blotting assay.

The siRNA for the *Smyd1* gene and the scrambled control were synthesized by Ambion (MA, USA). TransIT-X2 was purchased from Mirus Bio (WI, USA).

NIH/3T3 stable transfection

Stable expression of the *Smyd1*-overexpressing vector and control vector in NIH/3T3 cells was achieved as previously reported [34,35]. Briefly, 140,000 NIH/3T3 cells were placed in six-well plates and transfected with Lipofectamine™ 3000 (Invitrogen, CA, USA) with 2.0 µg *Smyd1*-containing plasmid (pCMV6Entry/SMYD1) or empty vector (pCMV6/Entry), both provided by OriGene (MD, USA). Cells were maintained in 10% FBS DMEM containing 0.7 µg/µl geneticin (G418 sulfate; Applied Biological Materials, Inc., WA, USA), and after 3 weeks, geneticin-resistant stable clones were picked up and transferred separately to dishes containing 0.4 µg/µl G418. Several NIH/3T3 clones transfected with either the *Smyd1*-containing vector or the empty vector were analyzed by RT-PCR to confirm the efficiency of transfection.

Oil Red O staining

3T3-L1 mature adipocytes were fixed with 4% paraformaldehyde for 15 min and then washed with 1× phosphate-buffered saline. Oil Red O (Sigma-Aldrich) staining was performed for 30 min at room temperature. After removal of excess stain, cells were photographed under a microscope at 40× magnification. To measure the accumulation of cellular lipid droplets, Oil Red O was dissolved in 100% isopropanol and the absorbance was measured at 540 nm using the Victor™ X4 (Perkin Elmer, CT, USA).

RNA isolation & qRT-PCR analysis

Total RNA was extracted from cells and tissues using TRIzol® (Invitrogen) according to the manufacturer's protocols, as previously described [36] cDNA was obtained by retrotranscription of 1.0 µg RNA using the LunaScript® RT SuperMix Kit (New England Biolabs, MA, USA) according to the manufacturer's instructions. RT-PCR was performed in triplicate using the Luna® Universal qPCR Master Mix (New England Biolabs) on a StepOnePlus Real-Time PCR System (Applied Biosystems, MA, USA). Quantification of gene expression was compared with

Table 1. List of primers used for quantitative real-time polymerase chain reaction analysis.

Gene	Forward sequence	Reverse sequence
ARBP	5′-CCGTGATGCCCAGGGAAGACAG-3′	5′-CCAACAGCATATCCCGAATCTCAG-3′
PPIA	5′-GCAGACAAAGTTCCAAAGACAG-3′	5′-CACCCTGGCACATGAATCC-3′
CyA	5′-CGCCACTGTCGCTTTTCG-3′	5′-AACTTTGTCTGCAAACAGCTC-3′
SMYD1	5′-GTGTGATCAACTGCAACGG-3′	5′-GCTCAATCCTCATCTGCGTG-3′
PGC1-α	5′-ATGTGCAGCCAAGACTCTGTA-3′	5′-CGCTACACCACTTCAATCCAC-3′
UCP1	5′-ACAGAGCTGGTAACATATGACCTC-3′	5′-CCGGCAACAAGAGCTGACAGTAAA-3′
C2TA	5′-GAAGTTCACCATTGAGCCATTT-3′	5′-GATCCTTGGCTGCATCTTCT-3′
MYST4	5′-TGATATTGGGCCACATGGAAA-3′	5′-TCCTCAGAAACCACAGCATTAG-3′
MT-ND5	5′-CAGCACAATTTGGCCTCCAC-3′	5′-TAGTCGTGAGGGGGTGGAAT-3′
ATP5O	5′-TCTCGACAGGTTCGGAGCTT-3′	5′-AGAGTACAGGGCGGTTGCATA-3′
MT-CO2	5′-AACCGAGTCCTTCTGCCAAT-3′	5′-CTAGGGAGGGGAACTGCTCAT-3′
UQCRC1	5′-AGACCCAGGTCAGCATCTTG-3′	5′-GCCGATTCTTTGTTCCCTTGA-3′
PPARγ	5′-CAGTGGAGACCGCCCAGGCT-3′	5′-TGGAGCAGGGGGTGAAGGCT-3′
cEBPα	5′-GGCTCGCCATGCCGGGAGAA-3′	5′-CCGGGTCGATGTAGGCGCTG-3′
-1 kb	5′-CAATGAGGGGTAATGCAGGT-3′	5′-GCCTGGAAGGGTTAAGTCTG-3′
-0.1 kb	5′-GACGCCAGTCAAGCTTTTTC-3′	5′-GACGTCAGGAGTTTGTGCAG-3′
+1 kb	5′-GGGTCCATCTCACCAGAGTC-3′	5′-ACACAAGCAGTTTCCCCGTA-3′
+2 kb	5′-ATGGAAGCTGCCCACACTAC-3′	5′-TCAGCGTCCAGCCTTAGATT-3′
+3 kb	5′-GTAACAATCCCAAGGCTCCA-3′	5′-TCTCCCCTCATCTCTGTGCT-3′

controls (equal to 1) and calculated using the comparative 2-ΔΔCt method based on cycle thresholds of target and housekeeping genes [37]. *Arbp*, *PpiA* and *CyA* were used as internal controls for 3T3-L1 cells, NIH/3T3 cells and mouse tissues. Table 1 contains the specific sequences of the primers used.

PCR array profiling for chromatin-modifying enzymes

Expression of epigenetic modifying enzymes in scWAT of HFD mice was examined using the RT^2 Profiler PCR array for the mouse epigenetic chromatin modification enzymes (PAMM-085Z, Qiagen, MD, USA). PCR data were analyzed using the web-based tool provided by the manufacturer (https://dataanalysis2.qiagen.com/pcr). Differentially expressed chromatin-modifying enzymes in scWAT of HFD mice were identified by a >2.5-fold change (FC) compared with StD mice. Statistical significance was determined by Student's *t*-test. A p-value < 0.05 was considered significant. The results obtained were validated by RT-PCR using primers different from those used in the array (Table 1) and normalized to *Arbp*.

In silico promoter analysis for searching CREB transcription factor binding sites

Analysis of CREB motifs in the murine *Smyd1* promoter region was performed as previously described [38]. Briefly, with the help of TFBIND (http:/tfbind.hgc.jp/), consensus CREB binding sites (ID V$CREB_01, AC M00039; ID V$CREB_02, AC M00113; ID V$CREB_Q2, AC M00177; ID V$CREB_Q4, AC M00178) with matrix similarity scores of 0.75 or higher (maximum 1.00) were identified in the upstream region of the murine *Smyd1* gene promoter. The positional analyses of all identified consensus CREB binding sites are shown in Supplementary Figure 1. The analysis was also performed using LASAGNA-Search 2.0, searching for transcription factor binding sites (https://biogrid-lasagna.engr.uconn.edu/lasagna_search/index.php), in which TRANSFAC, JASPAR, UniPROBE and ORegAnno matrices are included [38].

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as previously described [39]. Briefly, 3T3-L1 adipocytes in which the *Smyd1* gene was silenced out were crosslinked with 1% formaldehyde for 10 min at 37°C. After centrifugation, lysates were disrupted and sheared chromatin samples were used as input controls or immunoprecipitated overnight at 4°C with 3.0 μg anti-H3K4me3 (#39915; Active Motif, CA, USA) and normal rabbit IgG as negative controls. DNA fragments were recovered and analyzed by RT-PCR using a primer set that amplifies *Pgc1α* promoter regions from -1.0 to +3.0 Kb [29].

Protein extraction & immunoblotting

Cells were lysed for 30 min at 4°C in NP40 Lysis Buffer (Invitrogen) supplemented with 200.0 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (G135, Applied Biological Materials, Inc.). After centrifugation, the supernatant fraction was collected and quantified using a bicinchoninic acid protein assay kit (Thermo Scientific, MA, USA). Protein extracts were denatured by boiling in Laemmli sample buffer (CA, USA) containing 10% β-mercaptoethanol. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GVS Life Sciences, Milan, Italy). Filters were blocked for 1 h in TBS-Tween®/5% bovine serum albumin or non-fat dried milk, and then probed overnight at 4°C with the following primary antibodies for SMYD1 (ab32482, Abcam (Cambridge, UK); dilution 1:500), PGC1-α (ab54481, Abcam; dilution 1:1,000) and total OXPHOS rodent WB antibody cocktail (ab110413, Abcam; dilution 1:1,000). Anti-α-tubulin (sc-5546, Santa Cruz Biotechnology [CA, USA]; dilution 1:10,000), anti-histone H3 (#9715, Cell Signaling Technology, MA, USA; dilution 1:1,000) and anti-β-actin (D6A8) (#8457, Cell Signaling Technology; dilution 1:1,000) were used as loading controls for cells and tissues, respectively. Membranes were then hybridized for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit (#1706515) or anti-mouse (#1706516; both BioRad, CA, USA) secondary antibody (dilutions 1:3,000). Western blots were performed in triplicate, and densitometric analysis was performed using ImageJ software (NIH, MD, USA).

MitoTracker® staining & flow cytometry analysis

3T3-L1 adipocytes transiently transfected with either siSMYD1 or siCTR were stained for 30 min at 37°C with 200 nmol/l of a red fluorescent dye, MitoTracker Red FM (#M7512, Invitrogen), which passively diffuses across the plasma membrane and accumulates in active mitochondria. After centrifugation, the pellet was resuspended in serum-free DMEM because the probe is sensitive to potential oxidases in serum, and mitochondrial content was measured by FACS (BD FACSCanto™ flow cytometer, CA, USA). Results were analyzed using BD FACS Diva software (Duke University Flow Cytometry Shared Facility) and expressed as mean fluorescence intensity (%) of siSMYD1 cells compared with controls.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v. 9.4; GraphPad Software, Inc., CA, USA). All data are expressed as mean ± standard deviation. Relative mRNA and protein expression levels were compared using one-sample *t*-test with controls set at 100. Differences with a p-value < 0.05 were considered statistically significant.

Results

HFD alters gene expression profiles of epigenetic chromatin modification enzymes in WAT

To investigate the effects of HFD on chromatin changes, we compared the expression of epigenetic chromatin modification enzymes in adipose tissue of mice fed either HFD or StD. Figure 1A shows a schematic representation of our experimental setup. Analysis of RT^2 PCR array data, shown in scatter plots (Figure 1B), revealed upregulation of several genes encoding epigenetic chromatin modification enzymes after HFD. In particular, three genes – *C2ta*, *Myst4* and the histone methyltransferase *Smyd1* – were significantly upregulated in the scWAT of HFD mice compared with StD mice (*C2ta* FC = 4.7; *Myst4* FC = 2.7; *Smyd1* FC = 4.3) (Figure 1B). Subsequent expression analysis of scWAT from mice fed HFD for 8 weeks confirmed upregulation of only the *Smyd1* and *C2ta* genes (Figure 1C). No significant differences in the expression of these genes in skeletal muscle and liver were detected between HFD and StD mice (Figure 1D & E), suggesting that the effect of HFD on the expression of these genes is specific to scWAT. Western blotting analysis confirmed that SMYD1 increased in scWAT from mice fed HFD for 8 weeks (Figure 1F).

To further confirm these data, SMYD1 expression was also examined in BAT and various WATs from mice receiving HFD and StD for 8 weeks. As shown in Figure 2, SMYD1 expression was significantly increased at both mRNA and protein levels in the BAT (Figure 2A & B), scWAT (Figure 2C & D) and ingWAT (Figure 2E & F) of HFD-fed mice compared with StD-fed mice, while no changes were detected in the eWAT (Figure 2G & H). These results suggest that caloric intake controls the regulation of SMYD1 expression in BAT and subcutaneous ingWAT, which is characterized by a high propensity to transition to a BAT-like phenotype [10].

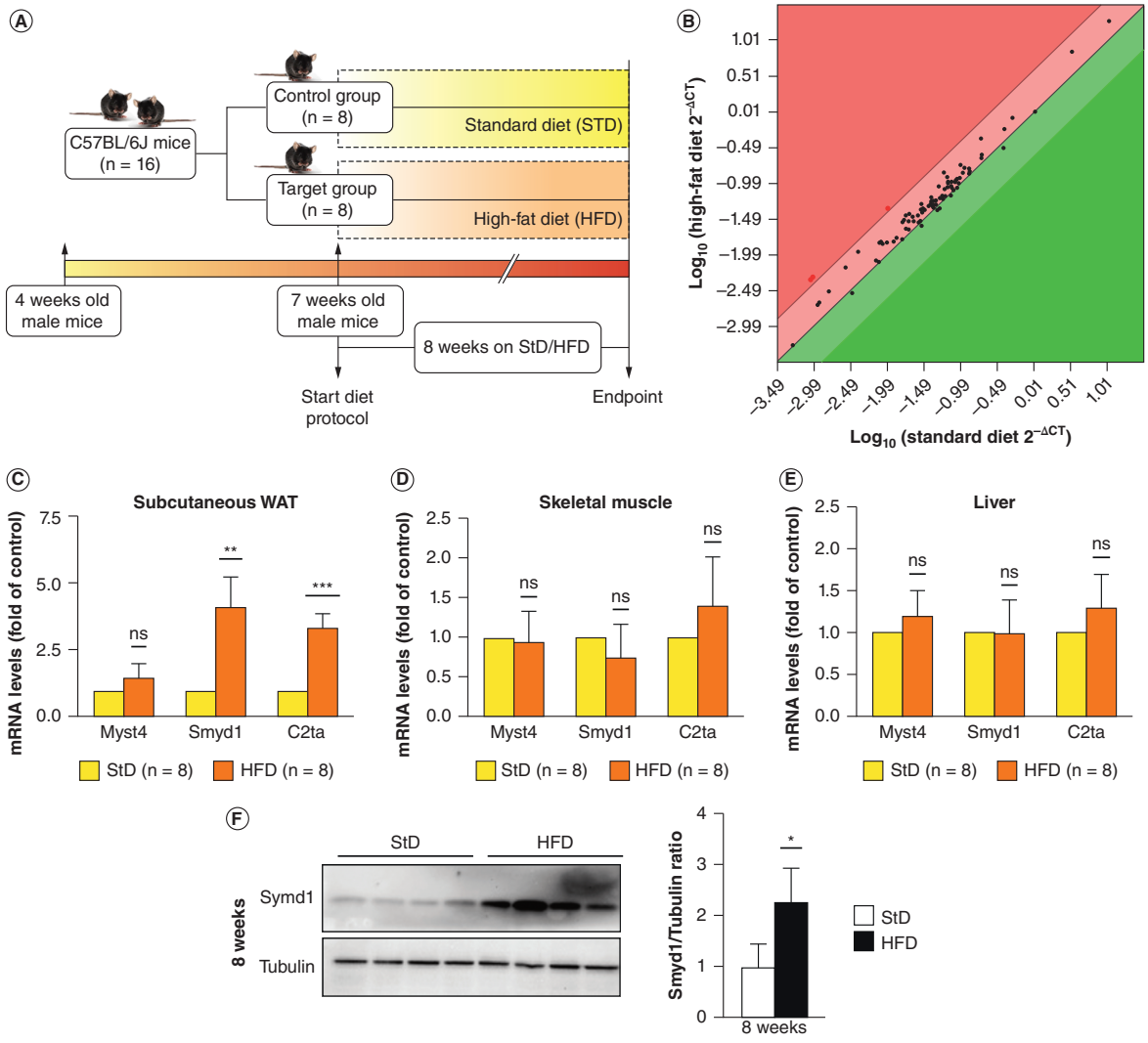

Figure 1. High-fat diet modulates the expression of epigenetic regulatory enzymes. (A) Protocol of the experiment. After a 3-week acclimatization period, mice were fed either STD (eight mice) or HFD (eight mice). After 8 weeks, animals were sacrificed for tissue sampling. **(B)** Expression of epigenetic enzymes assessed by RT^2-PCR array profiling. The scatter plot shows the normalized expression of genes from the subcutaneous WAT of HFD mice compared with the control group. The center line indicates unchanged gene expression, while the outer diagonal lines indicate the established thresholds (FC = 2.5). The red dots correspond to the three genes that were upregulated more than the thresholds: *Myst4* (FC = 2.7), *Smyd1* (FC = 4.3) and *C2ta* (FC = 4.7). **(C–E)** Validation by RT-PCR of *Myst4*, *Smyd1* and *C2ta* in **(C)** subcutaneous WAT, **(D)** skeletal muscle and **(E)** liver of mice fed either StD (yellow bars) or HFD (orange bars). mRNA levels were normalized to *Arbp* mRNA. Data are expressed as a fold increase over the corresponding tissue from StD mice. **(F)** Western blot of SMYD1 protein in subcutaneous WAT from 8-week HFD and StD mice. Tubulin was used as a loading control. Gels were quantified using ImageJ software, and the relative intensity of the bands, normalized for tubulin, is shown as a fold increase compared with controls (StD mice). Mean values ± standard deviations were calculated from eight mice/group *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
FC: Fold change; HFD: High-fat diet; STD: Standard diet; WAT: White adipose tissue.

Cold exposure increases SMYD1 expression in murine WAT & BAT

To investigate the possible involvement of SMYD1 in the thermogenic adaptation of WAT, C57BL/6J mice were exposed to cold temperature (4°C) for 16 h. Control mice were maintained at room temperature (Figure 3A). At the end of the experiments, the mice were sacrificed and BAT, eWAT, ingWAT and scWAT were collected (Figure 3B). SMYD1 expression was significantly increased at both the mRNA (left) and protein (right) levels in BAT (Figure 3C & D), eWAT (Figure 3E & F), ingWAT (Figure 3G & H) and scWAT (Figure 3I & J) of the cold-exposed mice compared with controls. The expression of *Ucp1* and *Pgc1α* genes was also examined in these

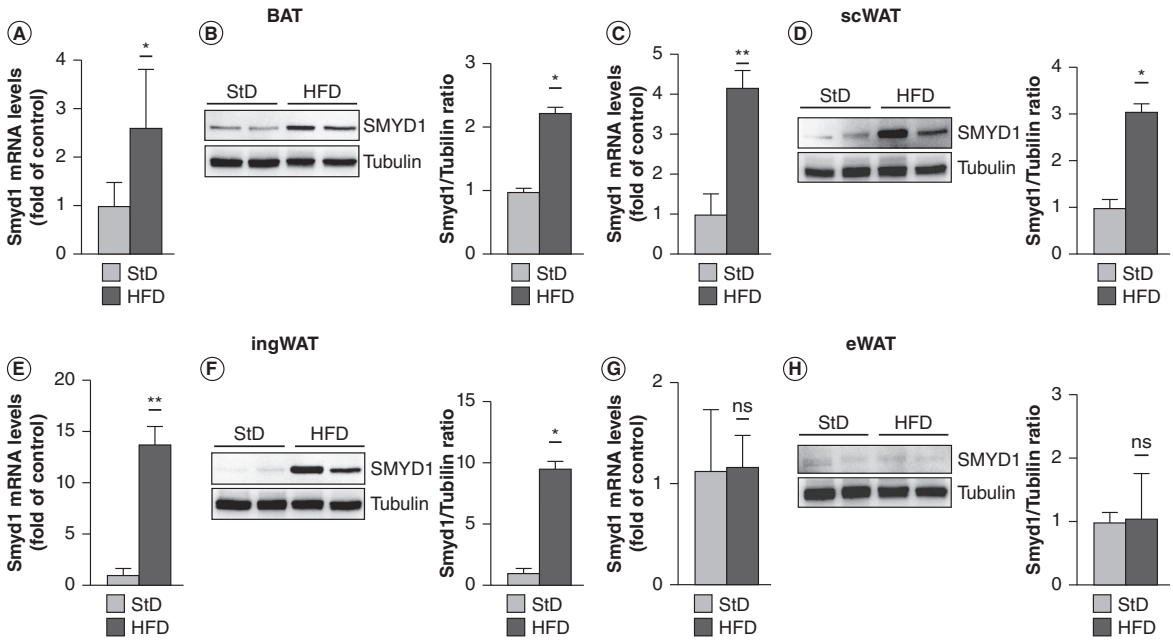

Figure 2. SMYD1 expression in brown and white adipose tissue of mice fed a high-fat diet. (A, C, E & G) The mRNA levels of *Smyd1* were analyzed by RT-PCR in **(A)** BAT, **(C)** scWAT, **(E)** ingWAT and **(G)** eWAT from mice fed HFD and StD for 8 weeks. Expression was normalized using *Cyclophilin-A* mRNA. The levels of the indicated genes are relative to StD-fed mice. **(B, D, F & H)** Western blot analysis of SMYD1 protein from HFD and StD mice in **(B)** BAT, **(D)** scWAT, **(F)** ingWAT and **(H)** eWAT. The images shown are representative of different experiments with very similar results. The histograms on the right show the relative quantification of SMYD1 protein compared with the corresponding adipose tissues from StD-fed mice. Tubulin was used as a loading control. Mean values ± standard deviations were calculated from data from eight separate animals. Statistical analyses were performed using the one-sample *t*-test compared with StD mice. *$p < 0.05$; **$p < 0.01$.
BAT: Brown adipose tissue; eWAT: Epididymal white adipose tissue; HFD: High-fat diet; ingWAT: Inguinal white adipose tissue; scWAT: Subcutaneous white adipose tissue; StD: Standard diet.

tissues as positive controls and showed increased expression after cold exposure, as expected (Figure 3C, E, G & I). These results indicate that SMYD1 levels increased in both BAT and WAT after cold exposure and followed a similar trend to the other major mitochondrial genes (*Ucp1* and *Pgc1α*). This suggests that SMYD1 might play a role in mitochondrial biogenesis and cold-induced browning.

SMYD1 expression is higher in beige adipocytes than in white adipocytes

Because SMYD1 changes in adipose tissue in response to thermogenic stimuli such as food intake and cold exposure, we compared its expression between beige (thermogenic) and white (storage) adipocytes. For this purpose, we used 3T3-L1 cells, an established cellular model of preadipocytes, which were induced to differentiate into white or beige adipocytes as described in the Methods section. As expected, differentiation of 3T3-L1 into beige adipocytes resulted in less accumulation of lipid droplets compared with white adipocytes, as shown by staining with Oil Red O (Figure 4A). SMYD1 expression increased during 3T3-L1 differentiation at both the mRNA (Figure 4B & C, left) and protein (Figure 4B & C, right) levels, with higher expression in beige compared with white 3T3-L1 adipocytes at days 6 and 12 of the differentiation period ($p < 0.05$). As expected, mRNA expression of *Ucp1* and *Pgc1α*, typical mitochondrial genes, also increased more during differentiation to beige adipocytes (Figure 4D) compared with white adipocytes (Figure 4E). Thus, SMYD1 shows a similar trend of expression as these two important regulators of mitochondrial biogenesis.

SMYD1 modulates the expression of *Pgc1α in vitro*

As previously reported, SMYD1 regulates mitochondrial metabolism in the heart through transcriptional control of *Pgc1α* [29]. Therefore, we investigated whether SMYD1 also modulates the expression of this key transcriptional metabolic regulator in adipose cells. To this end, 3T3-L1 adipocytes were transfected with siRNA targeting *Smyd1* (siSMYD1) or with a nonspecific siRNA (siCTR) and subsequently induced to differentiate into mature brown-like

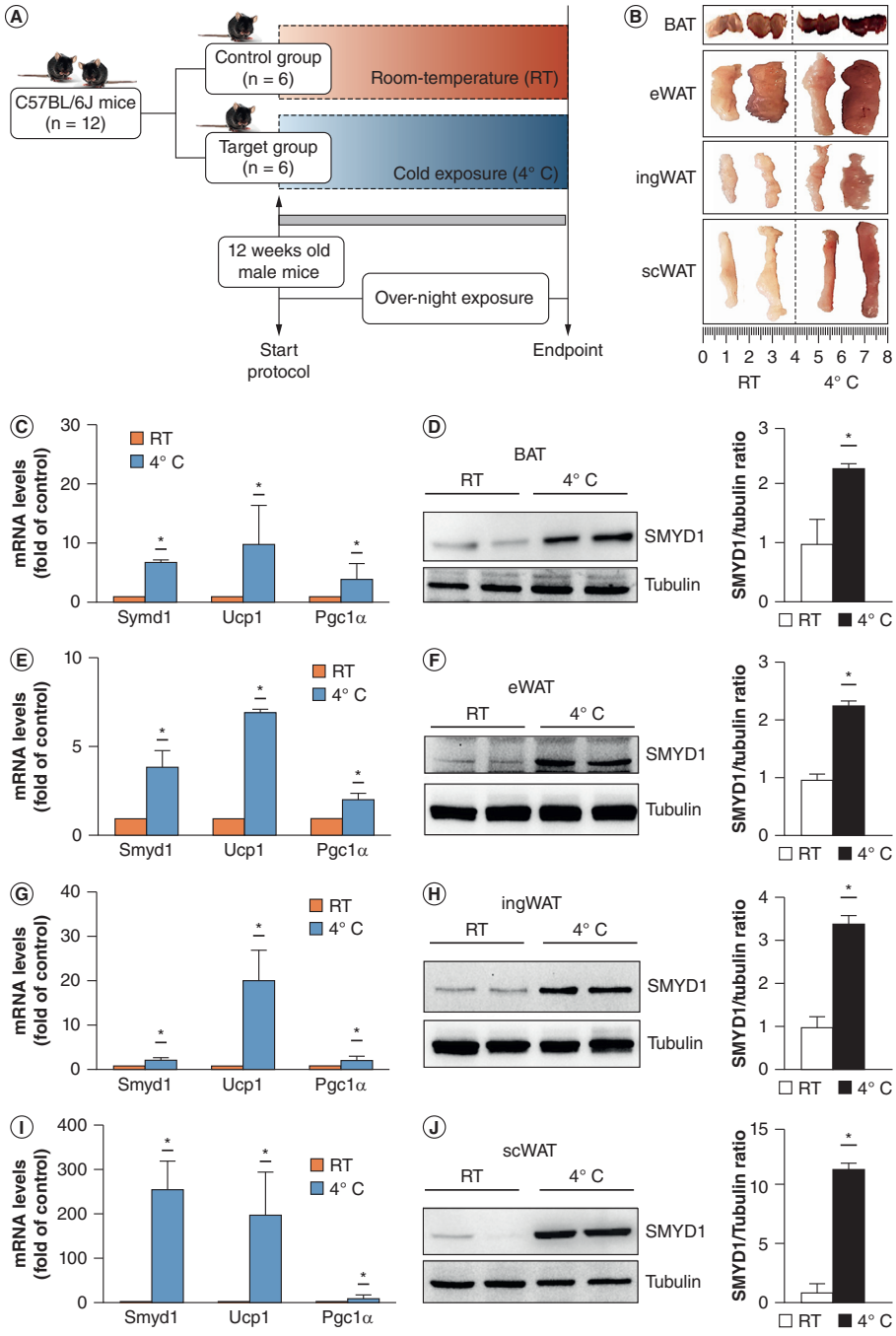

Figure 3. SMYD1 expression in cold-exposed mice. (A) Protocol of cold exposure experiments. 12 mice were randomly divided into two groups and exposed to 4°C (six mice) or RT (six mice) and sacrificed overnight (~16 h) after exposure. **(B)** Representative images of BAT, eWAT, ingWAT and scWAT from cold-exposed (4°C) and RT-exposed mice. **(C, E, G & I)** The mRNA levels of *Smyd1*, *Ucp1* and *Pgc1α* were analyzed by RT-PCR in **(C)** BAT, **(E)** eWAT, **(G)** ingWAT and **(I)** scWAT from cold-exposed mice compared with RT mice. Expression was normalized with *Cyclophilin-A* mRNA. The values of the indicated genes are shown as a fold increase compared with RT mice. **(D, F, H & J)** Western blot analysis of SMYD1 protein from cold-exposed and RT mice in **(D)** BAT, **(F)** eWAT, **(H)** ingWAT and **(J)** scWAT. The images shown are representative of different experiments with very similar results. The histograms on the right show the relative quantification of the indicated proteins compared with RT mice. Tubulin was used as loading control. Mean values ± standard deviations were calculated from data from six separate animals. Statistical analyses were performed using the one-sample *t*-test compared with RT mice.
*$p < 0.05$.
BAT: Brown adipose tissue; eWAT: Epididymal white adipose tissue; Inguinal white adipose tissue; RT: Room temperature; scWAT: Subcutaneous white adipose tissue.

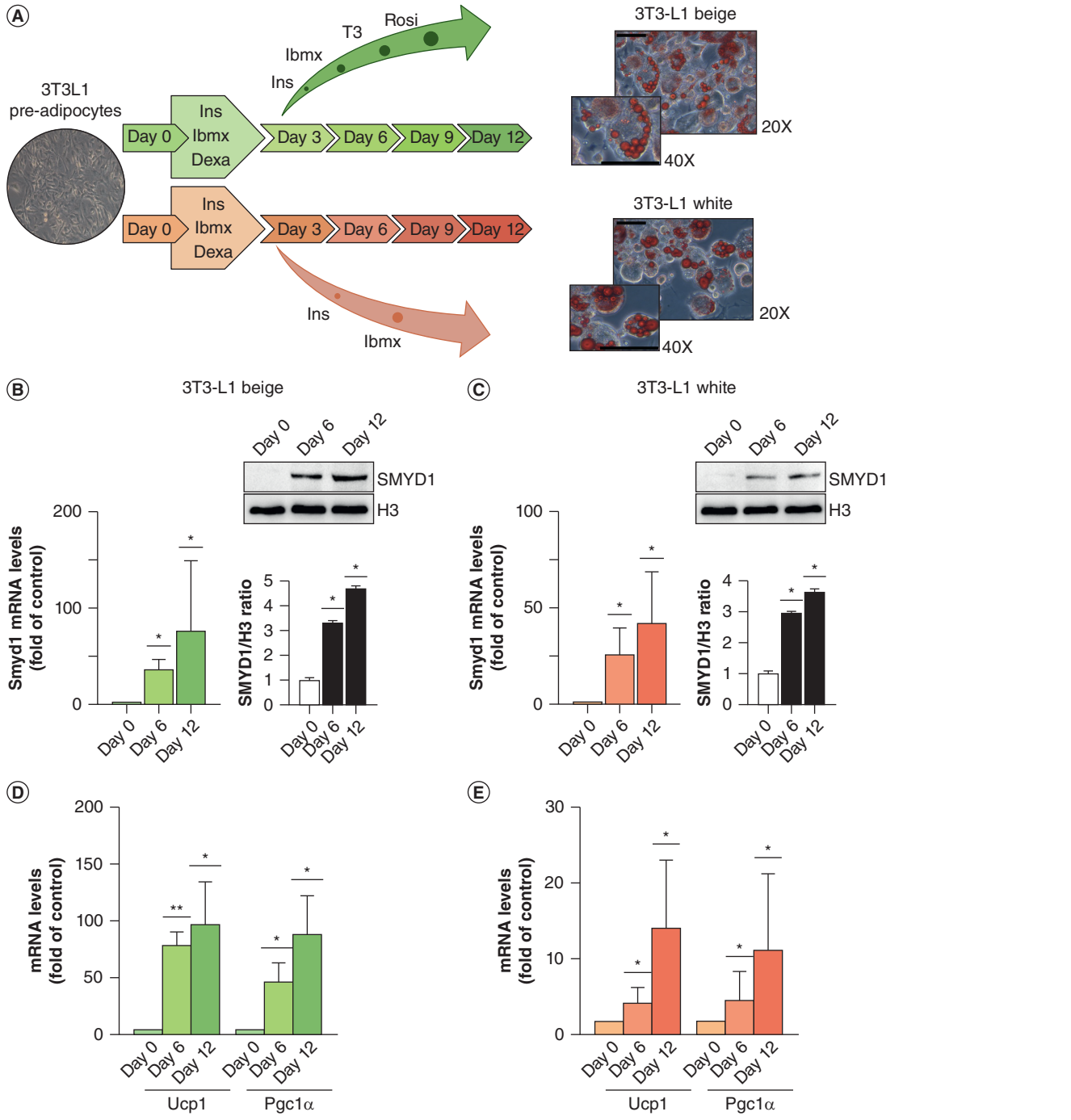

Figure 4. SMYD1 expression in 3T3-L1 cells differentiated into mature white and beige adipocytes. (A) Differentiation protocol of 3T3-L1 preadipocytes into mature white and beige adipocytes. Methodological details are provided in the Materials and methods section. Scale bars 50 μm, magnification 20×, zoom magnification 40×. **(B–E)** mRNA levels of *Smyd1*, *Ucp1* and *Pgc1α*: **(B & C, left)** *Smyd1* and **(D & E)** *Ucp1* and *Pgc1α* mRNA levels during differentiation of 3T3-L1 adipocytes into mature beige and white adipocytes. The mRNA levels were measured by quantitative RT-PCR, normalized for *Arbp*, and are presented as a fold increase compared with undifferentiated cells (day 0). **(B & C, right)** SMYD1 protein levels during differentiation in mature beige and white 3T3-L1 adipocytes. The blots shown are representative of three independent experiments with very similar results. Gels were quantified using ImageJ software, and the relative intensity of the band normalized for histone H3 is shown as a fold increase compared with undifferentiated cells. Mean values ± standard deviations were calculated from at least three independent experiments, each performed in triplicate. All results are expressed in comparison to day 0 with a one-sample *t*-test.
*$p < 0.05$; **$p < 0.01$.

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adipocytes. The Oil Red O experiment showed a lower accumulation of lipid droplets in *Smyd1*-silenced 3T3-L1 cells compared with control cells ($p < 0.05$) (Figure 5A). The analysis of mRNA expression levels revealed that transfection of 3T3-L1 cells with siSMYD1 reduced *Smyd1* expression to approximately 40% ($p < 0.05$) and 60% ($p < 0.01$) at day 6 and 12 of differentiation, respectively (Figure 5B, top left). *Pgc1α* mRNA levels were significantly reduced in *Smyd1*-silenced cells at both day 6 and day 12 of differentiation ($p < 0.05$) (Figure 5B, top right). In parallel, the expression of *Pparγ* and *cEBPα* genes, the two master regulators of adipogenesis, also decreased in these cells ($p < 0.01$ and $p < 0.05$, respectively) (Figure 5B, bottom left and right).

In addition, immunoblotting of cell lysates on day 6 of differentiation showed that both SMYD1 and PGC1-α protein levels decreased in *Smyd1*-silenced adipocytes (Figure 5C). Taken together, these results confirm that the expression of the master regulator of mitochondrial energetics, the *Pgc1α* gene, is modulated by SMYD1 protein in adipocytes, similar to cardiomyocytes.

To investigate whether the H3K4me3 profile of the *Pgc1α* gene promoter is altered after silencing *Smyd1* in adipocytes, we performed chromatin immunoprecipitation of *Smyd1*-silenced 3T3-L1 and control cells with an H3K4me3-specific antibody (Figure 5D). The results showed that the enrichment of H3K4me3 within the *Pgc1α* locus was significantly reduced in *Smyd1*-silenced 3T3-L1 cells compared with control cells ($p < 0.01$). We also analyzed H3K4me3 status at the *Pgc1α* promoter in white 3T3-L1 adipocytes and beige 3T3-L1 adipocytes and found a higher enrichment of H3K4me3 in beige compared with white 3T3-L1 adipocytes (Figure 5E). This suggests that SMYD1 regulates *Pgc1α* gene expression in adipocytes by modulating chromatin architecture, with a stronger effect in brown adipocytes.

To investigate the effects of SMYD1-dependent regulation of *Pgc1α* expression on the OXPHOS pathway, we analyzed the expression of genes encoding subunits of the electron transport chain when *Smyd1*-silenced 3T3-L1 cells were compared with siCTR cells. *Smyd1* silencing significantly reduced the mRNA and protein levels of OXPHOS complexes (Figure 5F & G).

To further investigate the direct role of SMYD1 on *Pgc1α* expression, a *Smyd1* expression vector (pCMV6/SMYD1) was stably transfected into NIH/3T3 cells. Figure 6A shows that overexpression of *Smyd1* resulted in activation of the *Pgc1α* gene compared with cells transfected with the empty vector (pCMV6) ($p < 0.05$).

To confirm that SMYD1 regulates energetics in adipocytes, we examined the effect of *Smyd1* silencing on differentiated cells using MitoTracker dye. Remarkably, differentiated, *Smyd1*-silenced 3T3-L1 cells showed a significant reduction in mitochondrial activity at days 3 and 5 of differentiation compared with cells transfected with the empty vector as control (Figure 6B). These data provide strong evidence supporting the role of SMYD1 as a positive regulator of mitochondrial metabolism in adipocytes.

Discussion

The SMYD family consists of five lysine methyltransferase proteins named for the presence of two highly conserved domains: the catalytic Su(var)3–9, Enhancer-of-zeste and Trithorax (SET), which is split by a Myeloid-Nervy-DEAF1 (MYND) domain. These proteins are characterized by their ability to methylate histone H3 at lysine 4, and SMYD1 represents their founding member. SMYD1 has been shown to function as a histone deacetylase-dependent transcriptional repressor that associates with HDAC1–3, NCoR and SMART [26] and can be inhibited by trichostatin A, a potent histone deacetylase inhibitor [28].

SMYD1 plays a critical role in cardiac and skeletal muscle development, homeostasis and pathology [25,28,29]. Recent findings have identified SMYD1 as a novel regulator of OXPHOS proteins in the heart [29]. In eukaryotes, OXPHOS occurs in mitochondria, which are the major functional organelles that distinguish brown or beige from white adipocytes. While the role of SMYD1 in mitochondrial bioenergetics in the heart is well understood, its functions in adipose tissue remain unexplored.

The present study highlights three important findings. First, in adipose tissue SMYD1 expression is regulated by thermogenic stimuli such as diet or cold temperature (Figures 1 & 2). Second, SMYD1 expression increases markedly during adipocyte differentiation and was higher in mature beige adipocytes than in white adipocytes (Figure 3). Third, silencing of *Smyd1* in 3T3-L1 cells decreased trimethylation of histone H3 on lysine 4 on the *Pgc1α* locus, resulting in a reduction in the expression of this gene. These data confirm that SMYD1, as in cardiomyocytes, regulates *Pgc1α* chromatin status [29]. In addition, *Smyd1*-silenced 3T3-L1 cells show a significant reduction in mitochondrial activity (Figure 4), highlighting the role of SMYD1 in mitochondrial biogenesis in adipocytes. Interestingly, silencing *Smyd1* leads to a downregulation of *Pgc1α* that persists until day 12 of differentiation, whereas the downstream effect on mitochondrial function is only observed until day 7. One

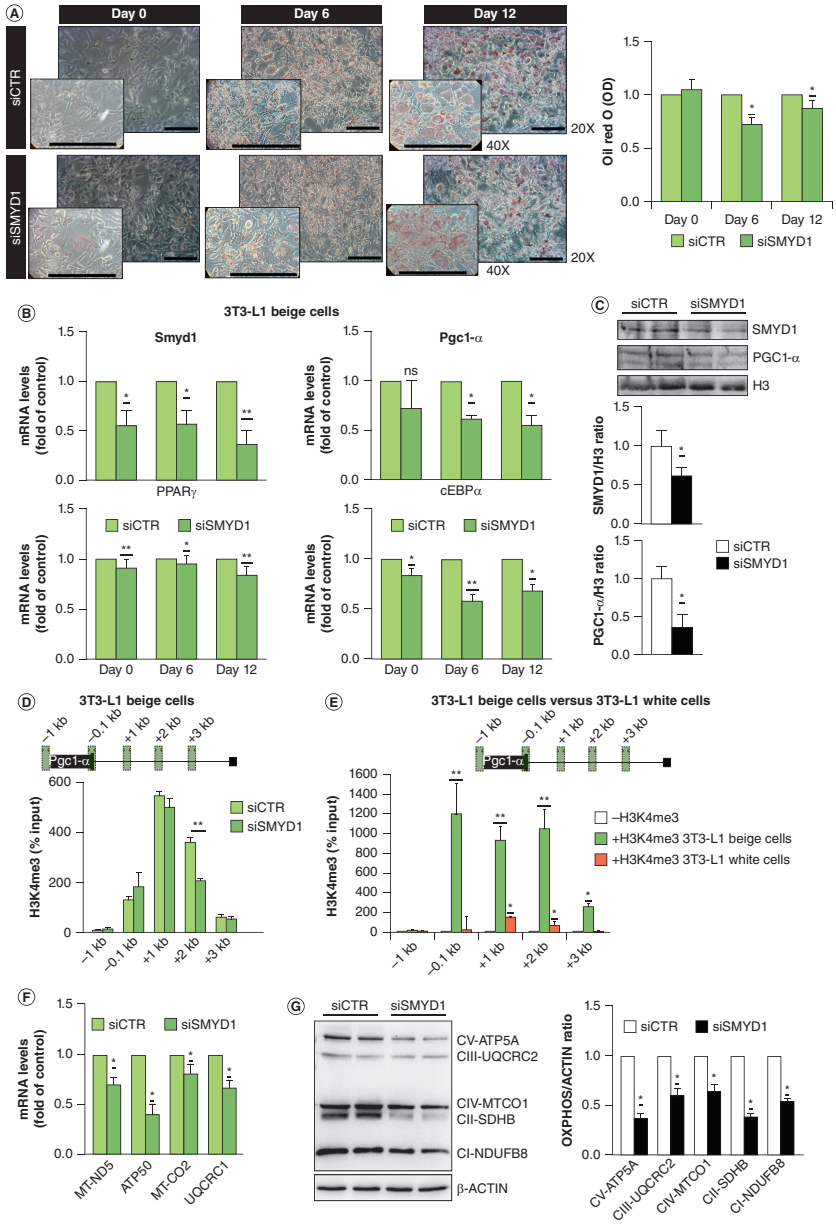

Figure 5. Regulatory effects of SMYD1 on the transcriptional activity of the *Pgc1α* gene in 3T3-L1 cells. (A) Intracellular lipids were stained with Oil Red O and quantified. The microphotographs were obtained with an optical microscope following staining. Scale bars 50 μm, magnification 20×, zoom magnification 40×. Lipid quantification test was expressed as optical density and reported as FC compared with control cells (siCTR). **(B)** mRNA expression of *Smyd1*, *Pgc1α*, *Pparγ* and *cEBPα* from 3T3-L1 cells transfected with either scrambled siRNA (siCTR) or *Smyd1*-specific siRNA (siSMYD1) during differentiation at the indicated time points. The mRNA levels were normalized for *Arbp* and expressed as a FC compared with control cells (siCTR). **(C)** Western blot analysis of SMYD1 and PGC1-α in 3T3-L1 cells transfected with siSMYD1 compared with siCTR cells at day 6 of differentiation. Histone H3 antibody was used as a loading control. Gels were quantified using ImageJ software, and the relative intensity of the band normalized for histone H3 is shown in the histograms. **(D)** Chromatin immunoprecipitation assays were used to measure H3K4me3 in the promoter regions from -1.0 to +3.0 Kb of the transcription start site of the *Pgc1α* gene in siSMYD1-3T3-L1 cells compared with siCTR cells at day 6 of differentiation. **(E)** Chromatin immunoprecipitation assays were used to measure H3K4me3 in the promoter regions from -1.0 Kb to +3.0 Kb of the transcription start site of the *Pgc1α* gene in beige 3T3-L1 adipocytes (green bars) and white 3T3-L1 adipocytes (orange bars) at day 6 of differentiation. The promoter region studied is shown schematically on the top of the panel. The results are expressed as a percentage (%) of enrichment relative to input DNA and are corrected for anti-IgG levels. **(F)** mRNA expression of a panel of OXPHOS genes from 3T3-L1 cells transfected with either scrambled siRNA (siCTR) or *Smyd1*-specific siRNA (siSMYD1) during differentiation at the indicated time points. The mRNA levels were normalized for *CyA* and expressed as a FC compared with control cells (siCTR). **(G)** Western blot analysis of OXPHOS complexes in 3T3-L1 cells transfected with siSMYD1 compared with siCTR cells at day 6 of differentiation. Histone H3 antibody was used as a loading control. Gels were quantified using ImageJ software, and the relative intensity of the band normalized for histone H3 is shown in the histograms.

FC: Fold change; OXPHOS: Oxidative phosphorylation.

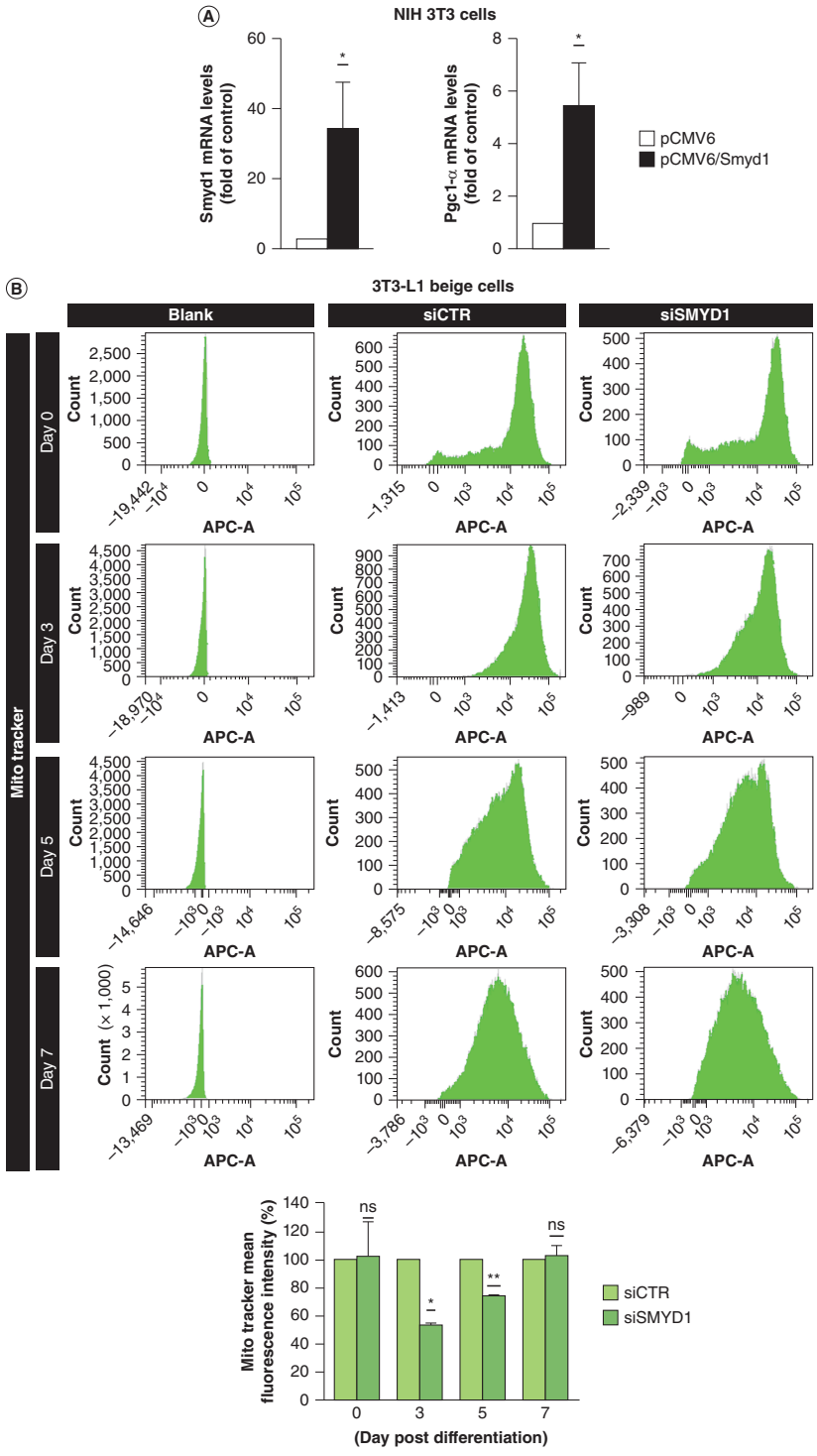

Figure 6. Role of SMYD1 in the regulation of mitochondrial activity in adipocytes. (A) NIH 3T3 cells were stably transfected with the pCMV6/SMYD1 expression vector. The empty pCMV6 vector was used as a control. The mRNA levels of *Smyd1* and *Pgc1α* were quantified by RT-PCR. The mRNA levels were normalized for *Ppia* and expressed as a fold increase compared with pCMV6-transfected cells. **(B)** 3T3-L1 beige cells transiently transfected with either scrambled siRNA or *Smyd1*-specific siRNA were stained with MitoTracker® Red FM at days 0, 3, 5 and 7. Cells were collected, and mitochondrial content was measured by fluorescence-activated cell sorting in *Smyd1*-silenced and control cells. The bar graphs on the right show the relative mean fluorescence intensity of siSMYD1 cells compared with controls. Results are given as means ± standard deviations of at least three separate experiments. Statistical analyses were performed using one-sample *t*-test.
*$p < 0.05$; **$p < 0.01$.

possible explanation for this discrepancy is the assumption that the reactivation of mitochondrial function is a physiological response of cells that utilize alternative methods to maintain mitochondrial respiration (e.g., reduction of mitochondrial turnover, reduction of mitophagy or potentiation of mitochondrial activity independent of the number of mitochondria).

As previously reported, methylation of H3K4 is the characteristic feature of SMYD1 protein, and histone methylation is considered to be an important epigenetic mark involved in the regulation of gene transcription and repression. Some methyl marks, such as H3K4, H3K36 and H3K79, are associated with gene activation, while others, such as H3K9, H3K27 and H4K20, typically mark gene repression [40]. The enzymes responsible for regulating these methyl marks in the development of BAT and in the thermogenic gene program are only now being identified. One example is the two histone methyltransferases MLL3/4 and EHMT1, both of which play a role in regulating thermogenesis [23]. EHMT1 is highly enriched in BAT, and its loss in adipose tissue of mice resulted in obesity and systemic insulin resistance associated with impaired adaptive thermogenesis. EHMT1 is part of the PRDM16 complex involved in BAT cell fate [23]. PRDM16 is a transcriptional coregulator that controls transcriptional activation of BAT-enriched genes such as *Ucp1*, *Dio2* and *Cidea* [41]. It promotes brown adipocyte differentiation and suppresses myogenesis [42–44]. In mice, transgenic expression of *Prdm16* promotes the formation of beige cells in WAT depots and increases their thermogenic capacity [45]. Interestingly, *Smyd1* conditional knockout mice showed decreased expression of *Prdm16* in addition to multiple skeletal muscle defects [46], suggesting that this critical coregulator is part of the SMYD1 regulatory pathway and may represent a link between SMYD1 and the thermogenic program in adipose tissue.

Increased expression of SMYD1 in the subcutaneous WAT of mice fed with HFD showed that SMYD1 is induced by overfeeding, a condition that stimulates the sympathetic nervous system [47]. These results may indicate that SMYD1 is a critical factor in the induction of adipose tissue thermogenesis, which is a natural defense mechanism to counteract excessive energy intake from caloric intake. Indeed, dietary thermogenesis is considered a potential buffer against weight gain [48]. Thus, the ability to modulate thermogenesis through the induction of specific thermogenic adipose tissue has several potentially beneficial effects on the control of body weight and glucose and lipid homeostasis. In addition, the evidence that SMYD1 expression is increased in cold- and HFD-exposed mice and the presence of multiple CREB binding sites at the *Smyd1* promoter suggests that the *Smyd1* promoter may be regulated by PKA-CREB (Supplementary Figure 1).

Epigenetic modulations have been shown to play an important role in regulating BAT gene expression, and understanding these epigenetic changes could help uncover the molecular mechanisms involved in controlling the thermogenic program and adipose tissue plasticity. This understanding could offer broader applications in the treatment of obesity, particularly in populations with low BAT levels, such as the elderly [49].

In this context, SMYD1 may represent an important component in the network that controls transcriptional modulation of gene expression specifically in brown adipocytes. Further studies are needed to gain a deeper understanding of the role of SMYD1 in adipose tissue thermogenesis, and certainly transgenic expression of *Smyd1* in mice is essential to include this gene in the epigenetic enzyme regulators of metabolic adaptation that have been uncovered in WAT. However, our observation that expression of SMYD1 is induced in WAT in diet-induced obesity and in cold-exposed mice suggests that this protein and the resulting epigenetic changes it orchestrates are critical for the regulation of energy modulation in the environment. This observation leads to the hypothesis that modulation of SMYD1 expression or activity could increase energy expenditure and potentially serve as a treatment approach for obesity and the metabolic syndrome.

Conclusion

Understanding the environmental conditions that promote the transition from a white adipocyte phenotype to a thermogenic phenotype through epigenetic mechanisms has promising therapeutic implications for obesity and diabetes. Epigenetic effectors are emerging as compelling targets for reprogramming WAT to a more thermogenic tissue. Consequently, more extensive research is needed to address these issues and uncover the pathways involving histone modifiers and readers that control the thermogenic adipose tissue program and browning of WAT. Such findings could lead to breakthrough strategies to combat obesity and diabetes by harnessing the power of epigenetic regulation in adipose tissue.

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Summary points

- SMYD1 is a histone methyltransferase that regulates energy metabolism in the heart. Its functions in adipose tissue have never been studied.
- Thermogenic stimuli, such as diet and cold, were used to induce beige adipocytes in mice. 3T3-L1 preadipocytes were used as an *in vitro* model for differentiation of preadipocytes into white and beige adipocytes.
- The expression of SMYD1 increased in the white adipose tissue of mice fed a high-fat diet for 8 weeks and in the white and brown adipose tissues of cold-exposed mice, indicating its role in diet- and cold-induced thermogenesis.
- SMYD1 expression increases during differentiation of preadipocytes into mature adipocytes, but levels are higher in beige adipocytes.
- Silencing of *Smyd1* in 3T3-L1 cells resulted in a decrease in the accumulation of H3K4me3, a marker of gene activation, at the *Pgc1α* locus, which contributed to a decrease in its expression.
- PGC1α is the master regulator of mitochondrial biogenesis, and its reduction led to a decrease in mitochondrial content.
- Our data suggest a novel role for SMYD1 as a positive regulator of energy control in adipose tissue.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2023-0381

Author contributions

A Cicatiello, A Nappi and F Franchini performed *in vitro* and *in vivo* experiments, contributed to writing a draft of the article and prepared figures. I Nettore performed PCR array analysis, analyzed the results and provided scientific interpretations. M Raia performed the FACS analysis studies. C Rocca and T Angelone provided critical reading of the manuscript and scientific interpretations of the data. M Dentice supervised the experiments, analyzed the results, provided scientific interpretations and contributed to writing the manuscript. P Macchia and P Ungaro designed the overall study, supervised the experiments, analyzed the results and wrote the manuscript. All authors discussed the results and provided input to the manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Animals were handled according to National and European Community guidelines, and protocols were approved by the Animal Research Committee of the University of Naples 'Federico II' (MIUR, approval code: 354/2019-PR).

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