BBA - Molecular and Cell Biology of Lipids 1865 (2020) 158594

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

Inhibition of class I HDACs imprints adipogenesis toward oxidative and brown-like phenotype☆

Alessandra Ferrari[a,1,2], Raffaella Longo[a,2], Carolina Peri[a], Lara Coppi[a], Donatella Caruso[a], Antonello Mai[b], Nico Mitro[a], Emma De Fabiani[a], Maurizio Crestani[a,*]

[a] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milano, Italy
[b] Dipartimento di Chimica e Tecnologie del Farmaco, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza Università di Roma, Roma, Italy

ARTICLE INFO

Keywords:
Histone deacetylases
Adipose differentiation
Oxidative metabolism
Browning

ABSTRACT

Obesity is characterized by uncontrolled expansion of adipose tissue mass, resulting in adipocyte hypertrophy (increased adipocyte size) and hyperplasia (increased number of adipocytes). The number of adipose cells is directly related to adipocyte differentiation process from stromal vascular cells to mature adipocytes. It is known that epigenetic factors influence adipose differentiation program. However, how specific epigenome modifiers affect white adipocyte differentiation and metabolic phenotype is still matter of research. Here, we provide evidence that class I histone deacetylases (HDACs) are involved both in the differentiation of adipocytes and in determining the metabolic features of these cells. We demonstrate that inhibition of class I HDACs from the very first stage of differentiation amplifies the differentiation process and imprints cells toward a highly oxidative phenotype. These effects are related to the capacity of the inhibitor to modulate H3K27 acetylation on enhancer regions regulating *Pparg* and *Ucp1* genes. These epigenomic modifications result in improved white adipocyte functionality and metabolism and induce browning. Collectively, our results show that modulation of class I HDAC activity regulates the metabolic phenotype of white adipocytes via epigenetic imprinting on a key histone mark.

1. Introduction

In recent years increasing evidences of a link between epigenetics, metabolism and related disorders have been reported [1]. Histone deacetylases (HDACs) are epigenome modifiers that compact chromatin and reduce the accessibility for transcription factors to target regions [2]. HDACs are divided into four classes: class I HDACs (HDAC1, 2, 3, and 8) [3] are located in the nucleus and exert the most relevant deacetylase activity, class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) shuttle from cytoplasm to nucleus and feature minimal histone deacetylase activity [4], class III (sirtuins) deacetylase activity requires NAD^+ hydrolysis as a cofactor [5], therefore sirtuins act as metabolic sensors of intracellular NAD^+/NADH ratio. HDAC11 is the only member of class IV HDACs although its function is still poorly understood [6]. Recent evidences demonstrated that HDACs participate in the regulation of energy balance and functionality of several metabolic organs, such as skeletal muscle, liver, and adipose tissues [7–10] suggesting that it is possible to rewire the metabolic capacity of these tissues through the modulation of HDAC activity.

Obesity is the consequence of an altered energy balance that results in excessive accumulation of white adipose tissue (WAT). Upon weight gain, different fat depots expand via hyperplasia (increase in the number of adipocytes) and hypertrophy (increase of adipocyte size) [11]. A pool of adipocyte precursors is present in adipose tissue even during adulthood and in humans 10% of adipocytes are renewed annually [12]. Adipocyte precursors also allow the expansion of adipose tissue in response to chronic caloric overload.

The literature of the last decade demonstrated that “browning” of WAT, a process whereby WAT acquires metabolic features typical of brown adipose tissue (BAT), increases energy expenditure and

☆ This research was supported by the European Commission grant # FP7 NR-NET PITN-GA-2013-606806, the Italian Ministry of University and Research (MIUR) grant # PRIN 2009K7R7NA, the CARIPLO Foundation Italy grant # 2015-0641 to MC and the Italian Ministry of University and Research (MIUR) Progetto Dipartimenti di Eccellenza.

* Corresponding author at: Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, 20133 Milano, Italy.
E-mail address: maurizio.crestani@unimi.it (M. Crestani).
[1] Current address: Department of Pathology and Laboratory Medicine, University of California Los Angeles, Los Angeles, CA, USA.
[2] These authors equally contributed to the manuscript.

https://doi.org/10.1016/j.bbalip.2019.158594
Received 22 November 2019; Accepted 18 December 2019
Available online 02 January 2020

attenuates the metabolic impairment consequent to the excessive accumulation of this depot [13]. Our group previously demonstrated that selective inhibition of class I HDACs is a successful strategy to counteract genetic and diet-induced obesity [14,15], pinpointing the role of these enzymes as key regulators of energy metabolism. We showed that the class I HDAC inhibitor MS-275 promotes functionality and oxidative metabolism of white fat in *db/db* and DIO (diet-induced obese) mice, determining a switch toward a brown-like phenotype. Although it has been shown that HDACs participate in the regulation of signaling pathways regulating adipogenesis [16], their role in adipose differentiation is still unclear, since different investigators provided conflicting evidences for both activating and repressing effect [17,18]. In this study, we provide new evidences of the role of class I HDACs in adipocyte differentiation and in the determination of their metabolic phenotype. We found that the class I selective inhibitor MS-275 is able to remodel white adipocyte phenotype, especially when adipose precursors are differentiated in the presence of the inhibitor. MS-275 in fact upregulates *Pparg* and *Ucp1* by increasing H3K27 acetylation in enhancer regions. This epigenome modification has been shown to mark activation of poised enhancers and concomitantly to establish promoter-enhancer loops, which favors co-activators recruitment and ultimately activation of target genes [19]. Moreover, MS-275 increases the expression of *Ppara* and its target genes and induces higher oxidative capacity. Altogether our results provide evidences of the role of class I HDACs in a crucial phase of differentiation that affects the metabolic signature of adipocytes, indicating how epigenome modifications impinge on the commitment toward a specific metabolic phenotype of fat cells.

2. Materials and methods

2.1. Cell culture

C3H/10T1/2, Clone 8 (ATCC® CCL-226™) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum (FBS, Euroclone), 1% glutamine (Life Technologies) and 1% penicillin/streptomycin (Pen/Strep, Life Technologies). During differentiation cells were maintained for 3 days in medium supplemented with 5 μg/ml insulin, 500 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich), 2 μg/ml dexamethasone (Sigma Aldrich), 5 μM rosiglitazone (Cayman Chemicals), and then switched to medium supplemented with 5 μg/ml insulin for 6 to 8 days. DMSO, 1 μM MS-275 or 10 μM garcinol (Cayman Chemicals) was added during differentiation or in the last 24 h of differentiation (i.e., terminally differentiated adipocytes). For experiments in Fig. 4D, MS-275 was purchased from Sigma Aldrich. All experiments were repeated at least 3 times, unless otherwise stated.

2.2. Determination of lipid droplet size in C3H/10T1/2 adipocytes

Lipid droplet size was assessed by using Photoshop CS6 (Adobe Systems Inc., San Jose, California): images were divided by a greed into 9 equal-sized boxes, and all lipid droplets in the three diagonal boxes from left to right were counted. For each condition were analyzed at least 4 pictures, from 3 different wells. Pictures were taken at 20× magnification using Axiovert Microscope (Zeiss).

2.3. Gene expression

RNA from cell cultures was isolated with TRIzol (Invitrogen) or RA1 lysis buffer (Macherey-Nagel) supplemented with C_2H_6OS, purified using the Nucleospin RNA II kit (Macherey-Nagel) and quantitated with Nanodrop (Thermo Scientific, Wilmington, DE). Specific mRNA was amplified and quantitated by real time PCR, using iScript™ One Step RT PCR for Probes (Bio-Rad Laboratories), following the manufacturer's instructions. Data were normalized to 36B4 mRNA and quantitated setting up a standard curve.

2.4. Quantification of lipid content

Quantification of lipid content was performed with Oil Red O (ORO) staining: staining was eluted from wells with 1.5 ml of 100% isopropanol (10 min incubation) and absorbance was measured (500 nm, 0.5 s reading). ORO staining quantification was normalized for total RNA or protein content.

2.5. Glycerol release

At the end of experiment, cell culture medium from C3H/10T1/2 cells grown and differentiated in 12-well plates was collected. Glycerol release in cell medium, as an index of lipolysis, was measured with Triglyceride Dosage Kit (Sentinel) and normalized for total RNA content.

2.6. Protein analysis

C3H/10T1/2 cells cultured in 6-well plate were lysed with SDS sample buffer (20% glycerol, 4% SDS, 100 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 0.002% bromophenol blue), 1 mM protease inhibitors (Sigma), and homogenized with TissueLyser (Qiagen). Tissue lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and sample loading and transfer evaluated by Ponceau staining. Membranes were then blocked in 5% non-fat dry milk in 1× TBS-t or 5% BSA in 1× TBS-t and incubated with PPARγ antibody (sc7273, Santa Cruz Biotechnology), ATGL antibody (#2138 s, Cell Signaling), HSP90α/β antibody (sc-69703, Santa Cruz) and β-actin antibody (A5441, Sigma). HRP-conjugated goat anti-mouse (Sigma-Aldrich), HRP-conjugated IgG goat anti-rabbit #7074 (Cell Signaling) and HRP-conjugated IgG horse anti-mouse #7076 (Cell Signaling) secondary antibodies were used for detection with chemiluminescence (ECL, Pierce).

2.7. Chromatin immunoprecipitation

C3H/10T1/2 cells differentiated for 12 h or for 9 days in presence of DMSO or MS-275 were cross-linked for 10 min with 1% formaldehyde. Chromatin was immunoprecipitated with H3K27ac antibody (ab4729, Abcam). After reversing the crosslinking by incubation overnight at 65 °C, the DNA was cleaned on QIAquick gel extraction kit columns (QIAGEN).

2.8. Statistical analyses

Statistical analysis was performed using the unpaired two-tailed Student *t*-test or One-way ANOVA with Tukey as post hoc test or Two-way ANOVA with Tukey as post hoc test with GraphPad PRISM (San Diego, CA).

3. Results

3.1. Class I HDAC inhibition stimulates adipogenic potential

Our previous results demonstrated that inhibition of class I HDACs counteracts metabolic impairments in a genetic and in a diet induced model of obesity (DIO) [14,15]. In *db/db* and DIO mice, inhibition of class I HDACs enhanced white adipose tissue functionality and promoted browning of this fat depot. These results raised new questions about the mechanism underlying the effects of MS-275 in WAT. This tissue is indeed a plastic organ [19] that contains both adipose precursors and mature adipocytes. To tease out the mechanisms underlying the effects on WAT, we asked whether the class I HDAC inhibitor MS-275 affects the phenotype of WAT by acting on adipocyte precursors or

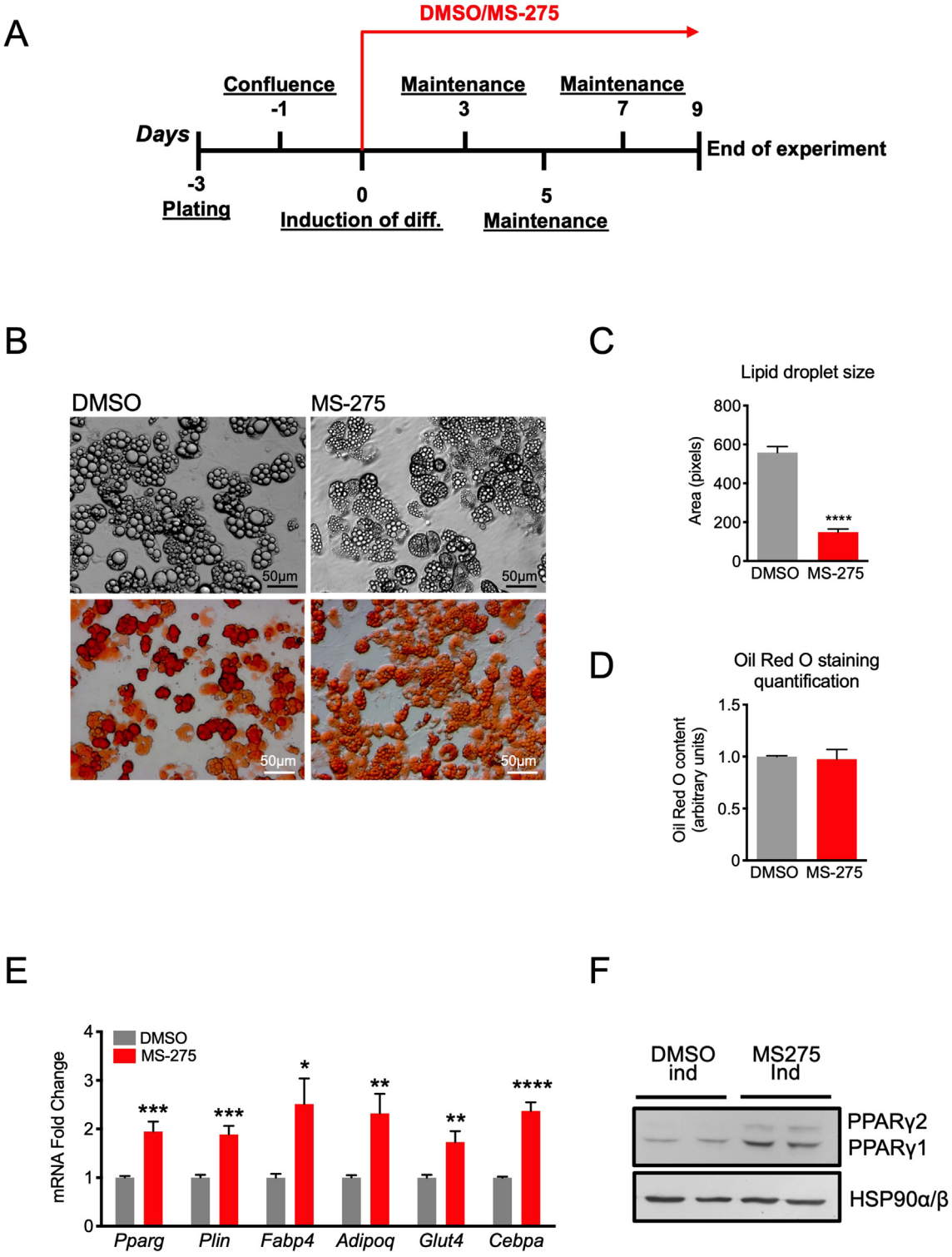


Fig. 1. Effect of class I HDAC inhibition in preadipocytes.

A) Experimental paradigm for C3H/10T1/2 cells exposed to vehicle/MS-275 during differentiation. B) Cellular morphology and Oil Red O staining of C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275. C) Quantification of lipid droplet size ($n = 3$ biological replicates). D) Oil Red O staining quantification ($n = 9$, from three independent experiments). E) Gene expression analysis of C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 12$, from four independent experiments). F) Western blot analysis of PPARγ in C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275.

Data are presented as mean ± SEM. Statistical analysis: Student's *t*-test, $*p < .05$, $**p < .01$, $***p < .001$.

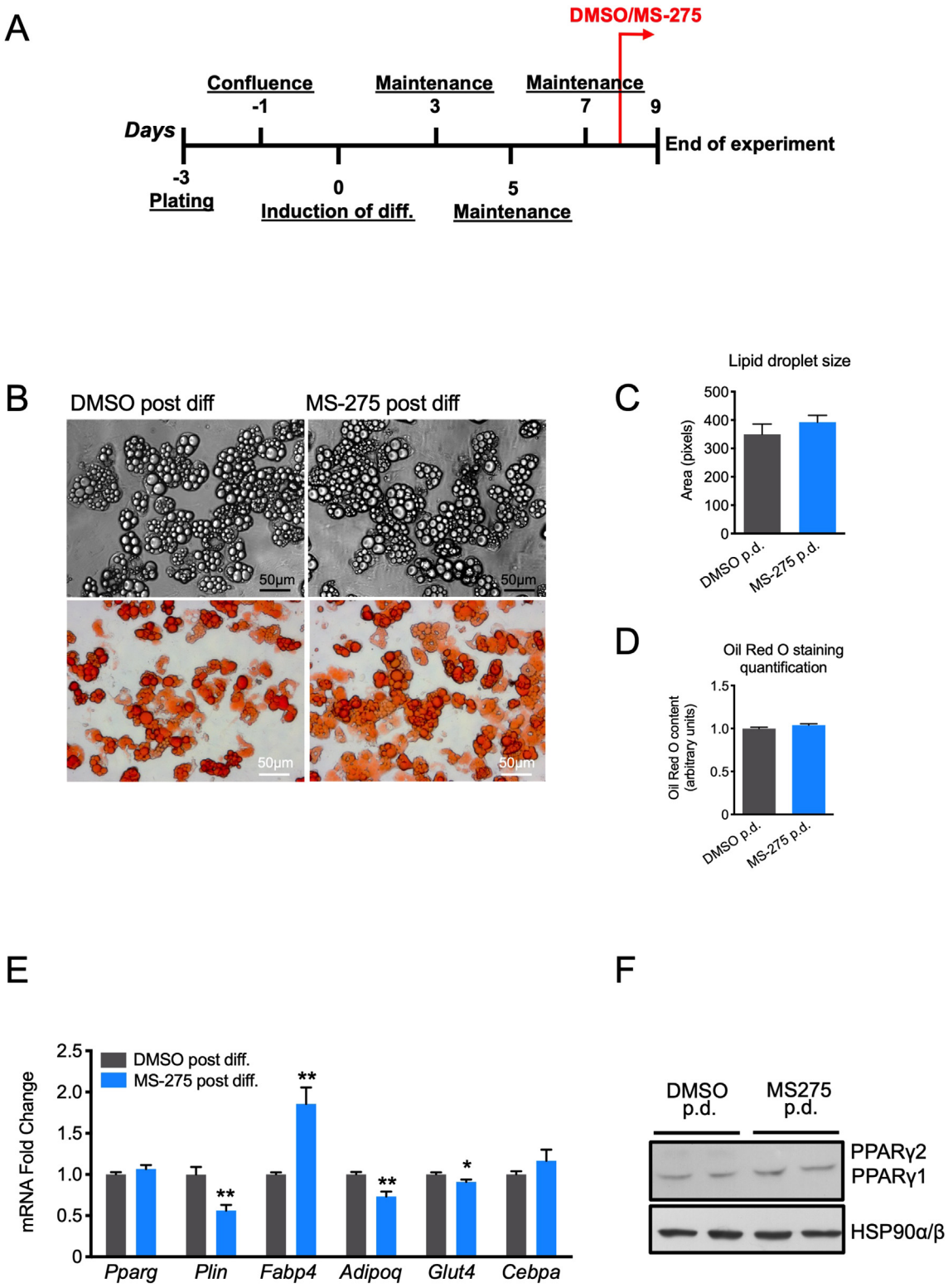


Fig. 2. Effect of class I HDAC inhibition in differentiated adipocytes.
A) Experimental paradigm for C3H/10T1/2 cells exposed to vehicle/MS-275 during the last 24 h of differentiation. B) Cellular morphology and Oil Red O staining of C3H/10T1/2 adipocytes exposed to vehicle/MS-275 during the last 24 h of differentiation. C) Quantification of lipid droplet size ($n = 3$ biological replicates). D) Oil Red O staining quantification ($n = 9$, from three independent experiments). E) Gene expression analysis of C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation ($n = 12$, from four independent experiments). F) Western blot analysis of PPARγ in C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation.
Data are presented as mean ± SEM. Statistical analysis: Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

on terminally differentiated adipocytes. To address this question, we treated C3H/10T1/2 mesenchymal stem cells at different stages of differentiation with the class I HDAC inhibitor MS-275. We chose C3H/10T1/2 cells as they are considered early adipocyte precursors [20], thus they represent an ideal model to investigate the effect of the treatment on pre-adipocytes. We treated cells with vehicle (DMSO) or with 1 μM MS-275 from the beginning of differentiation program (i.e., day 0 of the experiment, Fig. 1A). Strikingly, when cells were differentiated in the presence of MS-275 they showed a clear reduction of lipid droplet size (Fig. 1B, C). However, ORO staining revealed no differences in total lipid content under both tested conditions (Fig. 1B, D), suggesting that class I HDAC inhibition did not affect the capacity of adipocytes to store total lipids, rather it modified the characteristics of lipid droplets. Notably, when differentiated in the presence of MS-275, cells featured high expression of perilipin (*Plin*) (Fig. 1E), a PPARγ target gene crucial in the formation of lipid droplets [21]. Treatment of C3H/10T1/2 pre-adipocytes with the class I HDAC inhibitor during differentiation program, in fact, upregulated *Pparg* by 2-fold and, consequently, the adipose markers fatty acid binding protein 4 (*Fabp4*), adiponectin (*Adipoq*), the glucose transporter 4 (*Glut4*) and the transcription factor CAAT/enhancer binding protein α (*Cebpa*) (Fig. 1E). Accordingly, PPARγ protein accumulation was induced by treatment with MS-275 during differentiation (Fig. 1F).

Intriguingly, when we treated terminally differentiated adipocytes with vehicle (DMSO) or with 1 μM MS-275 (Fig. 2A) we found no differences on adipocyte morphology (Fig. 2B) and lipid accumulation (Fig. 2C, D). Analysis of expression of markers of adipocyte functionality revealed no changes in *Pparg* and *Cebpa*, a modest though statistical significant reduction in the expression of *Plin*, *Adipoq* and *Glut4* and an increase of *Fabp4* expression (Fig. 2E, F). This different trend in the expression of genes belonging to the same pathway suggests that *Pparg* transcriptional network is not fully activated when cells are treated with MS-275 at the end of differentiation. The increased expression of *Fabp4* in terminally adipocytes treated with MS-275 suggests that class I HDAC inhibition may directly target this gene.

These results reveal that class I HDAC inhibition influences adipose differentiation and expression of adipocyte functionality markers only if cells are exposed to the inhibitor from the beginning of differentiation program, suggesting that class I HDACs regulate the differentiation program of preadipocytes. Kinetic experiments demonstrated that inhibition of class I HDACs significantly increased *Pparg* expression from day 5 of differentiation (Fig. 3A), reaching a plateau at day 9. Accordingly, MS-275 boosted the expression of other adipogenic genes *Plin*, *Fabp4*, *Adipoq*, *Glut4* and *Cebpa* in most of the cases from day 5 of differentiation (Fig. 3A). To assess whether the early events of differentiation are crucial to determine the cell fate of adipocyte precursors, we exposed C3H10T1/2 cells to MS-275 only during the first 72 h of adipose differentiation. We noticed that the inhibitor was still able to upregulate the expression of adipose markers even after removing it (Fig. 3B). Collectively, these results suggest that MS-275 imprinted cells during the initial phase of differentiation and that class I HDACs impact key events during the early stage of adipogenesis.

3.2. Inhibition of class I HDACs stimulates oxidative metabolism and browning of differentiating adipocytes

The reduced lipid droplets size in cells differentiated in presence of MS-275 prompted us to ask whether inhibition of class I HDACs affected the expression of metabolic genes. Treatment with MS-275 from the beginning of differentiation increased expression of lipid internalization and lipolysis genes (e.g., *Cd36*, *Lpl*, *Lipe*, *Atgl*) (Fig. 4A). Accordingly, ATGL protein levels were increased in MS-275 treated cells (Fig. 4B). This was mirrored by increased glycerol release in the medium of these cells, a functional read-out of increased lipolysis (Fig. 4C). Moreover, MS-275 strongly stimulated pathways of mitochondrial fatty acid β-oxidation (e.g., *Cpt1b*, *Acadl*, *Acadm*, *Hadh*) and of *Ppara*, an important regulator of fatty acid oxidative metabolic pathways (Fig. 4D). In cells differentiated in presence of MS-275 we detected higher expression of genes belonging to the TCA cycle (*Idh3a*, *Suclg1*) and branched-chain amino acid catabolism (*Bckdhb*), electron transport chain (ETC) (*Cox6a1*, *Cox7a1*, *Etfdh*, *CytC*) and mitochondrial biogenesis (*Ppargc1a*) (Fig. 4E), suggesting increased mitochondrial activity. Indeed, these data were corroborated by increased signal of MitoTracker® Red CM-H_2Xros (Fig. 4F), a fluorescent dye staining mitochondria with high oxidative activity upon MS-275 treatment. Higher mitochondrial activity and *Ppara* expression in white adipocytes often correlates with a switch toward a "brown-like" phenotype. Moreover, we already demonstrated that MS-275 was able to induce browning of white fat when administered to obese mice. Thus, we measured the expression of browning markers in cells treated with the inhibitor during differentiation and, consistent with previous results, we detected higher expression of *Ucp1*, *Adrb3*, *Ppara*, *Cidea* and *Elovl3* in response to treatment with MS-275 during differentiation (Fig. 4G).

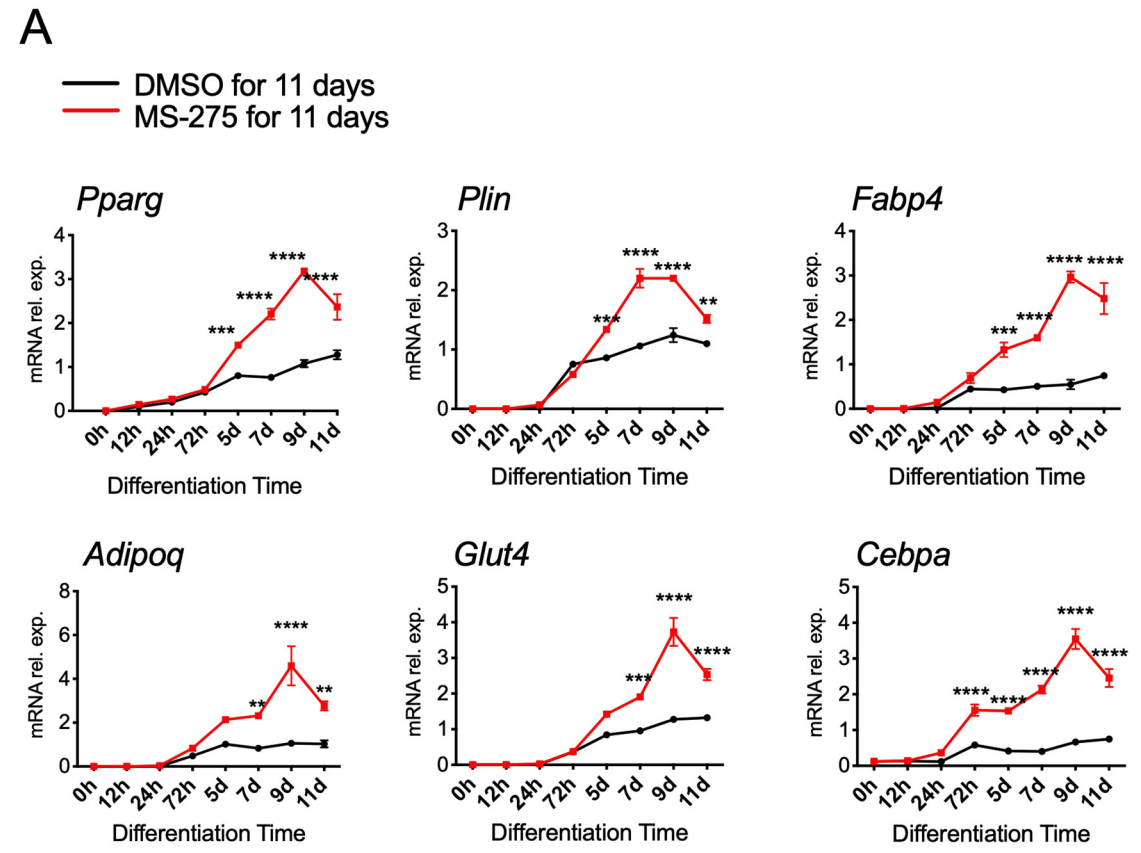
On the contrary, administration of MS-275 in terminally differentiated adipocytes yielded only mild effects on fatty acid β-oxidation genes (Fig. 5A) and on genes involved in oxidative metabolism (Fig. 5B), which was paralleled by the lack of changes in mitochondrial oxidative activity (Fig. 5C). Interestingly, also treatment of fully differentiated cells with MS-275 increased the expression of the BAT markers *Ucp1* and of *Elovl3* although the other genes were not affected (Fig. 5D).

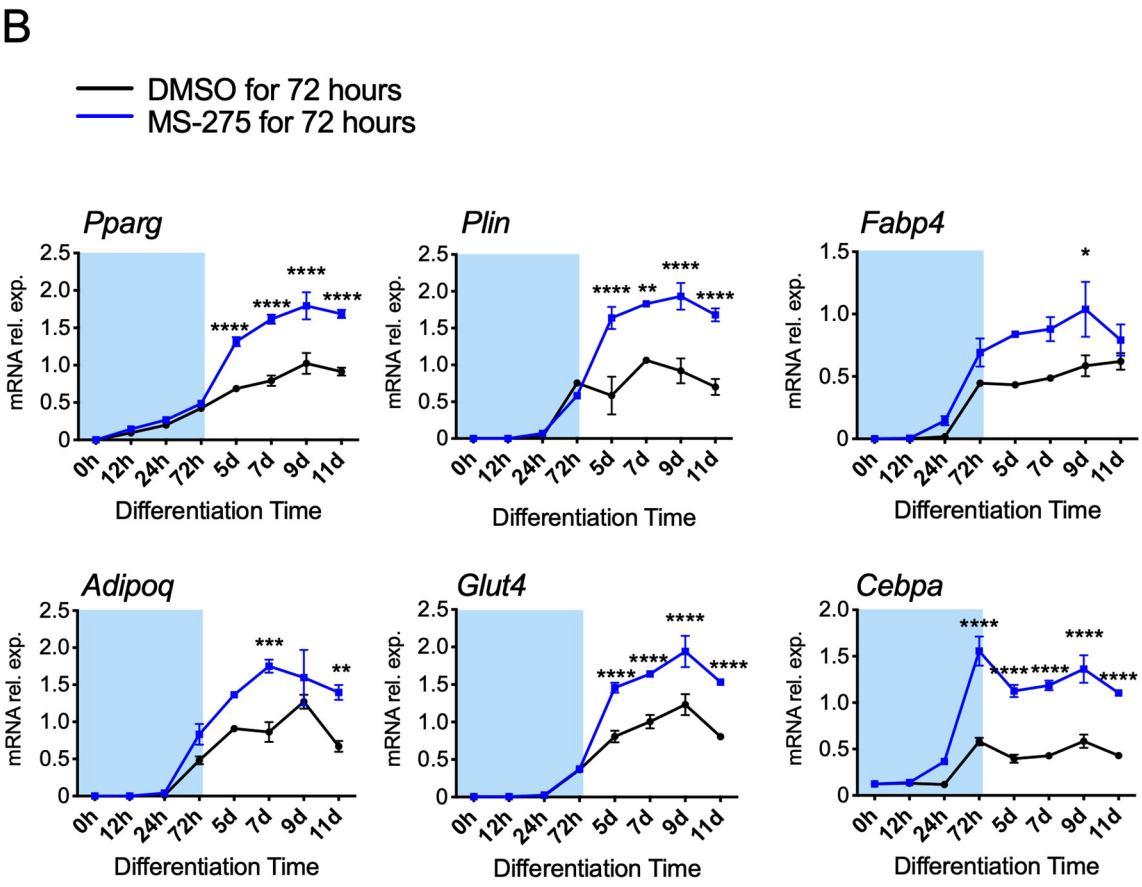
Altogether, our results indicate that MS-275 enhanced the oxidative capacity of C3H10T1/2 adipocytes and induced the expression of BAT markers gene. The effects of the class I HDAC inhibitor are more pronounced in differentiating cells than in terminally differentiated adipocytes, most likely because the inhibitor is able to remodel cell phenotype in the early phase of differentiation due to the higher plasticity of precursor cells.

3.3. Class I HDACs controls acetylation status of enhancer elements regulating Pparg and Ucp1 genes

To gain molecular insights on the transcriptional effects of MS-275 in white adipocytes, we performed chromatin immunoprecipitation experiments in C3H/10T1/2 cells focusing on acetylation of H3K27, a known histone mark of active enhancers which are crucial during the differentiation stages. The analysis of kinetic gene expression showed that *Pparg* and *Ucp1* genes were significantly up-regulated after 12 h of differentiation (Fig. 6A, C). We found that at this time point class I HDAC selective inhibitor was already able to increase H3K27 acetylation on a recently described potent enhancer element of *Pparg* gene, even though this increase did not reach the statistical meaning (Fig. 6B) [22]. Moreover, we also found increased H3K27ac at an enhancer region located 13 kb upstream of the transcription start site of *Ucp1* gene (Fig. 6D), which is typically hyper-acetylated during brown adipocyte differentiation [23]. The increased H3K27 acetylation in these regulator elements was even stronger in cells differentiated for 9 days in the presence of MS275 (Fig. 6E, F), correlating with stronger up-regulation of these genes (Figs. 1E and 5A). Furthermore, at this time point, the class I HDAC inhibitor was able to increase H3K27 acetylation also in another enhancer element located 5 kb upstream of the transcription start site of *Ucp1* gene (Fig. 6F), potentiating further the transcription of *Ucp1*.

Altogether, these results suggest that inhibition of class I HDACs in differentiating adipocytes increases the acetylation of H3K27 at *Pparg* and *Ucp1* enhancers, resulting in higher expression of genes governing adipocyte functionality and browning.





(caption on next page)

Fig. 3. Kinetic analysis of class I HDAC inhibition on gene expression.
A) Kinetics of gene expression in C3H/10T1/2 cells exposed to vehicle/MS-275 during all the differentiation program ($n = 3$). B) Kinetics of gene expression in C3H/10T1/2 cells exposed to vehicle/MS-275 only during the first 3 days of differentiation ($n = 3$).
Data are presented as mean ± SEM. Statistical analysis: Two-way ANOVA with Tukey's as post hoc test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ of cells treated with MS-275 vs. cells treated with vehicle (DMSO) at the corresponding time point. Although not indicated in the graph, statistical analysis indicates that the expression of the analyzed genes increased significantly during the differentiation both in the absence and in the presence of MS-275.

3.4. Inhibition of HAT activity prevents adipocyte differentiation and reduces the expression of genes for lipolysis, fatty acid β-oxidation, mitochondrial function and brown fat

The effects of the class I HDAC inhibitor on the phenotypic switch of adipocytes suggested that HATs could have a role on the phenotype of adipocytes. To test this hypothesis, we performed experiments to verify the effects of garcinol, a chemical inhibitor of HATs. C3H/10T1/2 preadipocytes were treated with garcinol at the beginning of differentiation until the end of the experiment at day 9 (Fig. 7A). At the end of the incubation, C3H/10T1/2 cultures treated with garcinol show a clearly diminished number of adipocytes while a greater fraction of cells did not differentiate (Fig. 7B). Quantification of ORO staining indicates strong reduction of lipid accumulation (Fig. 7C). Expression analysis of genes encoding markers of adipocyte function, lipolysis and fatty acid β-oxidation (FAO), mitochondria and brown fat reveals that the levels of these mRNAs were strongly reduced in cell treated with the HAT inhibitor garcinol. Altogether, these results highlight the central importance of HDACs and HATs in determining the metabolic phenotype of adipocytes.

4. Discussion

The role of HDACs in adipose tissue metabolism has emerged recently, however the mechanisms whereby they regulate adipose differentiation and functionality are not fully understood. The results of this study contribute to fill this gap, shedding light on how class I HDACs regulate early stages of adipose differentiation and the acquirement of brown-like phenotype.

The high plasticity [24] of fat depot is related to the presence of adipocyte precursors within adipose tissue [12], which determine the metabolic features in response to environmental cues. Our results demonstrate that inhibition of class I HDACs during differentiation strongly stimulates adipogenesis. An important finding of our study is that inhibition of class I HDACs in the early phase of adipose differentiation (72 h) imprints cell precursors toward a highly oxidative metabolic phenotype. These data suggest higher malleability of the epigenome in the first stage of adipose differentiation, allowing the HDAC inhibitor to deeply influence the metabolic features of the resulting adipocytes. On the contrary, when cells are terminally differentiated and epigenetic signature has already established, the impact of HDAC inhibition on adipose differentiation is modest. The reduced expression of *Plin* in fully differentiated adipocytes treated with MS-275 was not accompanied by reduction of lipid droplet size. Based on the results on gene expression and morphology analysis performed 24 h post-treatment with MS-275, we hypothesize that at this time point changes of gene expression may not be sufficient to determine a morphological rearrangement of lipid droplets. We cannot exclude that lipid droplets may change their shape later. Also, it should be noted that other proteins are associated to lipid droplets and they could compensate the reduced expression of *Plin*.

The effects of the class I HDAC inhibitor on adipose differentiation are mostly due to the earlier induction of the master regulator of adipogenesis PPARγ. Our results suggest that H3K27 hyperacetylation of *Pparg* enhancer, a histone modification that selectively marks active enhancers [25], is the "imprinting" event that primes and leads to the observed phenotype. These results are in line with recent evidences showing that during the first hours of differentiation H3K27ac is highly dynamic at already poised enhancer regions. Siersbæk et al. [26], in fact, demonstrated that when differentiation is induced with an adipogenic cocktail these regions loop together with promoters and recruit co-regulators. The authors demonstrate that dynamic chromatin looping of promoter-enhancer regions links reprogramming of enhancer activity to transcriptional activation of target genes. Histone acetylation levels are determined by the balance between the activity of HDACs and histone acetyltransferases (HATs). HATs-mediated acetylation plays a relevant role during adipogenesis [27]. As a matter of fact, treatment with garcinol, a natural compound which inhibits HATs, induced cell cycle arrest and reduced adipocyte differentiation of 3T3-L1 cells [28]. Thus, these evidences clearly demonstrate that the dynamic of chromatin rearrangement are crucial to modulate adipose cell fate.

Another main finding is that inhibition of class I HDACs induces *Ppara* expression and, consequently, potentiates oxidative metabolism by acting mostly in differentiating cells and, to a lesser extent, in fully differentiated adipocytes. The strong induction of lipolytic and fatty acid β-oxidation pathways, along with the induction of other genes like *Plin*, explains the reduction of lipid droplet size upon treatment with MS-275. PPARγ and PPARα induction emerged, in fact, as one of the key elements for the acquirement of the MS-275-mediated phenotype, as proved by the loss of effects in presence of PPARα/γ antagonists. Interestingly, Montgomery et al. [29] showed that pre-natal *Hdac3* cardiac-specific ablation over-activated PPARα and, consequently, increased the expression of genes important for fatty acid uptake and oxidation, electron transport chain and oxidative phosphorylation causing myocardial fatty acid accumulation, severe hypertrophy and lethality. Although higher PPARα activation is showed to be detrimental in this context, this work demonstrated a link between HDACs and PPARα activation. In parallel, post-natal *Hdac3* cardiac and skeletal muscle ablation was shown to be lethal only upon challenge with high fat diet [30]. This highlights how inactivation of HDACs in different stages during cell differentiation could result in different effects.

Moreover, in this study inhibition of class I HDACs caused hyperacetylation of H3K27 in two different enhancers regulating *Ucp1* expression, which is consequently increased. Hyperacetylation of these enhancers has been described in differentiated brown adipocytes [23]. Consequently, our results highlight an important browning effect. Furthermore, the higher expression of *Ucp1* could be in part mediated also by increased *Pparg* expression, whose role in the induction of browning in white adipocytes has been described [31]. Recently, it has been reported that the browning effect of rosiglitazone in human multipotent adipose-derived stem cells (hMADS) occurs only in mature white adipocytes, suggesting that PPARγ-mediated browning requires factors that are expressed late in the differentiation of white adipocytes [32]. Thus, our results highlight novel factors that promote browning in the early stages of adipocyte differentiation via inhibition of class I HDACs. Interestingly, the higher expression of *Ucp1* in response to MS-275 in both differentiating and terminally differentiated adipocytes suggests that class I HDACs may directly regulate this gene. However, the increased expression of *Ucp1* itself is not sufficient to establish browning, since in fully differentiated adipocytes treated with MS-275 we did not observe upregulation of all the other markers except *Elovl3* mRNA. In fact, release of fatty acids from lipid droplets in response to adrenergic stimulation, is required to activate UCP1 in mitochondria [33]. In the resting state, the activity of UCP1 in the mitochondrial inner membrane is inhibited by cytosolic purine nucleotides (i.e. ATP). We have previously demonstrated that browning of white adipocytes

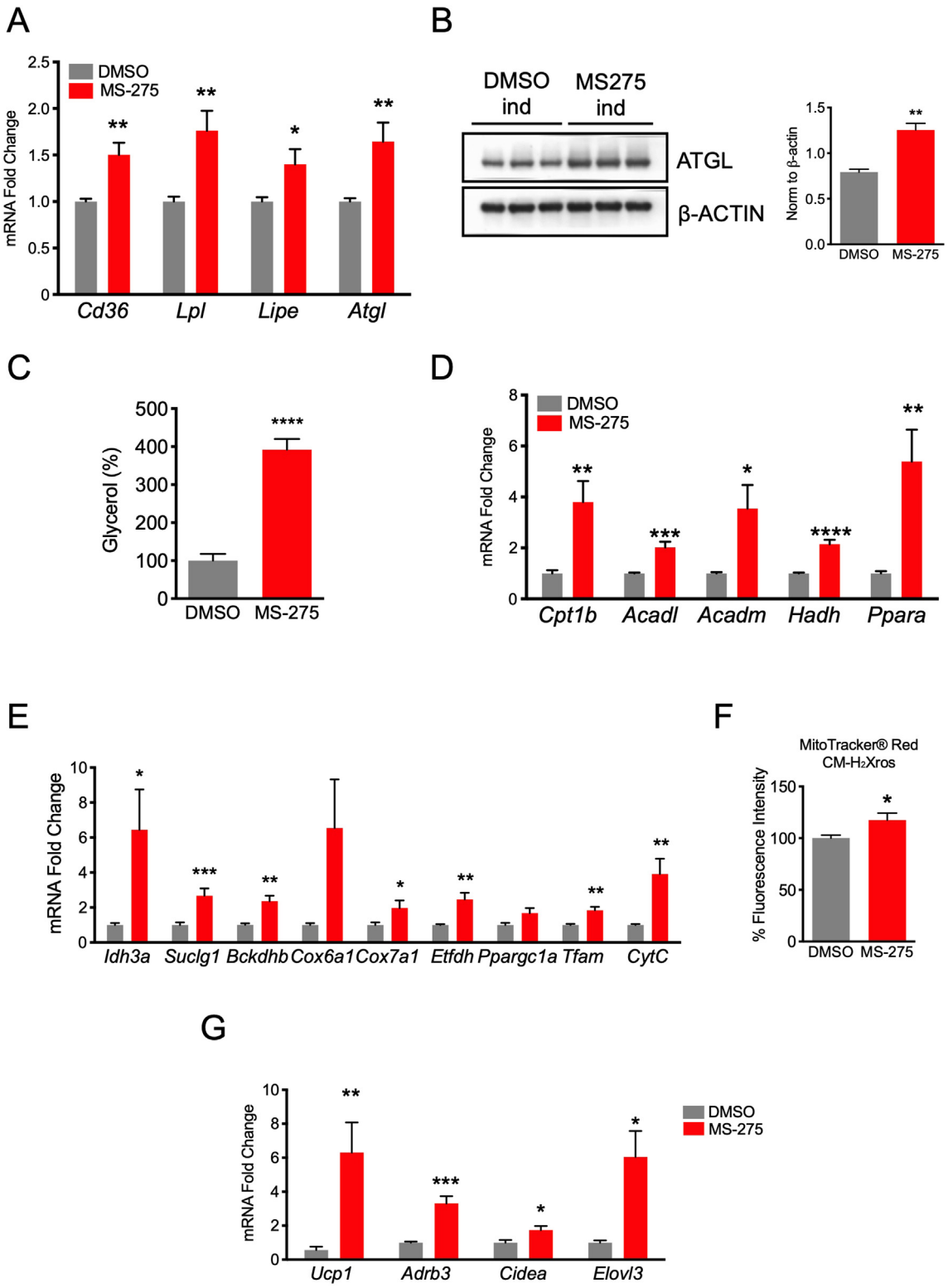


Fig. 4. Effects of class I HDAC inhibition in preadipocytes on lipolytic, fatty acid β-oxidation, mitochondrial, brown fat genes.
A) Expression of genes involved in lipolysis in C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 12$, from four independent experiments). B) Western blot analysis of ATGL protein levels and related quantification ($n = 3$, data from one single experiment). C) Glycerol release in medium from C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 6$). D) Expression of genes involved in fatty acid β-oxidation in C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 12$, from four independent experiments). E) Expression of oxidative metabolism genes in C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 12$, from four independent experiments). F) Mito Tracker RED in C3H/10T1/2 adipocytes at day 9 of differentiation in presence of vehicle/MS-275 ($n = 24$). G) Expression of browning genes in C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle/MS-275 ($n = 12$, from four independent experiments).
Data are presented as mean ± SEM. Statistical analysis: Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

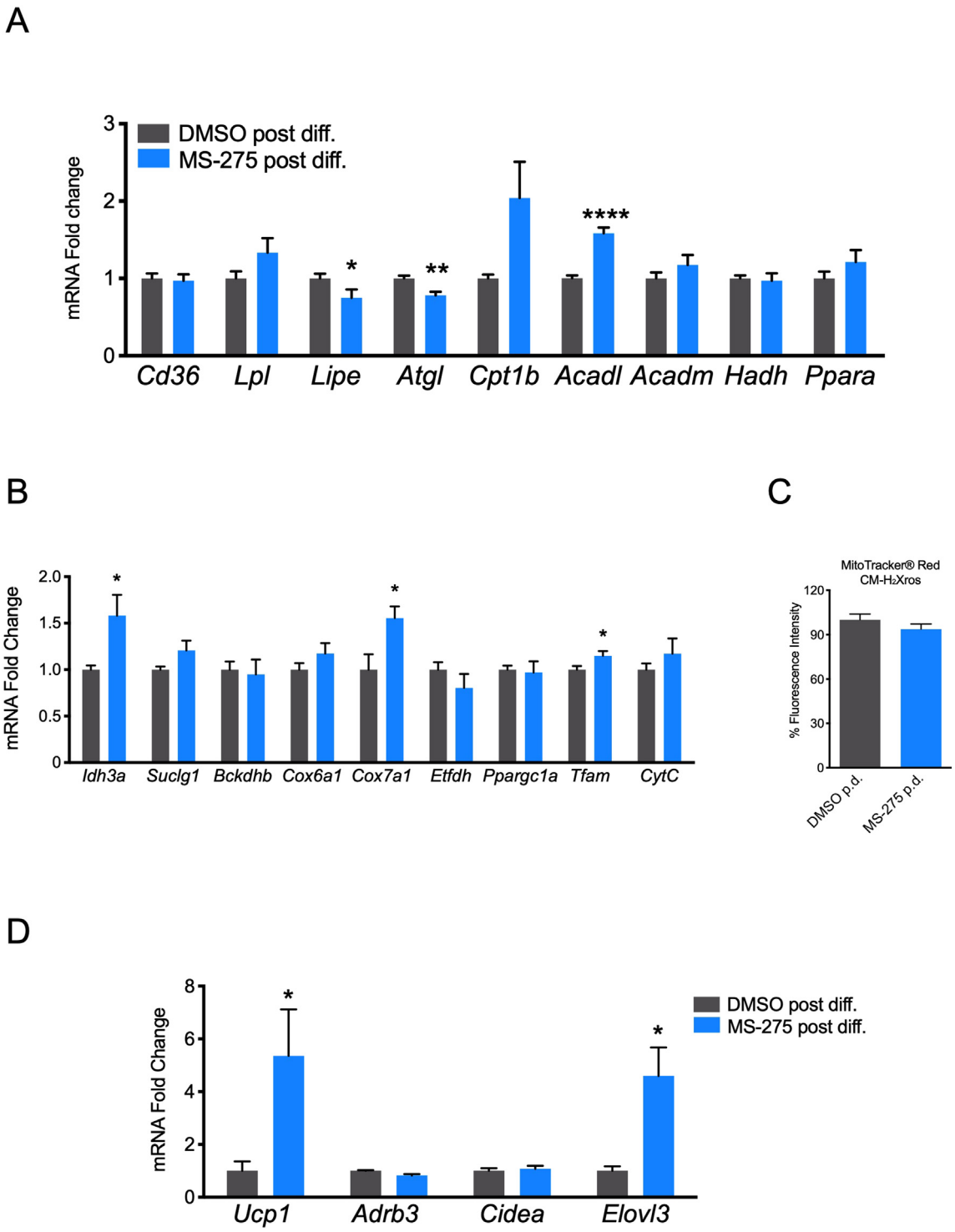


Fig. 5. Effects of class I HDAC inhibition in differentiated adipocytes on lipolytic, fatty acid β-oxidation, mitochondrial, brown fat genes.
A) Expression of genes involved in lipolysis and fatty acid β-oxidation in C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation ($n = 12$, from four independent experiments). B) Expression of oxidative metabolism genes in C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation ($n = 12$, from four independent experiments). C) Mito Tracker RED in C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation ($n = 24$). D) Expression of browning genes in C3H/10T1/2 adipocytes treated vehicle/MS-275 for 24 h at the end of differentiation ($n = 12$, from four independent experiments).
Data are presented as mean ± SEM. Statistical analysis: Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

requires metabolic rewiring to support the thermogenic program [34]. Therefore, the increased expression of *Ucp1* and *Elovl3* genes, observed in differentiated adipocytes treated with MS-275, may not be sufficient for the browning phenotype to fully display. Low temperature determines the release of norepinephrine (NE) from sympathetic nerves in BAT. UCP1 operates as a proton carrier activated by long chain fatty acids released after adrenergic-stimulated lipolysis [35,36]. The strong induction of lipolytic genes (*Lipe*, *Atgl*) by MS-275 indicates that inhibition of class I HDACs during the early phase of differentiation efficiently activates UCP1, while in terminally differentiated adipocytes,

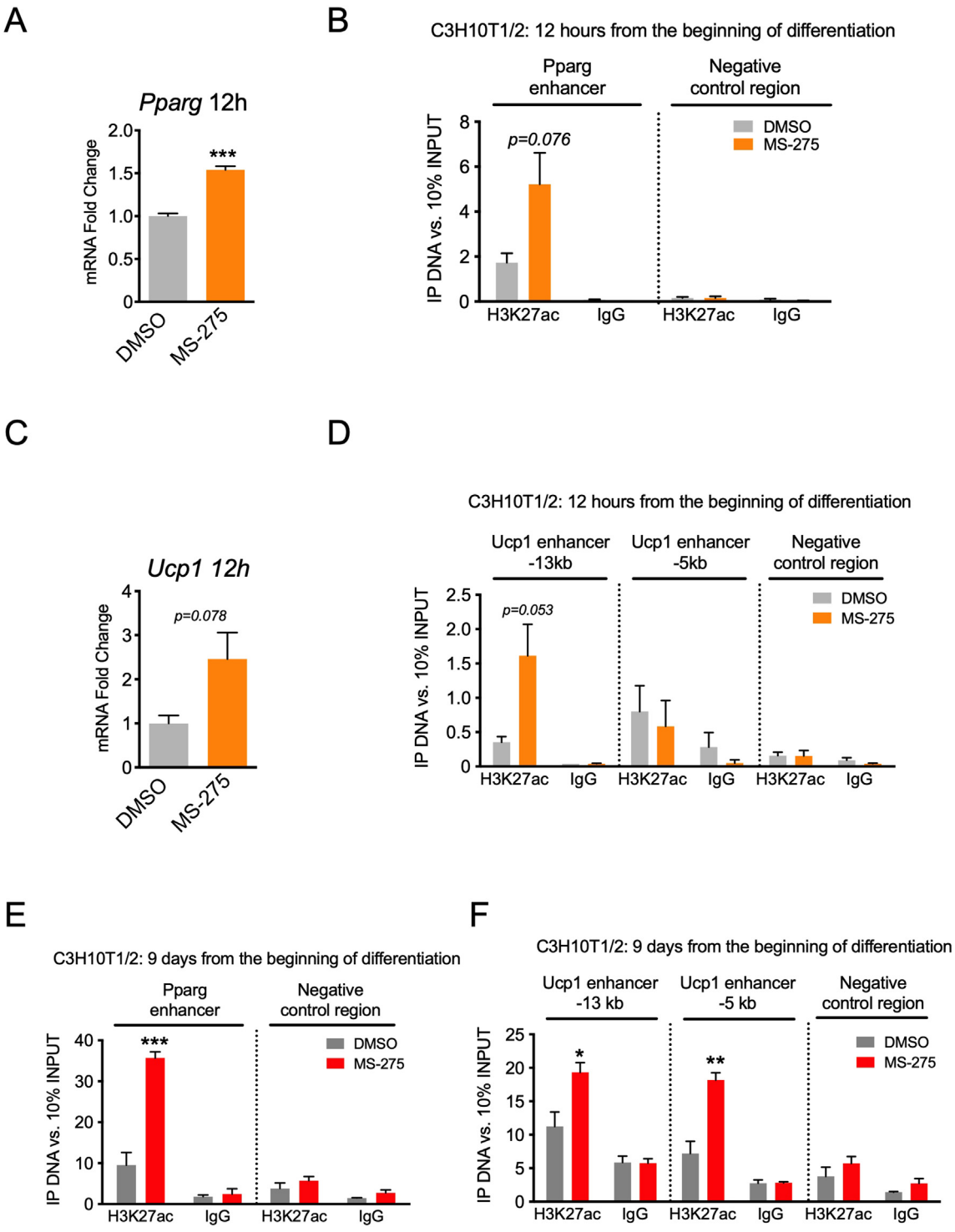


Fig. 6. ChIP analysis of the epigenetic modifications in the *Pparg* and *Ucp1* enhancers induced by the class I HDAC inhibitor MS-275.
A) *Pparg* expression in C3H/10T1/2 cells exposed to vehicle/MS-275 during the first 12 h of differentiation ($n = 3$). B) H3K27ac ChIP assay on *Pparg* enhancer region in C3H/10T1/2 cells exposed to vehicle/MS-275 during the first 12 h of differentiation ($n = 3$). C) *Ucp1* expression in C3H/10T1/2 cells exposed to vehicle/MS-275 during the first 12 h of differentiation ($n = 3$). D) H3K27ac ChIP on *Ucp1* enhancer regions in C3H/10T1/2 cells exposed to vehicle/MS-275 during the first 12 h of differentiation ($n = 3$). E) H3K27ac ChIP on *Pparg* enhancer region in C3H/10T1/2 adipocytes exposed to vehicle/MS-275 during the differentiation program (9 days) ($n = 3$); for the expression level of *Pparg* refer to Fig. 1E. F) H3K27ac ChIP on *Ucp1* enhancer regions in C3H/10T1/2 adipocytes exposed to vehicle/MS-275 during the differentiation program (9 days) ($n = 3$); for the expression of *Ucp1* refer to Fig. 4G.
Data are presented as mean ± SEM. Statistical analysis: Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

the inhibitor increased transcription of *Ucp1* but had no effect on lipolysis. It is worth to highlight that our investigations aimed to reveal new possible mechanisms that regulates adipogenesis and adipocyte metabolism, rather than propose HDAC inhibition as a new pharmacological approach to treat obesity. It should be noted that HDAC inhibitors are currently studied as chemotherapy in cancer. The main adverse effects associated with the use of HDAC inhibitors in anticancer therapies during clinical trials were gastrointestinal (nausea,

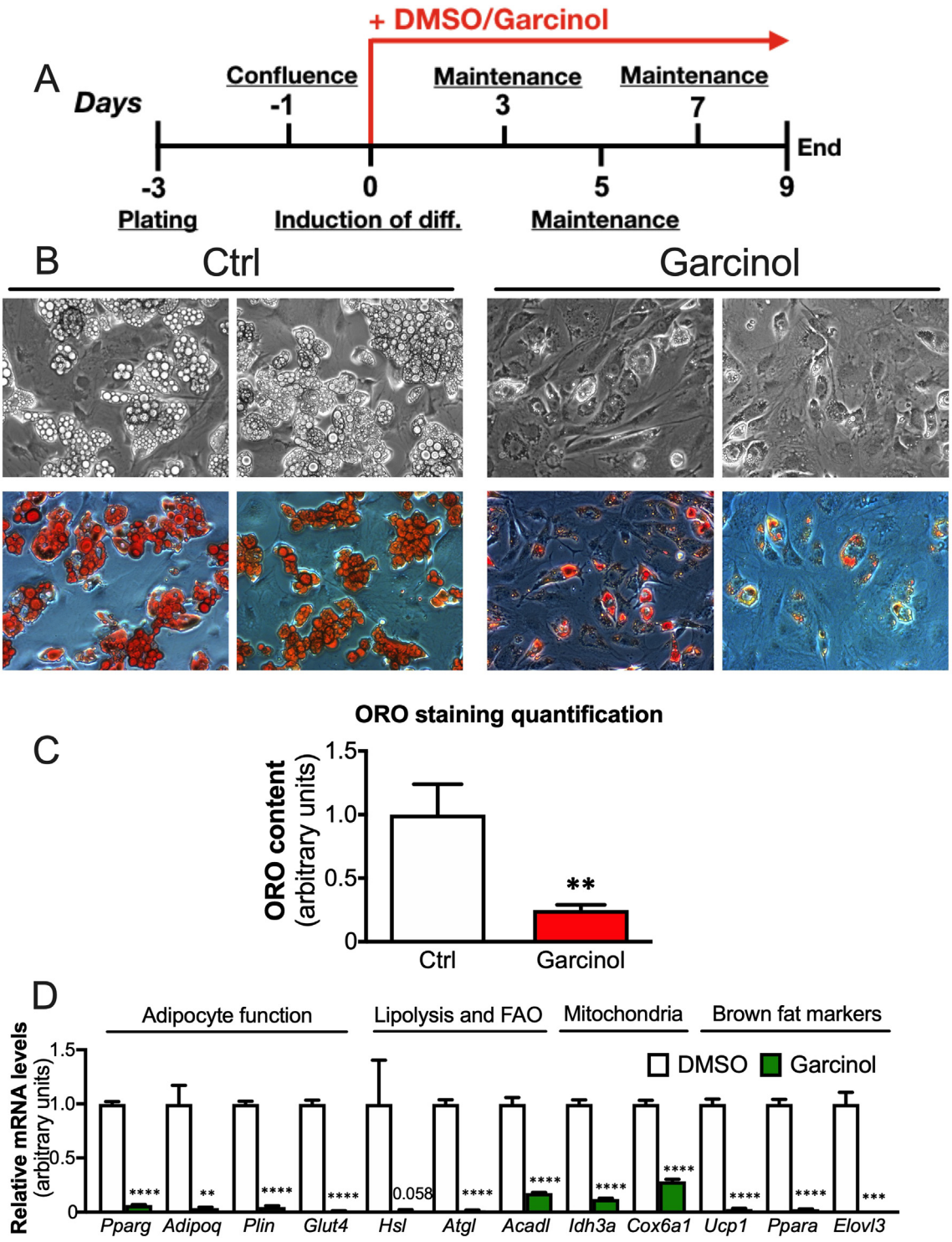


Fig. 7. Effect of the HAT inhibitor garcinol on adipocyte function, lipolysis and fatty acid β-oxidation, mitochondrial, brown fat genes on differentiating pre-adipocytes.

A) Experimental paradigm for C3H/10T1/2 cells exposed to vehicle/garcinol during differentiation. B) Cell morphology of C3H/10T1/2 adipocytes at day 9 of differentiation in the presence of vehicle (0.1% DMSO) or 10 μM garcinol. Top line of images shows unstained adipocytes; bottom line of images shows adipocytes stained with Oil Red O. C) Spectrophotometric quantification of ORO staining. D) Expression of genes for adipocyte function, lipolysis and fatty acid β-oxidation, mitochondrial functions, brown fat at day 9 of differentiation in the presence of vehicle (0.1% DMSO) or 10 μM garcinol.

Data are presented as mean ± SEM ($n = 6$ from two independent experiments). Statistical analysis: Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

vomiting, anorexia, constipation, diarrhea, dehydration and other less common symptoms), fatigue, weight loss, fever, hematological changes (thrombocytopenia, anemia and neutropenia), which resolved after the end of the treatment, cardiac, metabolic and other neurological, respiratory and infective. Also, deaths were reported to occur in clinical trials with HDAC inhibitors, but it is uncertain whether death incidence was higher than what usually reported in clinical trials in the field of cancer [37]. With respect to possible cardiac toxicity, a systematic

review reported that the use of HDAC inhibitors frequently caused the appearance of mild cardiac side effects, but it was not possible to state a clear incidence of cardiac side effect [38] due to the lack of the complete cardiac parameters and the concomitant use of other therapies. Of note, the clinical trial with MS-275 (Entinostat) included in this review showed 10.7% of incidence in heart failure. Altogether, these considerations warrant the evaluation of possible side effects of HDAC inhibitors in the field of metabolic diseases. At this regard, it is worth mentioning that our previously published data show amelioration of the metabolic alterations in pre-clinical models of obesity/insulin resistance (i.e., *db/db* and DIO mice) treated with MS-275 [14,15]. Furthermore, MS-275 improved hepatic functionality in *db/db* mice, as shown by reduced levels of ALT/AST in the blood.

In conclusion, our results suggest that the metabolic remodeling mediated by inhibition of class I HDACs during the early phase of differentiation stimulates browning of adipocytes and that epigenome alterations can affect the plasticity of precursors toward a different phenotype of terminally differentiated fat cells. This study unveils novel aspects of adipocyte differentiation and of mechanisms affecting their metabolic phenotype via dynamic epigenome modifications.

Author contributions

Alessandra Ferrari: Conceptualization, Methodology, Investigation, Writing - Original Draft, Visualization; **Raffaella Longo:** Investigation, Writing - Original Draft, Visualization, Writing- Reviewing and Editing; **Carolina Peri:** Investigation; **Lara Coppi:** Investigation; **Donatella Caruso:** Supervision; **Antonello Mai:** Resources; **Nico Mitro:** Supervision; **Emma De Fabiani:** Supervision; **Maurizio Crestani:** Conceptualization, Writing- Reviewing and Editing, Supervision, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Tommaso Laurenzi, Dalia Porro and Dalma Cricrí for their valuable help in culturing cells and performing ChIP analyses. The authors are grateful to Ms. Elda Desiderio Pinto and Dr. Marta Marchesi for administrative support.

References

[1] B.M. Herrera, S. Keildson, C.M. Lindgren, Genetics and epigenetics of obesity, Maturitas 69 (2011) 41–49.
[2] G. López-Rodas, G. Brosch, E. Georgieva, R. Sendra, L. Franco, P. Loidl, Histone deacetylase: a key enzyme for the binding of regulatory proteins to chromatin, FEBS Lett. 317 (1993) 175–180.
[3] A.J.M. de Ruijter, A.H. van Gennip, H.N. Caron, S. Kemp, A.B.P. van Kuilenburg, Histone deacetylases (HDACs): characterization of the classical HDAC family, Biochem. J. 370 (2003) 737–749.
[4] M. Haberland, R.L. Montgomery, E.N. Olson, The many roles of histone deacetylases in development and physiology: implications for disease and therapy, Nat. Rev. Genet. 10 (2009) 32–42.
[5] L. Guarente, Sirtuins as potential targets for metabolic syndrome, Nature 444 (2006) 868–874.
[6] L. Gao, M.A. Cueto, F. Asselbergs, P. Atadja, Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family, J. Biol. Chem. 277 (2002) 25748–25755.
[7] Z. Gao, J. Yin, J. Zhang, R.E. Ward, R.J. Martin, M. Lefevre, W.T. Cefalu, J. Ye, Butyrate improves insulin sensitivity and increases energy expenditure in mice, Diabetes 58 (2009) 1509–1517.
[8] M.M. Mihaylova, D.S. Vasquez, K. Ravnskjaer, P.D. Denechaud, R.T. Yu, J.G. Alvarez, M. Downes, R.M. Evans, M. Montminy, R.J. Shaw, Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis, Cell 145 (2011) 607–621.
[9] Z. Sun, R.A. Miller, R.T. Patel, J. Chen, R. Dhir, H. Wang, D. Zhang, M.J. Graham, T.G. Unterman, G.I. Shulman, C. Sztalryd, M.J. Bennett, R.S. Ahima, M.J. Birnbaum, M.A. Lazar, Hepatic Hdac3 promotes gluconeogenesis by repressing lipid synthesis and sequestration, Nat. Med. 18 (2012) 934–942.
[10] S.K. Knutson, B.J. Chyla, J.M. Amann, S. Bhaskara, S.S. Huppert, S.W. Hiebert, Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks, EMBO J. 27 (2008) 1017–1028.
[11] P. Chowdhury, Endocrine and metabolic regulation of body mass by nicotine: role of growth hormone, Ann. Clin. Lab. Sci. 20 (1990) 415–419.
[12] K.L. Spalding, E. Arner, P.O. Westermark, S. Bernard, B.A. Buchholz, O. Bergmann, L. Blomqvist, J. Hoffstedt, E. Naslund, T. Britton, H. Concha, M. Hassan, M. Ryden, J. Frisen, P. Arner, Dynamics of fat cell turnover in humans, Nature 453 (2008) 783–787.
[13] A. Bartelt, J. Heeren, Adipose tissue browning and metabolic health, Nat. Rev. Endocrinol. 10 (2014) 24–36.
[14] A. Galmozzi, N. Mitro, A. Ferrari, E. Gers, F. Gilardi, C. Godio, G. Cermenati, A. Gualerzi, E. Donetti, D. Rotili, S. Valente, U. Guerrini, D. Caruso, A. Mai, E. Saez, E. De Fabiani, M. Crestani, Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue, Diabetes 62 (2013) 732–742.
[15] A. Ferrari, E. Fiorino, R. Longo, S. Barilla, N. Mitro, G. Cermenati, M. Giudici, D. Caruso, A. Mai, U. Guerrini, E. De Fabiani, M. Crestani, Attenuation of diet-induced obesity and induction of white fat browning with a chemical inhibitor of histone deacetylases, Int. J. Obes. 41 (2017) 289–298.
[16] S.R. Farmer, Transcriptional control of adipocyte formation, Cell Metab. 4 (2006) 263–273.
[17] M. Haberland, M. Carrer, M.H. Mokalled, R.L. Montgomery, E.N. Olson, Redundant control of adipogenesis by histone deacetylases 1 and 2, J. Biol. Chem. 285 (2010) 14663–14670.
[18] S.-N. Kim, H.-Y. Choi, Y.K. Kim, Regulation of adipocyte differentiation by histone deacetylase inhibitors, Arch. Pharm. Res. 32 (2009) 535–541.
[19] A. Vitali, I. Murano, M.C. Zingaretti, A. Frontini, D. Ricquier, S. Cinti, The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes, J. Lipid Res. 53 (2012) 619–629.
[20] Q.-Q. Tang, T.C. Otto, M.D. Lane, Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage, Proc. Natl. Acad. Sci. 101 (2004) 9607–9611.
[21] N. Arimura, T. Horiba, M. Imagawa, M. Shimizu, R. Sato, The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes, J. Biol. Chem. 279 (2004) 10070–10076.
[22] M.K. Ramlee, Q. Zhang, M. Idris, X. Peng, C.K. Sim, W. Han, F. Xu, Histone H3 K27 acetylation marks a potent enhancer element on the adipogenic master regulator gene Pparg2, Cell Cycle 13 (2014) 3414–3422.
[23] Y. Abe, R. Rozqie, Y. Matsumura, T. Kawamura, R. Nakaki, Y. Tsurutani, K. Tanimura-Inagaki, A. Shiono, K. Magoori, K. Nakamura, S. Ogi, S. Kajimura, H. Kimura, T. Tanaka, K. Fukami, T.F. Osborne, T. Kodama, H. Aburatani, T. Inagaki, J. Sakai, JMJD1A is a signal-sensing scaffold that regulates acute chromatin dynamics via SWI/SNF association for thermogenesis, Nat. Commun. 6 (2015) 7052.
[24] W.P. Cawthorn, E.L. Scheller, O.A. MacDougald, Adipose tissue stem cells meet preadipocyte commitment: going back to the future, J. Lipid Res. 53 (2012) 227–246, https://doi.org/10.1016/j.conbuildmat.2016.09.076.
[25] M.P. Creyghton, A.W. Cheng, G.G. Welstead, T. Kooistra, B.W. Carey, E.J. Steine, J. Hanna, M.A. Lodato, G.M. Frampton, P.A. Sharp, L.A. Boyer, R.A. Young, R. Jaenisch, Histone H3K27ac separates active from poised enhancers and predicts developmental state, Proc. Natl. Acad. Sci. 107 (2010) 21931–21936.
[26] R. Siersbæk, J.G.S. Madsen, B.M. Javierre, R. Nielsen, E.K. Bagge, J. Cairns, S.W. Wingett, S. Traynor, M. Spivakov, P. Fraser, S. Mandrup, Dynamic rewiring of promoter-anchored chromatin loops during adipocyte differentiation, Mol. Cell 66 (2017) 420–435 (e5).
[27] M.M. Musri, R. Gomis, M. Párrizas, A chromatin perspective of adipogenesis, Organogenesis 6 (2010) 15–23.
[28] C.L. Hsu, Y.J. Lin, C.T. Ho, G.C. Yen, Inhibitory effects of garcinol and pterostilbene on cell proliferation and adipogenesis in 3T3-L1 cells, Food Funct. 3 (2012) 49–57.
[29] R.L. Montgomery, M.J. Potthoff, M. Haberland, X. Qi, S. Matsuzaki, K.M. Humphries, J.A. Richardson, R. Bassel-Duby, E.N. Olson, Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice, J. Clin. Invest. 118 (2008) 3588–3597.
[30] Z. Sun, N. Singh, S.E. Mullican, L.J. Everett, L. Li, L. Yuan, X. Liu, J.A. Epstein, M.A. Lazar, Diet-induced lethality due to deletion of the Hdac3 gene in heart and skeletal muscle, J. Biol. Chem. 286 (2011) 33301–33309.
[31] N. Petrovic, T.B. Walden, I.G. Shabalina, J.A. Timmons, B. Cannon, J. Nedergaard, Chronic peroxisome proliferator-activated receptor γ (PPARγ) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes, J. Biol. Chem. 285 (2010) 7153–7164.
[32] A. Loft, I. Forss, M.S. Siersbæk, S.F. Schmidt, A.-S.B. Larsen, J.G.S. Madsen, D.F. Pisani, R. Nielsen, M.M. Aagaard, A. Mathison, M.J. Neville, R. Urrutia, F. Karpe, E.-Z. Amri, S. Mandrup, Browning of human adipocytes requires KLF11 and reprogramming of PPARγ superenhancers, Genes Dev. 29 (2015) 7–22.
[33] V. Peirce, S. Carobbio, A. Vidal-Puig, The different shades of fat, Nature 510 (2014) 76–83.
[34] A. Ferrari, R. Longo, E. Fiorino, R. Silva, N. Mitro, G. Cermenati, F. Gilardi, B. Desvergne, A. Andolfo, C. Magagnotti, D. Caruso, E. De Fabiani, S.W. Hiebert, M. Crestani, HDAC3 is a molecular brake of the metabolic switch supporting white adipose tissue browning, Nat. Commun. 8 (2017) 93.
[35] J. Nedergaard, B. Cannon, The changed metabolic world with human brown

adipose tissue: therapeutic visions, Cell Metab. 11 (2010) 268–272.
[36] A. Fedorenko, P.V. Lishko, Y. Kirichok, Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria, Cell 151 (2012) 400–413.
[37] S. Subramanian, S.E. Bates, J.J. Wright, I. Espinoza-Delgado, R.L. Piekarz, Clinical toxicities of histone deacetylase inhibitors, Pharmaceuticals 3 (2010) 2751–2767.
[38] G.G. Schiattarella, A. Sannino, E. Toscano, F. Cattaneo, B. Trimarco, G. Esposito, C. Perrino, Cardiovascular effects of histone deacetylase inhibitors epigenetic therapies: systematic review of 62 studies and new hypotheses for future research, Int. J. Cardiol. 219 (2016) 396–403.