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Histone Deacetylase 1 (HDAC1) Negatively Regulates Thermogenic Program in Brown Adipocytes via Coordinated Regulation of H3K27 Deacetylation and Methylation

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Running Title: HDAC1 Negatively Regulates Brown Fat Gene Expression

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Abstract

Inhibiting class I histone deacetylases (HDACs) increases energy expenditure, reduces adiposity and improves insulin sensitivity in obese mice. However, the precise mechanism is poorly understood. Here, we demonstrate that HDAC1 is a negative regulator of brown adipocyte thermogenic program. HDAC1 level is lower in mouse brown fat (BAT) than white fat, is suppressed in mouse BAT during cold exposure or β3-adrenergic stimulation, and is down-regulated during brown adipocyte differentiation. Remarkably, overexpressing HDAC1 profoundly blocks, whereas deleting HDAC1 significantly enhances β-adrenergic activation-induced BAT-specific gene expression in brown adipocytes. β-adrenergic activation in brown adipocytes results in a dissociation of HDAC1 from promoters of BAT-specific genes, including uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), leading to increased acetylation of histone H3 lysine 27 (H3K27), an epigenetic mark of gene activation. This is followed by dissociation of the polycomb repressive complexes, including the H3K27 methyltransferase enhancer of zeste homologue (EZH2), suppressor of zeste 12 (SUZ12), and ring finger protein 2 (RNF2) from, and concomitant recruitment of H3K27 demethylase ubiquitously transcribed tetratricopeptide repeat on chromosome X (UTX) to UCP1 and PGC1α promoters, leading to decreased H3K27 trimethylation, a histone transcriptional repression mark. Thus, HDAC1 negatively regulates brown adipocyte thermogenic program, and inhibiting HDAC1 promotes BAT-specific gene expression through a coordinated control of increased acetylation and decreased methylation of...
H3K27, thereby switching transcriptional repressive state to active state at the promoters of UCP1 and PGC1α. Targeting HDAC1 may be beneficial in prevention and treatment of obesity by enhancing BAT thermogenesis.

Introduction

Obesity develops when a persistent imbalance between energy intake and energy expenditure occurs (1). While white adipose tissue (WAT) is involved in energy storage, that of brown adipose tissue (BAT) is to dissipate energy as heat due to its unique expression of uncoupling protein 1 (UCP1) (2-4). In rodents, there exist two types of brown adipocytes. Traditional brown adipocytes are located in discrete areas; whereas “inducible” beige adipocytes are dispersed in WAT (5-8), and can be induced by cold exposure or β3-adrenergic receptor activation (9-13). The ability of brown/beige adipocytes to produce adaptive thermogenesis depends on the unique expression of UCP1 in the inner mitochondrial membrane, which serves to uncouple oxidative phosphorylation from ATP synthesis, thereby profoundly increasing energy expenditure (2-4). Recent reports demonstrate that adult humans also possess metabolically active brown fat; the amount of brown fat is inversely correlated with body weight but positively correlated with energy expenditure (14-16). This important discovery provides new insight into the mechanisms regulating energy homeostasis in adult humans and suggests that increasing functional brown/beige adipocytes in humans is a novel and promising target in treating obesity.

Although the genome is fixed and identical in all cells, the epigenome, the combination of all genome-wide DNA and chromatin modifications, is continuously modified in response to developmental, environmental, physiological and pathological cues (17-19).

Epigenetic modifications, including DNA methylation, histone acetylation and methylation, result in organization of the chromatin structure on different hierarchical levels, which regulate gene expression (17-19). Recent evidence suggests that epigenetic mechanisms have emerged as an important link between environmental factors and obesity (20-24). For example, UCP1 promoter activity is regulated by changes in DNA methylation status (25). The H3K9 demethylase JHDM2a directly regulates UCP1 expression, and genetic deletion of JHDM2a in mice results in obesity (26). This is a new emerging research area; however, much remains to be discovered regarding how epigenetic mechanisms regulate metabolism and energy homeostasis.

Histone acetylation and deacetylation are regulated by the balanced action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (17-19). HDACs consist of four major classes: class I (1, 2, 3 and 8), class II (4, 5, 6, 7, 9, 10), class III (SIRTs 1-7) and class IV (11). The class I, II and IV HDACs act to remove acetyl groups from lysine residues in histone as well as cellular proteins thereby regulating gene expression and cellular protein activity; whereas class III HDACs (Sirt1–Sirt7) form a structurally distinct class of NAD-dependent enzymes and can be inhibited by nicotinamide (17,18). Recent data suggest that HDACs have emerged as important players in the regulation of energy and glucose homeostasis (27). For example, it is reported that class I HDAC inhibitor (HDACi) MS-275 ameliorates obesity and diabetes in animal models through stimulation of oxidative phosphorylation and mitochondrial function in muscle and fat (28). However, the precise mechanism by which class I HDACi exerts these effects is poorly understood.

In the current study, we have identified HDAC1 as a prominent epigenetic target in regulating the thermogenic program in brown...
adipocytes. Using loss- and gain-of-function approaches, we demonstrated that HDAC1 deficiency activated, whereas HDAC1 overexpression repressed transcription of brown adipocyte-specific gene expression through regulating acetylation and methylation status of histone H3 lysine 27 (H3K27) on promoter and enhancer regions of UCP1 and peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1-α (PGC1α). Thus, our data suggest that epigenetics plays an important role in brown adipocyte thermogenesis, and HDAC1 may be an important regulator during this process.

**Materials and Methods**

**Mice.** C57BL/6J (B6) and AJ mice (Jackson Laboratories, Bar Harbor, ME) were housed with a 12/12 h light–dark cycle in temperature- and humidity-controlled rooms with free access to water and food. To study the role of HDAC1 in BAT thermogenic function, 7-8-week-old mice were exposed to cold condition (4°C) or intraperitoneally (i.p.) injected with β3-adrenergic agonist [CL316243 (Sigma Aldrich, St. Louis, MO), dose: 1mg/kg] for up to 7 days. Mice were then sacrificed and BAT was harvested for gene expression or chromatin immunoprecipitation (ChIP) analysis. All aspects of animal care were approved by Georgia State University’s Animal Care and Use Committee.

**Cell Culture.** All cells were maintained at 37°C with 5% CO₂. Immortalized brown preadipocytes BAT1 (kindly provided by Dr. Patrick Seale, University of Pennsylvania) (29,30) and HIB-1B (31,32) were maintained in growth medium (DMEM/F12 (BAT1) or DMEM (HIB-1B) containing 10% fetal bovine serum and 1% Penicillin/Streptomycin). Brown adipocyte differentiation was induced as described previously (29,30). Briefly, cells were grown into 90% confluence in growth medium and were then induced to differentiate with a differentiation medium (growth medium plus 20nM insulin, 1nM triiodothyronine (T3), 125μM indomethacin, 500μM isobutylmethylxanthine and 0.5μM dexamethasone). After 2 days, cells were cultured in maintenance medium (growth medium plus 20nM insulin and 1nM T3). At day 6, all of cells were differentiated as previously described (29,30). To induce the thermogenic program, brown adipocytes were treated with 1μM isoproterenol or norepinephrine (NE) for 3-4 hours.

**Small interfering RNA (siRNA) and plasmid DNA transfection.** The MGC fully sequenced mouse cDNA expression plasmids for HDAC1 (Clone ID 4976514), enhancer of zeste homologue (EZH2) (Clone ID 3586689), suppressor of zeste 12 (SUZ12) (Clone ID 6821922), ring finger protein 2 (RNF2) (Clone ID 4021046), the ON-TARGET plus Mouse HDAC1 siRNA–SMART pool (L-040287-02-0005), and HDAC1-11 siRNAs for our initial screening were purchased from GE Healthcare (Lafayette, CO). The sequences for HDAC1-11 siRNAs were listed in Table 1. All other plasmids were in mammalian expression vector pSPORT6 except for SUZ12, which was in a non-expressing vector pXY-Asc. To clone SUZ12 into pSPORT6 vector, PciI and SalI were used to release SUZ12 cDNA insert from pXY-Asc and sub-cloned into pSPORT6. The overexpressing plasmids or siRNAs were transfected into BAT1 or HIB-1B brown adipocytes by Amaxa Nucleofector II Electroporator (Lonza) using Amaxa cell line nucleofector kit L according to manufacturer’s instructions (Lonza). Briefly, at day 4-6 of differentiation, cells (2×10⁶ cells per sample) were trypsinized and centrifuged at 90×g for 5 minutes at room temperature. Cell pellet was resuspended in nucleofector solution (100μl per sample) with 2μg plasmid DNA or 20pmol
siRNA and seeded into 24-well plates. Cells were treated with NE or Isoproterenol 2 days after transfection.

For co-immunoprecipitation (co-IP) experiment, HEK293T cells were electroporated with pSPORT6 vector or co-transfected with HDAC1, EZH2, SUZ12 and RNF2 cDNA for 2 days. Cell lysates from transfected HEK293T cells or endogenous BAT1 brown adipocytes were collected to detect protein interactions by co-IP.

Immunoprecipitation (IP) and Immunoblotting (IB). IP and IB were performed as we previously described (33-36). Briefly, tissue or cell samples were homogenized in a modified radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1mM Sodium Orthovanadate, 1% protease inhibitor mixture (Sigma), and 1% phosphatase inhibitor mixture (Sigma). Cell homogenates were incubated on ice for 45 min to solubilize all proteins, and insoluble portions were removed by centrifugation at 14,000 g at 4 °C for 15 min. Two mg of cell lysates was incubated overnight with the appropriate antibodies (Table 2) and protein A/G-agarose (Santa Cruz Biotechnology) at 4 °C with constant gentle mixing. Agarose beads were collected by centrifugation, washed with ice-cold RIPA lysis buffer and then with phosphate-buffered saline, and then boiled in Laemmli sample buffer. Protein from IP or whole cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The transferred membranes were blocked, washed, and incubated with various primary antibodies (Table 2), followed by incubating with Alexa Fluor 680-conjugated secondary antibodies (Life Technologies). The fluorescent signal was visualized with a Li-COR Imager System (Li-COR Biosciences, Lincoln, NE).

Quantitative real-time RT-PCR. Total RNA was extracted with TRI Reagent according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). RNA expression was quantified by real-time RT-PCR with TaqMan one-step RT-PCR Master mix (Life Technologies, Foster City, CA) using a Stratagene Mx3000p thermocycler (Stratagene, La Jolla, CA) and normalized to cyclophilin. The primer and probe pairs used in the assays were purchased from Applied Biosystems or designed using Primer Express software (Life Technologies) (Tables 3 and 4).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using a ChIP assay kit (Upstate) as we previously described (37). For tissue ChIP assays, tissue samples were cut into small pieces and fixed with 1% of formaldehyde. The samples were then homogenized in cell lysis buffer (5 mM PIPES, 85 mM KCl, and 0.5% NP-40, supplemented with protease inhibitors, pH 8.0) using a dounce homogenizer to isolate nuclei. The nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl, 10 mM EDTA, and 1% SDS, supplemented with protease inhibitors, pH 8.1) and sonicated to shear genomic DNA to an average fragment length of 200–1,000 bp with a Diagenode Bioruptor (Diagenode, Denville, NJ). Lysates were centrifuged, and the supernatants were collected. Fifty microliters of each sample was removed as the input control. The supernatants underwent overnight immunoprecipitation, elution, reverse cross-linking, and protease K digestion, according to the manufacturer’s manual. A mock immunoprecipitation without antibody was also included for each sample. Eluted DNA was analyzed by real-time PCR using SYBR green quantitative PCR (Life Technologies). Primer
sequences used in this study were as follows: UCP1 proximal promoter, 5’-CCCACTAGCAGCTCTTTGGA-3’ and 5’-CTGTGGAGCAGCTCAAAGGT; UCP1 enhancer region, 5’-CTCCTCTACAGCGTCACAGAGG-3’ and 5’-AGTCTGAGGAAAGGGTTGA-3’; PGC1α CRE region, 5’-CAAAGCTGGCTTCAGTCACA-3’ and 5’-AAAAGTAGGCTGGGCTGTCA-3’.

Statistical Analysis. Data were expressed as mean±SEM. Statistical tests were performed using SPSS software (version 16.0, SPSS Inc, Chicago, IL, USA). One-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test was used to determine multiple comparisons. Statistical significance was accepted at \( P<0.05 \).

Results

**HDAC1 is enriched in WAT vs. BAT, and is down-regulated in BAT during \( \beta\)-adrenergic stimulation, and during brown adipocyte differentiation.**

Recent studies reported that class I but not class II HDACi enhanced whole body energy expenditure and attenuated high fat diet-induced insulin resistance through increased mitochondrial biogenesis in skeletal muscle and adipose tissues (28). However, little is known whether this is exerted directly through activating brown fat thermogenesis and which HDAC is responsible for these beneficial effects. Thus, we first knocked down individual HDACs1-11 in brown adipocyte cell line HIB-1B cells using siRNA approach. Interestingly, whereas knocking down of other HDACs exerted minimal effects, reducing the expression of the class I HDAC family member HDAC1 by siRNA knockdown significantly enhanced NE-stimulated UCP1 expression (**Figure 1A**). This promoted us to investigate the role of HDAC1 in regulating brown adipocyte thermogenic program.

We first measured HDAC1 expression pattern in brown and white adipose tissues. As expected, UCP1 protein was enriched in BAT but not detectable in epididymal WAT (Epi WAT) in adult mice housed at ambient temperature (**Figure 1B**). Interestingly, in contrast to UCP1 expression, HDAC1 protein level was enriched in WAT, but much lower in BAT (**Figure 1B**). BAT1 and HIB-1B brown adipocyte differentiation was marked with significantly increased UCP1 mRNA and protein levels (**Figure 1C-D**), which was accompanied by decreased mRNA and protein levels of HDAC1 (**Figure 1C-D**).

It is well documented that cold temperature triggers sympathetic discharge, leading to the release of NE in BAT and WAT (3,38). Thus, we tested whether HDAC1 expression in BAT was regulated by sympathetic stimulation. A/J mice were exposed to cold (4ºC) or intraperitoneally injected with \( \beta\)-adrenergic agonist (CL-316,243) for up to 7 days. HDAC1 expression was profoundly reduced in BAT after 6 hours of cold exposure, which tended to stay reduced up to 7 days of cold exposure (**Figure 1E**). Similar reduction of HDAC1 expression was also observed in BAT after CL-316,243 injection for 7 days (**Figure 1F**). These data indicate that decreased HDAC1 may be a marker of mature brown adipocytes and that HDAC1 may negatively regulate BAT thermogenic program.

**HDAC1 regulates brown adipocyte thermogenic gene expression.**

To investigate the role of HDAC1 in the regulation of brown fat specific gene expression, we performed gain- or loss- of function experiments in differentiated BAT1 cells. It has been reported that HDAC1 regulates the early steps of adipocyte differentiation (39). Thus, to avoid the confounding effects of HDAC1 on differentiation, we have focused on the role of
HDAC1 in mature brown adipocyte gene expression by knocking down or overexpressing HDAC1 in BAT1 cells after 4-6 days of differentiation. HDAC1 mRNA in knockdown cells was reduced by 80% as measured by real time RT-PCR, and HDAC1 protein expression was also significantly decreased, as assessed by immunoblotting (Figure 2A). As expected, knocking down HDAC1 in mature BAT1 cells did not affect the mRNA levels of general adipocyte differentiation markers including PPARγ, C/EBPα, Sterol regulatory element-binding protein α (SREBP1C), adipocyte protein 2 (aP2) and adiponectin (AdipoQ) (Figure 2B). Remarkably, HDAC1 knockdown in BAT1 cells significantly increased basal and Isoproterenol-stimulated expression of brown adipocyte-specific genes, including UCP1, PGC1α (Figure 2C), PGC1β, PR-domain containing protein 16 (PRDM16), PPARα, type 2 deiodinase (DIO2), acyl CoA oxidase 1 (ACOX1), cytochrome c oxidase 1 (COX1), epithelial V-like antigen 1 (EVA1) and otopetrin 1 (OTOP1) (Figure 2D). In addition, isoproterenol significantly stimulated UCP1 protein expression in BAT1 cells, which was further enhanced by HDAC1 knockdown (Figure 2E).

We then over-expressed HDAC1 in BAT1 brown adipocytes, as shown by increased HDAC1 protein expression (Figure 3A). Overexpression of HDAC1 in BAT1 brown adipocytes significantly suppressed basal and/or Isoproterenol-stimulated brown adipocyte-specific gene expression, including UCP1, PGC1α, PGC1β, PRDM16, C/EBPβ, fibroblast growth factor 21 (FGF21), PPARα, carnitine palmitoyltransferase 1B (CPT1B), ACOX1, COX1, OTOP1 and cell death-inducing DNA fragmentation factor α subunit-like effector A (CIDEA) (Figure 3B-C).

Similar results were observed in HIB-1B brown adipocyte cell line. In HIB-1B cells with HDAC1 siRNA knockdown, HDAC1 mRNA was reduced by more than 80% (Figure 4A), and knocking down HDAC1 significantly increased basal and/or NE-stimulated brown adipocyte-specific gene expression, including UCP1 (Figure 4B), PGC1α, PGC1β, COX1, ACOX1, CIDEA, PRDM16, CPT1B and PPARα (Figure 4C-D). Similarly, HDAC1 overexpression significantly reduced NE-stimulated UCP1 expression in HIB-1B cells (Figure 4E). These data demonstrate that HDAC1 is a negative regulator of the thermogenic program in mature brown adipocytes.

**HDAC1 deficiency mediates the beneficial effects of class I HDACi on improving metabolic phenotypes.**

It is reported that the pan-HDACi SAHA and class I HDACi MS-275, but not class II HDACi MC-1568 ameliorate obesity and diabetes in animal models, possibly through stimulation of oxidative phosphorylation and mitochondrial function in muscle and fat (28). To our surprise, treating BAT1 brown adipocytes with the pan-HDACi TSA and SAHA resulted in complex effects on brown-fat-specific gene expression. Whereas TSA and SAHA enhanced isoproterenol-stimulated expression of genes involved in mitochondria oxidative activity, such as PGC1α and ACOX1, they significantly suppressed isoproterenol-stimulated other BAT-specific gene expression, including UCP1, PRDM16, PPARγ and OTOP1 (Figure 5A-B). In contrast, the class I HDACi MS-275 significantly enhanced isoproterenol-stimulated BAT-specific gene expression, including UCP1, ELOVL3 and PGC1α, whereas it had minimal effects on PRDM16 and PPARγ expression (Figure 5C).

We also tested whether HDAC1 might be the molecular target of class I HDACi MS-275 in regulating BAT-specific gene expression in BAT1 cells. As expected, MS275 or HDAC1
siRNA knockdown enhanced isoproterenol-stimulated gene expression of UCP1 and PGC1α in BAT1 cells to a similar extent when treated individually; however, they did not exert any additive effects on these gene expressions when treated in combination in BAT1 cells (Figure 5D). Our data suggest that HDAC1 may be the molecular target of the class I HDACi MS-275 in stimulating brown fat function.

Sympathetic activation in brown adipocytes reduces HDAC1 binding and increases histone H3 lysine 27 (H3K27) acetylation at UCP1 and PGC1α promoters.

HDACs repress gene expression by removing acetyl groups from lysine residues in histone proteins at specific gene promoters (18). We reasoned that if HDAC1 indeed negatively regulates brown fat functions, sympathetic activation in BAT may trigger HDAC1 dissociation from promoter regions of key genes regulating brown fat thermogenesis, which should allow transcriptional activation of these genes and subsequently the full activation of brown adipocyte thermogenic program. UCP1 is responsible for mitochondrial uncoupling in BAT (2-4), whereas PGC1α is the central regulator of brown adipocyte thermogenesis (40,41). A ~220bp upstream enhancer element along with the proximal promoter that closer to the transcriptional start site have been identified at the UCP1 promoter that mediates sympathetic-stimulated, and tissue-specific expression of UCP1 (42-44). In addition, PGC1α expression is highly induced via β-adrenergic receptor activated protein kinase A (PKA)/cAMP response element (CRE) binding protein (CREB) pathway (45,46) through the CRE cis-element on PGC1α promoter (28,47). We therefore examined the binding of HDAC1 at the enhancer and proximal promoter regions of UCP1 and the CRE cis-element on PGC1α promoter (Figure 6A) upon sympathetic activation. As expected, in BAT of mice treated with β3-adrenergic agonist CL-243,316, binding of HDAC1 was significantly reduced at the enhancer and proximal promoter regions of UCP1 (Figure 6B-C), and the CRE cis-element region at PGC1α promoter (Figure 6D). In addition, isoproterenol treatment also significantly reduced HDAC1 binding to these promoter/enhancer regions in differentiated BAT1 brown adipocytes (Figure 6E). These data indicate that β-adrenergic stimulation in brown adipocytes dissociates HDAC1 from promoters of key genes regulating brown adipocyte thermogenesis, including UCP1 and PGC1α. This may facilitate subsequent chromatin modifications and DNA unwinding, which can eventually lead to increased accessibility to transcription factor binding and gene activation (17-19).

Acetylation at histone H3 lysine 27 (H3K27ac) results in transcriptional activation (48). We found that isoproterenol treatment in BAT1 cells significantly increased H3K27ac levels at the enhancer and proximal promoter regions of UCP1 and the CRE region at PGC1α promoter (Figure 6F-G). Importantly, reducing HDAC1 expression by siRNA knockdown induced a similar increase in H3K27ac levels and further enhanced isoproterenol-stimulated increase in H3K27ac levels (Figure 6F); whereas HDAC1 overexpression completely blocked isoproterenol-induced increase in H3K27ac levels at these promoter/enhancer regions (Figure 6G).

On the other hand, the acetylation levels at histone H3 lysine 14 (H3K14ac) and histone H3 lysine 9 (H3K9ac), two other lysine residues that can be modified by acetylation (18,49), did not exhibit similar responsiveness to HDAC1 knockdown and/or isoproterenol stimulation in BAT1 cells (Figure 6H). Thus, our data suggest that H3K27 may be the major target of HDAC1 in brown adipocytes regulating brown-specific gene expression.
HDAC1 regulates UCP1 and PGC1α expression by further modifying H3K27 methylation.

Except for acetylation, histone lysine residues can also be mono-, di- and tri-methylated, which are associated with either gene repression or activation, depending on the lysine residues that are methylated and the degree of methylation. Tri-methylation of H3K27 (H3K27me3) is a hallmark of gene repression, whereas trimethylation of H3K4 (H3K4me3) marks transcriptional activation (17-19). Importantly, histone acetylation and methylation mutually affects each other in the regulation of transcriptional process (49). We thus tested whether modulation of histone acetylation by HDAC1 led to further alterations in histone methylation. ChIP assay analysis demonstrated that isoproterenol treatment in BAT1 brown adipocytes resulted in a significant decrease in H3K27me3 levels at UCP1 and PGC1α promoters (Figure 7A-B). Reducing HDAC1 expression by siRNA knockdown mimicked isoproterenol’s effect by reducing H3K27me3 levels at these promoter/enhancer regions to a similar extent as isoproterenol, and did not further decrease isoproterenol-suppressed H3K27me3 levels (Figure 7A). In contrast, HDAC1 overexpression significantly blocked isoproterenol-suppressed H3K27me3 levels at these promoter/enhancer regions (Figure 7B).

H3K27 methylation is dynamically regulated by both histone methyltransferases and demethylases (18,50,51). EZH2 is a methyltransferase that specifically di- and tri-methylates H3K27 (18,50,51), whereas lysine-specific demethylase 6A (KDM6A)/ubiquitously transcribed tetricopeptide repeat on chromosome X (UTX) is a di- and tri-methyl-H3K27 demethylase (52). ChIP assay demonstrated that isoproterenol treatment in BAT1 brown adipocytes resulted in a significant decrease in EZH2 binding at UCP1 and PGC1α promoters (Figure 7C-D). Reducing HDAC1 expression by siRNA knockdown mimicked isoproterenol’s effect by reducing EZH2 binding at these promoter/enhancer regions to a similar extent as isoproterenol, and did not further decrease isoproterenol-suppressed EZH2 binding (Figure 7C); whereas HDAC1 overexpression completely reversed isoproterenol-induced suppression of EZH2 binding (Figure 7D).

In contrast, isoproterenol treatment in BAT1 brown adipocytes resulted in a significant increase in UTX binding at UCP1 and PGC1α promoters (Figure 7E-F). Reducing HDAC1 expression by siRNA knockdown also induced a similar increase in UTX binding to UCP1 enhancer region, and further enhanced isoproterenol-induced binding of UTX to UCP1 proximal promoter and PGC1α CRE region (Figure 7E); whereas HDAC overexpression completely prevented isoproterenol-induced increase in UTX binding to these promoter/enhancer regions (Figure 7F). Overall, our data demonstrated that HDAC1 may coordinately regulate H3K27ac and H3K27me3 levels at UCP1 and PGC1α promoters through differential recruitment of the H3K27 methyltransferase EZH2 and the H3K27 demethylase UTX, thus regulates these genes expression.

EZH2 is a component of the polycomb group (PcG) proteins, which are known to mediate gene silencing by regulating chromatin structure (51). Two major PcG proteins exist in mammals, namely polycomb repressive complex 1 and 2 (PRC1 and PRC2). PRC1 comprises three main components: ring finger protein 1 (RING1), RNF2 and Bmi1 polycomb ring finger oncogene (BMI1). PRC2 comprises three major components: EZH2, SUZ12 and embryonic ectoderm development (EED). PRC2 promotes H3K27 methylation through the methyltransferase activity of EZH2, and also facilitates the recruitment of PRC1 onto
methylated H3K27, which in turn leads to further gene repression (51). It has been shown that HDAC1 may be associated with PRC2 (51). We thus investigated whether HDAC1 regulates the recruitment of components of PRC1/2 to UCP1 and PGC1α promoters. ChIP assay demonstrated that isoproterenol treatment in BAT1 cells decreased binding of SUZ12 (Figure 8A, 8C) and RNF2 (Figure 8B, 8D) to UCP1 and PGC1α promoters. Importantly, reducing HDAC1 expression by siRNA knockdown mimicked isoproterenol’s effect by inducing a similar decrease in SUZ12 and RNF2 binding, and did not further reduce isoproterenol-suppressed SUZ12 and RNF2 binding at these promoters (Figure 8A-B); whereas HDAC1 overexpression completely prevented isoproterenol-suppressed SUZ12 and RNF2 binding to these promoters (Figure 8C-D).

We further performed co-IP experiments to test whether HDAC1 physically interacts with components of PRC1 and PRC2 complexes. Our data demonstrated that HDAC1 interacts with the PRC2 complex components EZH2 and SUZ12, and the PRC1 component RNF2 in HEK293T cells overexpressing HDAC1, EZH2, SUZ12 and RNF2 (Figure 8E) and, most importantly, in endogenous BAT1 brown adipocytes (Figure 8F).

Discussion

Recent data suggest that HDACs have emerged as important players in the regulation of energy and glucose homeostasis (27). Pan-HDAC inhibitors sodium butyrate and trichostatin A increases energy expenditure, reduces adiposity and improves insulin sensitivity in diet-induced obese (DIO) mice (53). This is possibly exerted through the inhibition of class I HDACs, as the specific class I HDACi exerts similar effects in DIO mice, whereas class II HDACi has no effects (28). However, the precise mechanism by which class I HDACi exerts these beneficial effects is poorly understood. Here, we demonstrate that the class I HDAC1 negatively regulates brown adipocyte thermogenic program. This is based on the following observations. Firstly, in contrast to UCP1 expression, which is usually enriched in BAT, HDAC1 is enriched in WAT, but its expression is much lower in BAT. Secondly, brown adipocyte differentiation is associated with significantly up-regulated UCP1 expression, which is concomitantly associated with reduction of HDAC1 RNA and protein levels. Thirdly, sympathetic activation of BAT is associated with down-regulation of HDAC1 expression. These data suggest that HDAC1 may be a negative regulator of brown adipocyte thermogenic program. Indeed, knocking down HDAC1 in brown adipocytes significantly up-regulates BAT-specific gene expression, whereas overexpressing HDAC1 significantly suppresses BAT-specific gene expression, suggesting a causative link between HDAC1 inhibition and up-regulation of brown adipocyte thermogenic program. Activating brown/beige adipocyte thermogenesis alleviates obesity and its associated metabolic diseases (9,11,54-56). Thus, our data suggest that HDAC1 may be important in regulating energy homeostasis in animal models, and inhibiting HDAC1 in brown adipocytes may contribute to the beneficial effects of class I HDACi in DIO mice (28). Further studies using genetic animal models with brown fat-specific deletion or overexpression of HDAC1 will be needed to study the role of HDAC1 in energy homeostasis and metabolism in whole animals.

Interestingly, we found that whereas both the pan-HDACi TSA and SAHA and the class I-specific HDACi MS-275 (57) stimulate the expression of genes involved in mitochondrial function and oxidative activity, such as PGC1α, the effect of these different classes of HDACis on the expression of other genes that are
important for brown adipocyte differentiation and determination is different. For example, the class I HDACi MS-275 potently stimulates; whereas the pan-HDACis TSA and SAHA significantly suppress, isoproterenol-stimulated UCP1 expression in brown adipocytes. In addition, TSA also significantly inhibited PRDM16 and PPARγ expression; whereas MS-275 had minimal effects on these gene expressions. These TSA-suppressed genes are either key players in brown adipocyte differentiation and determination (PPARγ and PRDM16), or marker of brown adipocyte (UCP1). Since TSA and SAHA inhibit both class I and II HDACs (57), and since it has been shown that class II HDACs are mainly involved in the regulation of cellular differentiation (58), the difference in the gene regulation between the pan-HDACi TSA and the class I HDACi MS-275 may be mainly due to the different function of class I and class II HDACs. Thus, further studies are warranted to elucidate the differential effects of class I and II HDACis on brown adipocyte function. Nonetheless, our data suggest that the class I HDACi MS-275 may contribute to its beneficial effect on improving metabolic phenotypes in DIO mice (as reported by Galmozzi et al (28)) at least partly by directly stimulating brown adipocyte function, and HDAC1 may mediate this effect. Thus, class I HDACi such as MS-275 and more specifically, HDAC1, may serve as promising therapeutic targets in treating obesity and associated metabolic syndrome.

Histone acetylation and methylation are epigenetic mechanisms that regulate gene expression by remodeling chromatin structure (17-19). Whereas H3K27ac is a histone transcriptional activation mark, H3K27me3 serves as a histone transcriptional repression mark (17-19,48). Histone acetylation is regulated by balanced action of HATs and HDACs, whereas histone methylation is regulated by histone methyltransferases and demethylases (17-19). Specifically, the di- and tri-methylation of H3K27 is catalyzed by the PRC2 complex. Within PRC2, EZH2 is the catalytic subunit that possesses methyltransferase activity towards H3K27, and its activity also requires binding to the two other PRC2 protein components, SUZ12 and EED (51). The di- and tri-methylated H3K27 further recruits PRC1 to the target genes, which, through the E3 ubiquitin ligase activity of the PRC1 components RING1 and RNF2, induces further chromatin compaction through histone 2A ubiquitination (51). On the other hand, UTX is a histone demethylase that specifically demethylates di- and tri-methylated H3K27 (52). Interestingly, we find that HDAC1 physically interacts with the PRC2 components EZH2 and SUZ12, and the PRC1 catalytic subunit RNF2, suggesting a role of HDAC1 in connecting H3K27 deacetylation to methylation, and possibly further chromatin compaction by histone ubiquitination. Indeed, we find that knocking down HDAC1 in brown adipocytes results in not only an increase in H3K27ac, it also leads to reduced H3K27me3 in promoters of BAT-specific genes, including UCP1 and PGC1α. This is exerted through the dissociation of the PRC2 components EZH2 and SUZ12, and PRC1 catalytic subunit RNF2 from these promoters and a reciprocal recruitment of the H3K27 demethylase UTX to these promoters. Thus, our data demonstrate that HDAC1 negatively regulates brown adipocyte thermogenic program through interaction with PRC1/2 complexes, thereby promoting H3K27 deacetylation and methylation at promoters of BAT-specific genes, such as UCP1 and PGC1α. In addition, our data also demonstrate that β-adrenergic activation induces dissociation of HDAC1 along with PRC complexes from UCP1 and PGC1α promoters, which in turn leads to gene activation.

Sympathetic signaling is essential in brown/beige adipocyte activation (9-13). We find that β-adrenergic activation in BAT1 adipocytes is associated with increased H3K27ac
and decreased H3K27me3 through dissociation of HDAC1 and PRC complexes and concomitant recruitment of UTX on BAT-specific gene promoters, including UCP1 and PGC1α. These data indicate that HDAC1 may be one of the epigenetic modulations triggered by sympathetic signaling in brown adipocytes, which eventually leads to thermogenic activation. It would be interesting to study how β-adrenergic stimulation triggers the dissociation of HDAC1 from BAT-specific gene promoters in brown adipocytes. HDAC1 is a part of the catalytic core of several multimeric corepressor complexes including SIN3A, nucleosome remodeling deacetylase (NuRD) and corepressor of R1-silencing transcription factor (CoREST) (59-61), and is also a part of the PRC2 complex (51). The recruitment of these multiprotein complexes is usually triggered by cell-specific transcriptional factors or the histone-recognition motifs found within the complex components (59-61). In this context, Retinoblastoma protein (pRB) and Receptor interacting protein 140 (RIP140) are potent negative regulators of BAT-specific gene expression (62). It has been reported that pRB and RIP140 silences promoter activity and gene expression through recruiting HDACs, including HDAC1 (63-65). Thus, it is possible that HDAC1 may be recruited to BAT-specific promoters through association with negative transcriptional regulators of BAT, such as pRB and RIP140.

In addition, HDAC1 itself is subjected to various post-transcriptional modifications. For example, phosphorylation of HDAC1 by casein kinase II (CKII) up-regulates its activity, whereas acetylation of HDAC1 by the acetyltransferase p300 suppresses its activity (61). Interestingly, recent studies using phosphoproteomics have identified CKII as a negative regulator of BAT function through phosphorylating and regulating HDAC1 activity (66). Thus, our data fall in line with the results from Shinoda et al, and further demonstrate the importance of HDAC1 in the regulation of BAT function.

Moreover, HDAC1 activity or protein levels can also be regulated by ubiquitination, SUMOylation, nitrosylation and carbonylation (61). Further study is required to decipher the cellular signaling cascades and mechanisms that regulate HDAC1 activity and recruitment to BAT-specific promoters in response to sympathetic and other stimuli in brown adipocytes.

In the present study, we have focused on the epigenetic regulation of PGC1α by HDAC1, as it is well established that PGC1α plays a central role in regulating important pathways involved in mitochondrial biogenesis and thermogenesis(40,41). However, we have found that HDAC1 regulates H3K27 acetylation and methylation at the promoter and enhancer regions of UCP1, a brown adipocyte terminal differentiation marker. Thus, it would be interesting to know whether HDAC1 also regulates H3K27 deacetylation and methylation at other genes’ promoters to regulate their transcription. Unbiased approaches such as ChIP sequencing will be required to explore the gene profile HDAC1 regulates through H3K27 deacetylation.

Although both express UCP1 and share striking similarities in morphological and biological properties, traditional brown fat and inducible beige adipocytes are derived from distinct cell origins during embryonic development (7,8,67). In rodents, traditional brown adipocytes are originated from the skeletal muscle lineage (7), while at least a subset of beige cells arise from the smooth muscle origin (8). A recent study shows that human brown adipocytes possess molecular features similar to those of rodent beige cells (68). We used the brown fat cell BAT1 in this study. Thus, it is not clear whether the role of HDAC1 in regulating brown adipocyte function can be extrapolated to beige cells. Additional
studies, involving knocking down or overexpression of HDAC1 in beige lineage cells, will be warranted to determine the role of HDAC1 in the regulation of beige cell function.

In the present study, we investigated the role of HDAC1 in brown fat gene expression at the mature adipocyte stage. It has been reported that HDAC1 inhibits white adipocyte differentiation by deacetylating H4 at the promoter of C/EBPα, an important regulator of adipogenesis (39). In addition, we find that HDAC1 RNA and protein levels are down-regulated during brown adipocyte differentiation. Thus, it would be interesting to study whether HDAC1 also regulates brown adipogenesis and subsequently affects brown fat thermogenic program.

In summary, we have identified HDAC1 as a negative regulator of brown adipocyte thermogenic program. Our data show that HDAC1 is down-regulated during brown adipocyte differentiation and is also suppressed by sympathetic activation in BAT. Overexpressing HDAC1 blocks, whereas knocking down HDAC1 further enhances β-adrenergic agonist-stimulated BAT-specific gene expression in brown adipocytes. Remarkably, HDAC1 physically interacts with PRC1/2 complexes, and activation of β-adrenergic signaling dissociates HDAC1 along with PRC1/2 complexes from UCP1 and PGC1α promoters, and concomitantly recruits UTX to these promoters, leading to increased H3K27 acetylation and decreased H3K27me3 levels in these promoters. These coordinated changes switch transcriptional repressive state to transcriptional active state at the promoters of UCP1 and PGC1α, which in turn activates brown thermogenic program. Thus, our data demonstrate that HDAC1 negatively regulates brown adipocyte gene expression by coordinated regulation of H3K27 deacetylation and methylation and inhibiting HDAC1 promotes brown adipocyte thermogenic program. Targeting HDAC1 may be a novel therapeutic target in the treatment of obesity by promoting brown adipocyte thermogenesis and energy dissipation.

Acknowledgements
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions
F.L., R.W., X.C., and L.Z. performed most experiments. L.Y. contributed to discussion and reviewed/edited the manuscript. B.X., H.S. and F.L. conceived the hypothesis, designed the study, analyzed the data and wrote the manuscript.

References


**Figure legends**

**Fig.1 The expression pattern of HDAC1.** (A) Reducing HDAC1 expression by siRNA knockdown in HIB-1B brown adipocytes up-regulates norepinephrine (NE) stimulated UCP1 expression. HIB-1B cells were transfected with scramble siRNA or siRNAs targeting individual HDACs. After 48 hours, cells were treated with or without 1μM NE for 4 hours and RNA was isolated for gene expression measurements. Class I HDACs were in blue. (B) UCP1 and HDAC1 protein levels in brown adipose tissues (BAT) and epididymal (Epi) white adipose tissues (WAT) from C57BL/6J (B6) mice. (C-D) UCP1 and HDAC1 RNA (C) and protein (D) levels in BAT1 and HIB-1B brown adipocytes during differentiation. (E-F) HDAC1 expression in A/J mouse BAT tissues exposed to 4°C at indicated time (E) or treated with β3-agonist CL-316,243 for 7 days. Data were expressed as mean ± SEM. *P< 0.05 versus undifferentiated samples (C), Time 0 (E) or Control (Ctrl) (F).

**Fig.2 Reducing HDAC expression promotes brown specific gene expression in BAT1 brown adipocytes.** BAT1 brown adipocytes were transfected with scramble or HDAC1 siRNA. Two days later, cells were treated with isoproterenol (Isop, 1μM) for 3 hours and RNA and protein were isolated for gene expression and protein level measurements. (A) HDAC1 RNA and protein levels in scramble or HDAC1 siRNA transfected cells. (B-D) The mRNA levels of various genes in scramble or HDAC1 siRNA transfected cells. n=6-8 per group. (E) UCP1 protein levels in scramble or HDAC1 siRNA transfected cells. Left: UCP1 immunoblot; right: quantitation of UCP1 protein levels normalized to α-tubulin. n=3 per group. Data are expressed as mean ± SEM. * p<0.05. ns: not statistically significant.

**Fig.3 HDAC1 over-expression attenuates mRNA expression of brown specific genes.** BAT1 brown adipocytes were transfected with pSPORT vector or HDAC1 cDNA. Two days later, cells were treated with isoproterenol (isop, 1μM) for 3 hours and RNA and protein were isolated for gene expression and HDAC1 protein level measurements. (A) HDAC1 protein levels in BAT1 cells transfected with pSPORT Vector or HDAC1 cDNA. (B-C) Expression levels of BAT-specific genes in BAT1 cells transfected with pSPORT Vector or HDAC1 cDNA. Data are expressed as mean ± SEM. n=6-8 per group. *p<0.05. ns: not statistically significant.

**Fig.4 HDAC1 negatively regulates brown specific gene expression in HIB-1B brown adipocytes.** (A-D) Reducing HDAC1 expression by siRNA knockdown in HIB-1B cells promotes brown specific gene expression. HIB-1B brown adipocytes were transfected with scramble or HDAC1 siRNA. Two days later, cells were treated with 1μM NE for 4 hours and RNA was isolated for gene expression analysis, including HDAC1 (A), UCP1 (B), PGC1α, PGC1β, COX1, ACOX1 (C), CIDEA, PRDM16, CPT1B and PPARα (D). (E) HDAC1 overexpression attenuates UCP1 mRNA levels. HIB-1B cells were transfected with pSPORT vector or HDAC1 overexpressing plasmids. Two days later, cells were treated with 1μM NE for 4 hours and mRNA levels of UCP1 were measured by RT-PCR. Data are expressed as mean ± SEM. n=6-8 in (A-D) and 4-6 in (E). *p<0.05. ns: not statistically significant.

**Fig. 5 HDAC1 mediates the effects of class I HDACi MS-275 on BAT-1 brown adipocyte gene expression.** (A-C) Isoproterenol (Isop)-stimulated gene expression in BAT1 cells treated with
different concentration of pan-HDACi Trichostatin A (TSA) (A), SAHA (B) and the class I HDACi MS-275 (C). (D) Isoproterenol-stimulated gene expression in BAT1 cells treated with MS-275 (5μM) or HDAC1 siRNA individually or in combination. Data are expressed as mean ± SEM. n=6. *p<0.05

Fig.6 HDAC1 regulates H3K27 acetylation (H3K27ac) at PGC1α and UCP1 promoters. (A) Schematic illustration of promoter/enhancer regions of UCP1 and PGC1α genes. (B-D) β3 adrenergic agonist CL-316,243 treatment dissociates HDAC1 from UCP1 and PGC1α promoters in BAT of AJ mice. AJ mice were treated with CL-316,243 for indicated time and BAT was collected for ChIP assay to measure HDAC1 binding to UCP1 and PGC1α promoters as described in Materials and Methods. (E) Isoproterenol treatment dissociates HDAC1 from UCP1 and PGC1α promoters in BAT1 brown adipocytes. BAT1 cells were treated with isoproterenol (Isop, 1μM) for 3 hours. Cells were collected, and ChIP assays were performed to measure HDAC1 binding to UCP1 and PGC1α promoters. (F-G) H3K27ac levels in UCP1 and PGC1α promoters in BAT1 brown adipocytes. In (F), BAT1 cells were transfected with scramble or HDAC1 siRNA for 2 days; in (G), BAT1 cells were transfected with pSPORT6 or pSPORT6-HDAC1 overexpressing plasmids for 2 days. Cells were then treated with 1μM isoproterenol for 3 hours. ChIP assay was performed as described in Materials and Methods. (H) H3K14ac and H3K9ac levels in UCP1 and PGC1α promoters. Data are expressed as mean ± SEM. n=6. *p<0.05. ns: not statistically significant.

Fig.7 HDAC1 regulates tri-methylation of H3K27 (H3K27me3) at UCP1 and PGC1α promoters through coordinated regulation of EZH2 and UTX binding to BAT-specific promoters. H3K27me3 levels (A-B), EZH2 (C-D) and UTX (E-F) binding at UCP1 and PGC1α promoters in BAR1 cells. BAT1 brown adipocytes were transfected with scramble or HDAC1 siRNA (A-C, E) or pSPORT6 or pSPORT6-HDAC1 overexpressing plasmids (B, D, F). Two days later, cells were treated with or without isoproterenol (Isop, 1μM) for 3 hours and ChIP assay was performed as described in Materials and Methods. Data are expressed as mean ± SEM. n=4-6. *p<0.05. ns: not statistically significant.

Fig.8 HDAC1 regulates the recruitment of polycomb repressor complexes to UCP1 and PGC1α promoters. (A-D) SUZ12 and RNF2 binding at UCP1 and PGC1α promoters in BAT1 cells. BAT1 brown adipocytes were transfected with scramble or HDAC1 siRNA (A-B) or pSPORT6 or pSPORT-HDAC1 overexpressing plasmids (C-D). Two days later, cells were treated with or without isoproterenol (Isop, 1μM) for 3 hours and ChIP assay was performed as described in Materials and Methods. (E-F) HDAC1 interacts with EZH2, SUZ12 and RNF2. In (E), HEK293T cells were transfected with pSPORT vector or co-transfected with HDAC1, EZH2, SUZ12 and RNF2 cDNA. Two days later, cell lysates were collected to detect protein interactions by co-immunoprecipitation (co-IP). Left: overexpression of HDAC1, EZH2, SUZ12 and RNF2 was verified by immunoblotting using whole cell lysates. Right: IP of HDAC1 pulls down EZH2, SUZ12 and RNF2. In (F), the interaction between HDAC1 and components of the polycomb repressor complexes in endogenous BAT1 cells was measured by co-IP. Data are expressed as mean ± SEM. n=4-6 in (A-D). Blots in (E-F) are representative of two-three independent experiments. *p<0.05. ns: not statistically significant.
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Figure 1

A) UCP1 mRNA expression levels in different siRNA treated groups. The y-axis represents fold change relative to control.

B) Western blot analysis of BAT, Epi (WAT), UCP1, HDAC1, and Tubulin. Bars represent bands for each sample.

C) Quantification of UCP1 and HDAC1 mRNA expression levels in BAT1 and HIB-1B cells. * indicates statistical significance.

D) Time course of BAT1 differentiation. Western blot analysis showing UCP1, HDAC1, and Tubulin expression levels at different time points.

E) Time course of HDAC1 mRNA expression levels in BAT1 cells exposed to cold. * indicates statistical significance.

F) Effect of β3 adrenergic agonist on HDAC1 mRNA expression levels in BAT cells. * indicates statistical significance.

Figure 1
Figure. 2
Figure 3

A

HDAC1

Tubulin

- - + + pSPORT Vector
- + - + HDAC1
- - + + Isop

B

Relative mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>pSPORT6, Basal</th>
<th>HDAC1, Basal</th>
<th>pSPORT6, Isop</th>
<th>HDAC1, Isop</th>
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</thead>
<tbody>
<tr>
<td>UCP1</td>
<td></td>
<td>*</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>PGC1α</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>PGC1β</td>
<td></td>
<td>*</td>
<td>n.s</td>
<td>n.s</td>
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<tr>
<td>PRDM16</td>
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<td></td>
<td></td>
<td>*</td>
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<tr>
<td>CEBPβ</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

C

Relative mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>pSPORT6, Basal</th>
<th>HDAC1, Basal</th>
<th>pSPORT6, Isop</th>
<th>HDAC1, Isop</th>
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<td>FGF21</td>
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<tr>
<td>PPARα</td>
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<tr>
<td>OTOP1</td>
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<tr>
<td>CIDEA</td>
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<td></td>
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</tbody>
</table>
Figure 5
Figure 7

A) H3K27me3 ChIP

B) H3K27me3 ChIP

C) EZH2 ChIP

D) EZH2 ChIP

E) UTX ChIP

F) UTX ChIP

* indicates significant differences.

Legend:
- Scramble, Basal
- HDAC1 siRNA, Basal
- Scramble, Isopropyl
- HDAC1 siRNA, Isopropyl

Figure 7
Histone Deacetylase 1 (HDAC1) Negatively Regulates Thermogenic Program in Brown Adipocytes via Coordinated Regulation of H3K27 Deacetylation and Methylation
Fenfen Li, Rui Wu, Xin Cui, Lin Zha, Liqing Yu, Hang Shi and Bingzhong Xue
J. Biol. Chem. published online January 5, 2016

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