HDAC3 is a molecular brake of the metabolic switch supporting white adipose tissue browning

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White adipose tissue (WAT) can undergo a phenotypic switch, known as browning, in response to environmental stimuli such as cold. Post-translational modifications of histones have been shown to regulate cellular energy metabolism, but their role in white adipose tissue physiology remains incompletely understood. Here we show that histone deacetylase 3 (HDAC3) regulates WAT metabolism and function. Selective ablation of Hdac3 in fat switches the metabolic signature of WAT by activating a futile cycle of de novo fatty acid synthesis and β-oxidation that potentiates WAT oxidative capacity and ultimately supports browning. Specific ablation of Hdac3 in adipose tissue increases acetylation of enhancers in Pparg and Ucp1 genes, and of putative regulatory regions of the Ppara gene. Our results unveil HDAC3 as a regulator of WAT physiology, which acts as a molecular brake that inhibits fatty acid metabolism and WAT browning.

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White (WAT) and brown (BAT) adipose tissues differ in their morphology and functionality and are marked by distinct metabolic signatures. However, upon specific environmental cues (for example, cold exposure), WAT switches to a brown-like phenotype in a phenomenon called browning. The identification of factors that may contribute to control browning is still a matter of intense investigations. Several studies demonstrated that HDACs participate in metabolic regulation of different organs, especially highlighting a pivotal role played by histone deacetylase 3 (HDAC3) as modulator of metabolic reprogramming in liver, heart, and adipose tissue physiology; furthermore, genetic ablation in adipose tissue of NCoR1, a co-repressor interacting with HDAC3, improved adipose tissue functionality, and insulin resistance. Several groups reported evidences about a link between metabolism and post-translational modifications of chromatin, sparking new interest in the role of epigenome modifiers in determining the metabolic signature of specific tissues. However, the links between metabolic phenotype and chromatin modifications (for example, histone acetylation), and the underlying regulators of the epigenome dynamics in adipose tissue have started to emerge only recently.

By using a combination of transcriptomic, metabolomic, and proteomic approaches coupled with genetic manipulation of mouse and cellular models, we show that HDAC3 regulates the metabolic phenotype of BAT by acting as a molecular brake of the cellular metabolic rewiring that sustains WAT browning.

**Results**

**Differential expression of metabolic genes in BAT vs. WAT.** To characterize the phenotype of fat specific Hdac3 KO mice, we first verified that floxed mice, here used as controls for comparison vs. H3atKO mice, display the typical molecular signature underlying the distinct morphological and functional features of WAT and BAT. To this end, we conducted a focused gene expression profiling of inguinal WAT (IngWAT) and BAT in floxed C57Bl/6j mice. As expected, this analysis showed that BAT is

![Fig. 1](image-url)
characterized by remarkably high mRNA levels of brown fat specific genes such as those encoding for uncoupling protein 1 (Ucp1), β3-adrenergic receptor (Adrb3), deiodinase 2 (Dio2), vascular endothelial growth factor 2a (Vegfa2), PR domain containing 16 (Prdm16) and peroxisome proliferator-activated receptor α (Ppara) (Fig. 1a). However, this comparison revealed that BAT also showed exceptional upregulation of several genes involved in lipid, glucose, and citrate metabolism (Fig. 1a), highlighting brown fat as an organ with “high metabolic rate”, with a concurrent activation of lipolytic/fatty acid (FA) β-oxidation and lipogenic gene programs in BAT, to meet the energy needs related to thermogenic function and lipogenic gene programs in BAT, to meet the energy needs related to thermogenic function

Fig. 2 Differential transcriptome profile in IngWAT of floxed vs. H3atKO mice. a Down-regulated pathways in IngWAT of H3atKO mice (n = 3 per group, where each sample represents a pool from 2 individual mice); b Upregulated pathways in IngWAT of H3atKO mice (n = 3 per group, where each sample represents a pool from 2 individual mice); c, d qPCR gene expression analysis of browning genes and adipose markers in IngWAT of floxed and H3atKO mice (n = 9–11 per group). Data in c and d are presented as mean ± s.e.m. Statistical analysis: Student’s t-test, *p < 0.05, **p < 0.01, and ***p < 0.001.

Adipose ablation of HDAC3 induces browning of white fat. Several studies demonstrated that HDACs participate in metabolic regulation of different organs4–7. We previously showed that chemical class I HDAC inhibitor MS-275 improves obesity and glucose tolerance in db/db and diet-induced obese mice, via induction of WAT browning8, 9. Since it has been demonstrated that genetic ablation of NCoR1 in adipose tissue11, a co-repressor interacting with HDAC3, improved adipose tissue functionality and insulin resistance12, we decided to investigate the role of HDAC3 in adipose tissue physiology and metabolism. To this end, we deleted Hdac3 in adipose tissues by breeding Hdac3 floxed mice with mice expressing the Cre recombinase under the control of the adiponectin gene promoter (Adipoq). Selective deletion of Hdac3 in adipose tissues (H3atKO) was confirmed by PCR and western blot (Fig. 1b, Supplementary Fig. 1b, Supplementary Fig. 2a, b, Supplementary Fig. 3a, b). Interestingly, we observed that, despite no differences in total body weight gain (Supplementary Fig. 1c), H3atKO mice showed reduction of epididymal WAT (EpiWAT; Supplementary Fig. 2c), accompanied by a mild, though not statistically significant, reduction of IngWAT (Supplementary Fig. 1d). Surprisingly, IngWAT appeared reddish (Fig. 1c) and hematoxylin/eosin staining showed a clear difference of cellular morphology, characterized by smaller, though not statistically significant, cell size and by the presence of multilocular adipocytes (Fig. 1d–f). The immunohistochemical analysis of IngWAT revealed strong increase of UCP1 staining (Fig. 1g). Most importantly, when exposed to cold H3atKO mice were able to maintain body temperature to values higher than those of floxed mice (Fig. 1h), thus suggesting browning of WAT in H3atKO mice, which would
Fig. 3 Adipose ablation of Hdac3 remodels WAT metabolism. a Heatmap of IngWAT metabolome in floxed and H3atKO mice (n = 7–8 per group); b Volcano plot of IngWAT metabolome in floxed and H3atKO mice (n = 7–8 per group); c Levels of acetyl-CoA and citrate in IngWAT of floxed and H3atKO mice (n = 7–8 per group); d Expression analysis of genes involved in citrate mitochondrial export in IngWAT of floxed and H3atKO mice (n = 9–11 per group); e Levels of glycolysis intermediates in IngWAT of floxed and H3atKO mice (n = 7–8 per group); f Expression analysis of genes involved in glucose metabolism in IngWAT of Hdac3 floxed and H3atKO mice (n = 9–11 per group); g Western blot and quantification of pSer-293 PDHE1α, pSer-300 PDHE1α, and PDHE1α. *-Tubulin in IngWAT of Hdac3 floxed and H3atKO mice (n = 3 per group). Data are presented as mean ± s.e.m. Statistical analysis: Student’s t-test, *p < 0.05, and ***p < 0.001.
contribute to improve the response to cold. RNA sequencing was performed in IngWAT from a subset of floxed and H3atKO animals fed standard diet for an initial characterization of transcriptome. Interestingly, we found that several genes were differentially regulated in IngWAT in response to adipose ablation of HDAC3 (Fig. 2a, b). Among upregulated transcripts we found genes belonging to functional annotations related to mitochondria, FA metabolism and Krebs cycle, indicating activation of exceptional oxidative program in white adipocytes upon HDAC3 deletion. Of note, several other genes were down-regulated in
H3atKO mice, indicating that ablation of this transcriptional repressor did not de-repress globally the entire transcriptome (1429 upregulated transcripts and 2246 down-regulated transcripts). Based on the results of RNA sequencing we decided to conduct quantitative PCR (qPCR) analysis on specific panels of genes, playing a role in adipose physiology and in WAT browning. We confirmed increased expression of classical brown fat marker genes (Ucp1, Dio2, Ppara, Prdm16, Adbr3, and Vegfa; Fig. 2c) and, concomitantly, higher expression of markers of adipocyte functionality (Pparg, Glut4, Fabp4, Adipoq, and Ptim; Fig. 2d) as well as of genes involved in oxidative metabolism (Idh3a, Bckdhb, Cox7a1, and Ppargc1a; Supplementary Fig. 1e) that clearly indicate transcriptional remodeling of IngWAT. Likewise, EpiWAT of H3atKO mice showed similar though milder phenotype, with no significant differences in adipocyte morphology compared to floxed mice (Supplementary Fig. 2d) but increased expression of browning genes, adipose markers, and oxidative metabolism genes (Supplementary Fig. 2e–g). Of note, levels of serum triglycerides (Supplementary Fig. 1f) and non-esterified FAs (NEFA) (Supplementary Fig. 1g) were reduced in H3atKO mice, while serum cholesterol levels did not differ (Supplementary Fig. 1h). Finally, livers from H3atKO, in which Hdac3 was not ablated (Supplementary Fig. 1i), did not show signs of lipid steatosis, as shown by hematoxylin and eosin staining (Supplementary Fig. 1j).

The increased thermogenic activity during cold exposure in H3atKO mice prompted us to analyze BAT from floxed and H3atKO mice. Surprisingly, we found that Hdac3 ablation in brown fat of mice housed at room temperature did not reprogram the expression of brown markers, except Dio2, nor that of genes involved in glucose and lipid metabolism (Supplementary Fig. 2f). More importantly, the expression of Ucp1 protein was not ablated (Supplementary Fig. 1i), did not show signs of lipid steatosis, as shown by hematoxylin and eosin staining (Supplementary Fig. 1j).

To explain these metabolic changes, we measured the expression of genes involved in citrate metabolism: we detected increased expression of citrate synthase (Cs) in H3atKO mice (Fig. 3d), suggesting that oxaloacetate could condensate to acetyl-CoA to form citrate. Further gene expression analysis also demonstrated induction of the gene encoding the mitochondrial citrate carrier Slc25a1, which exports citrate from mitochondria to cytosol. The expression of the gene encoding the ATP citrate lyase (Acly) cleaving citrate to acetyl-CoA and oxaloacetate (Fig. 3d) increased in H3atKO mice, explaining the reduction of citrate observed in these mice. Further metabolomic analysis also revealed reduced levels of glucose, glucose 6-phosphate, fructose 1,6-bisphosphate, and dihydroxyacetonate phosphate/glyceraldehyde 3-phosphate in H3atKO (Fig. 3e), indicating reduced accumulation of glycolysis intermediates, potentially consistent with greater conversion of glucose to pyruvate. A similar gene expression profile was observed in EpiWAT from H3atKO mice (Supplementary Fig. 4a, b). In analogy to BAT (Fig. 1a), the expression of pyruvate kinase Pkm and Pdk4 was highly augmented in IngWAT of H3atKO mice (Fig. 3f). Western blot analysis of pyruvate dehydrogenase (PDHE1α) showed increased phosphorylation at serine 293 and 300 (Fig. 3g) in IngWAT of H3atKO mice, correlating with reduced activity of the protein.20 These results indicate that Hdac3 deletion stimulates glycolysis, but at the same time prevents the conversion of pyruvate to acetyl-CoA. Metabolome analysis showed that both levels of pyruvate and lactate (Supplementary Fig. 5a) were reduced in WAT from H3atKO, excluding that in our model pyruvate is converted to lactate. In this regard, increased expression of Mpc1 and Pcx in knock out mice (Fig. 3i) suggested that pyruvate entered into mitochondria, where it is converted to oxaloacetate. The expression of cytosolic PEPCK (Pck1) showed a robust increase in H3atKO mice (Fig. 3f). Of note, this enzyme has been proposed to play a relevant role in BAT metabolism and non-shivering thermogenesis.21 We also found that intermediates of the pentose phosphate pathway were reduced in H3atKO mice (Supplementary Fig. 5b). Moreover, we found lower levels of TCA cycle intermediates in KO mice (Supplementary Fig. 5c), possibly as a consequence of considerable citrate export from mitochondria. These results point out that adipose ablation of Hdac3 profoundly remodeled metabolite in WAT, most likely as a consequence of reset gene expression program.

**Fig. 4** Hdac3 ablation activates a futile cycle of FA metabolism in adipocytes. a IngWAT fatty acid quantification by mass spectrometry in floxed and H3atKO mice (n = 7–8 per group); b Malonyl CoA levels in IngWAT of floxed and H3atKO mice (n = 7–8 per group); c Expression of lipolysis and FA β-oxidation genes in IngWAT of floxed and H3atKO mice (n = 9–11 per group); d Western blot analysis and quantification of ATGL and ACADL in IngWAT of floxed and H3atKO mice (n = 3 per group); e Expression of lipogenic genes in IngWAT of floxed and H3atKO mice (n = 9–11 per group); expression of Acolsy gene was the same reported in Fig. 3d; f Experimental paradigm of differentiation and infection of 3C/H10T1/2 cells; g Western blot analysis of HDAC3, showing the depletion of the protein in cells infected with adenovirus expressing a shRNA targeted to Hdac3 (shHdac3) vs. cell infected with a scrambled control shRNA (scramble; n = 3); h Expression of browning genes in scramble and shHdac3 infected cells (n = 4 biological replicates per group); i Morphology of scramble and shHdac3 infected cells, scale bar is 50 μm.; j Expression of lipolytic and lipogenic genes in scramble and shHdac3 infected cells (n = 4 biological replicates per group); k Glycerol release in scramble and shHdac3 infected cells (n = 10 per group); l–n 13C-labeled acetyl-CoA, citrate, and palmitate in scramble and shHdac3 infected cells (n = 6 per group). Data are presented as mean ± s.e.m. Statistical analysis: Student’s t-test, *p < 0.05, **p < 0.01, and ***p < 0.001.
Lack of HDAC3 elicits a futile cycle of FA metabolism in WAT. A distinctive hallmark of BAT metabolism is the concomitant activation of lipolytic and lipogenic pathways to sustain energy demand related to thermogenic potential. Interestingly, cold exposure activates a similar reprogramming of lipid metabolism in WAT via β-adrenergic receptor. Thus, we completed the metabolomics approach with lipodomics analysis in WAT of floxed and H3atKO mice, which revealed a reduction of saturated and unsaturated FAs in H3atKO (Fig. 4a) in the IngWAT. Decreased levels of FAs in adipose tissue may be suggestive of a higher utilization of fat as energy source in this depot. In this regard, mass spectrometry analysis showed that the allosteric inhibitor of CPT1 malonyl-CoA was reduced in IngWAT of H3atKO mice (Fig. 4b), thus favoring mitochondrial FA transport and β-oxidation. Accordingly, we detected an upsurge of the expression of lipolytic genes (Lipe and Agrp), periliposomal FA β-oxidation (Acx1) and mitochondrial FA β-oxidation (Acsl1, Cpt1b, Srebp1, Acad1, Acadm, and Hdh) (Fig. 4c). Western blot analysis confirmed significant increased protein expression of adipose tissue triglyceride lipase (ATGL) and of long chain acyl-CoA dehydrogenase (ACADL; Fig. 4d). The expression of genes involved in de novo FA synthesis (H3atKO, Mc3, G6pd2, and Me3) was upregulated in H3atKO mice (Fig. 4e), corroborating the hypothesis that the browning effect observed in these mice is sustained by a metabolic restructuring that phenocopies BAT metabolism. Similar, albeit less pronounced, effects in gene expression and FA profile occurred in epididymal fat (Supplementary Fig. 4c–e). As expected, cold exposure increased the expression of browning markers in IngWAT from mice exposed to 4°C for 24 h (Supplementary Fig. 6), and similarly to Hdc3 ablation, increased expression of glucose-related genes (Glu4 and Pdk4) and of genes involved in citrate export and FA synthesis/β-oxidation (Supplementary Fig. 6). Interestingly, H3atKO mice exposed to cold showed lowered effects when compared to floxed under the same temperature condition (Supplementary Fig. 6).

It has been demonstrated that the high rate of FA β-oxidation correlates with increased levels of short-chain acylcarnitines. Nonetheless, mass spectrometry analysis yielded unexpected results as the levels of most acylcarnitines in both inguinal (Supplementary Fig. 7a) and epididymal WAT (Supplementary Fig. 7b) showed a reduction in H3atKO mice. To explain the decreased levels of acylcarnitines in H3atKO mice we hypothesized that FAs are shortened via periliposomal β-oxidation and that acetyl-CoA from FA β-oxidation in adipose tissue of H3atKO mice is immediately used for de novo FA synthesis. Consistent with increased periliposomal FA β-oxidation we also detected increased expression of Acox1 (Fig. 4c) and Catalase, the latter involved in detoxification of H2O2, resulting from periliposomal β-oxidation. To demonstrate the onset of a futile cycle of FA metabolism consequent to Hdc3 inactivation, we used a cellular model of preadipocytes, C3/1H10T1/2. Preadipocytes were infected with scramble adenovirus or with adenovirus delivering short hairpin RNA to silence Hdc3 (shHdc3; Fig. 4f). Silencing of Hdc3 in cells was assessed by western blot (Fig. 4g). This cellular model recapitulated the in vitro effects of Hdc3 deletion: in vitro inactivation of Hdc3 caused the induction of browning genes (Fig. 4h), and promoted adipocyte differentiation, as demonstrated by the greater number of differentiated cells (Fig. 4i) and by the increased lipid accumulation in ORO staining (Supplementary Fig. 8a). Accordingly, shHdc3 treated cells showed increased expression of adipocyte markers (Supplementary Fig. 8b). Gene expression analysis also confirmed that silencing of Hdc3 in C3/1H10T1/2 adipocytes increased the expression of genes of glucose and citrate metabolism (Supplementary Fig. 8c) and of lipolysis, FA β-oxidation and FA synthesis genes (Fig. 4j). The increased rate of lipolysis was confirmed by higher glycerol release in shHdc3 cells (Fig. 4k). Administration of [U-13C]-Palmitate (where U denotes uniformly labeled) demonstrated that silencing of Hdc3 determined higher β-oxidation rate, as confirmed by increase of 13C-Acetyl-CoA (+1) (Fig. 4l), coming from β-oxidation of labeled palmitate (Supplementary Fig. 9). No differences in the levels of 13C-Acetyl-CoA (+2) between scramble and shHDC3-silenced cells were detected (Fig. 4l). Higher levels of 13C-Acetyl-CoA (+1) were paralleled by decreased levels of 13C-Citrate (+1) (Fig. 4m), confirming that silencing of Hdc3 promoted the activity of ATP citrate lyase, which converts citrate into acetyl-CoA. This result was in line with the upregulation of the gene (Acly) encoding for this enzyme (Fig. 4j). 13C-isotopologue labeling experiments also demonstrated that shHDC3 silenced cells showed higher levels of 13C-Citrate (+4) (Fig. 4m), whose formation requires that 2 molecules of 13C-Acetyl-CoA (+2), coming from labeled palmitate, entered the TCA cycle (Supplementary Fig. 9). No differences in the levels of 13C-Citrate (+2) and 13C-Citrate (+5) between scramble and shHDC3-silenced cells were detected (Fig. 4m). Increased 13C-Acetyl-CoA (+1) and 13C-Citrate (+4) levels confirmed that silencing of Hdc3 boosted mitochondrial FA β-oxidation and TCA cycle. Moreover, we found higher level of 13C-Palmitate (+2) in shHDC3 cells (Fig. 4n and Supplementary Fig. 9) and a trend of increased of 13C-Malonyl-CoA (+2, +3) (Supplementary Fig. 8d), corroborating de novo FA synthesis from β-oxidized FAs under this condition. 13C-Acetyl-CoA (+2) is incorporated in 13C-Palmitate (+2), thus explaining the lack of this isotope in shHDC3 cells. These in vitro experiments demonstrated that adipocyte ablation of HDAC3 triggers FA futile cycle in white fat.

HDAC3 deletion selectively remodels histone acetylation. To investigate the molecular mechanisms underlying the effect of HDAC3 ablation in white adipocytes, we focused on histone post-translational modifications as read out of HDAC3 activity. First we tested whether HDAC3 deletion correlated with increased histone acetylation. Consistent with evidences reported by other authors, acid extraction of histones resulted in no differences in global pan- and lysine 27 acetylation on histone H3 in mice and cells lacking HDAC3 (Supplementary Fig. 10a, b). Additionally, in order to investigate whether acetyl-CoA coming from FA β-oxidation could be a source of acetyl groups for histone acetylation, we performed a mass spectrometry analysis of histone H3 (acid extracted) from C3H10T1/2 cells infected with scramble or HDAC3 short hairpin RNAs (shRNAs), incubated with [U-13C]-Palmitate. Consistent with the western blots, mass spectrometry analysis showed no statistical significant differences in acetylation status of 9–17, 18–26, 27–40, 54–63, 73–83, and 117–128 peptides (Supplementary Fig. 10c). More interestingly, we were able to show incorporation of labeled carbons (13C acetylation) on 9–17, 18–26, and 27–40 peptides, with no differences between scrambled and knock down cells. Peptide 27–40 contains 3 lysine residues that could be acetylated (Lys 27, 36, and 37). Therefore, we specifically investigated the acetylation status of lysine 27, a known mark of active enhancers, and quantified the amount of 13C- vs. 13C-acetylation in scrambled or HDAC3 silenced cells (Supplementary Fig. 10d). Since we detected that most acetyl groups showed labeled carbons (13C-acetylation) in both conditions, we confirmed that acetyl-CoA, derived from FA β-oxidation, was a source of acetyl groups for histone acetylation on lysine 27. This result corroborated data by 13C-isotopologue labeling experiments (Fig. 4l–n): knock down of HDAC3 in fact increased β-oxidation and amount of labeled acetyl-CoA, but concomitantly this was in part used for
de novo synthesis of FA. This possibly caused the lack of increased $^{13}$C acetyl-CoA incorporation on histone H3.

It has been reported that selective deletion of HDAC3 increased acetylation of specific chromatin regions, even though global histone acetylation did not change. Thus, we focused on “hot regions”, whose epigenomic regulation might be crucial for the transcriptional and metabolic phenotype of H3atKO mice. Since Hdac3 deletion increased expression of the master regulator of adipocyte differentiation, Pparg, and of its main targets, we looked at acetylation status of this gene. It has been recently demonstrated that H3K27ac marks a potent enhancer of Pparg gene, thus we performed a ChIP analysis of H3K27ac and found increased acetylation on Pparg enhancer in shHDAC3 treated cells and in IngWAT of H3atKO mice (Figs. 5b, c). Another key gene upregulated upon Hdac3 inactivation is Ucp1: two enhancers located 13 and 5 kb upstream of the transcription start site (TSS) of Ucp1 are hyperacetylated (H3K27ac) in differentiating brown adipocytes (Fig. 5a). ChIP analysis showed increased H3K27 acetylation in cells lacking HDAC3 (Fig. 5b), while only −5 kb enhancer resulted in hyperacetylated in IngWAT of H3atKO mice (Fig. 5c). Moreover, by reviewing published H3K27ac ChIP-Seq data (GSE63964), we

![Fig. 5 Adipose ablation of HDAC3 remodels chromatin acetylation in “hot regions”.](image-url)

The diagram shows the acetylation status of Pparg and Ucp1 enhancers in C3H/10T1/2 adipocytes and IngWAT of H3atKO mice, highlighting the increased H3K27ac enrichment on these enhancers in the absence of HDAC3 activity.
noted hyperacetylation of H3K27 in regions located 15, 11, 3.5, and 1.6 kb upstream of the TSS of $Ppara$ gene in BAT samples, while the same regions were not acetylated in WAT samples (Fig. 5d, Supplementary Fig. 11a). PPARα is in fact highly expressed in BAT and directly regulates $Pdk4$ and FA β-oxidation genes. Thus, we analyzed H3K27 acetylation in these putative regulatory regions and found that −15 and −11 kb regions showed significant increase in H3K27ac in shHDAC3 cells and in IngWAT of mice lacking HDAC3 (Fig. 5e, f). Moreover, H3K27 acetylation increased in the −1.6 kb region in IngWAT of H3atKO mice (Fig. 5f).

ACLY is required for the establishment of H3atKO phenotype. In WAT from H3atKO mice we detected increased expression of Acly gene (Fig. 4e), encoding the cytosolic enzyme belonging to the FA biosynthetic pathway that converts citrate to acetyl-CoA and oxaloacetate. However, the reaction catalyzed by ACLY also represents the major source of acetyl-CoA for histone acetylation in mammalian cells. Notably, immunofluorescence analysis of ACLY in IngWAT from floxed and H3atKO mice showed higher nuclear localization in knock out mice (Fig. 6b). Nuclear ACLY catalyzes the reaction that could locally provide acetyl groups to sustain chromatin hyperacetylation.

**Fig. 6** ACLY is required for the establishment of H3atKO phenotype. a, b Immunofluorescence staining and quantification of ACLY, showing nuclear localization in Hdc3 knock out mice fed LFD (n = 3 per group), scale bar is 40 μm; c Morphology of cells infected with adenovirus expressing shRNA targeted to Hdc3 (shHDAC3), to Acly (shACLY) or both (shHDAC3 + shACLY) or to a scrambled control shRNA (scramble), scale bar is 50 μm; d Gene expression analysis in knock down cells (n = 3 per group); e H3K27ac ChIP analysis in knock down cells (n = 6 per group). Data are presented as mean ± SEM. Statistical analysis: One way ANOVA, Tukey as post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001 vs. scramble, §§p < 0.01, §§§p < 0.001 vs. shHDAC3.
Fig. 7 High fat diet overcomes the effects of adipose ablation of HDAC3. a Body weight gain of floxed and H3atKO mice fed HFD (n = 12–14 per group); b Glucose tolerance test of floxed and H3atKO mice (n = 12–14 per group), performed after 20 weeks of HFD; c Volcano plot of IngWAT metabolome in floxed and H3atKO mice fed HFD (n = 6 per group); d Heatmap of IngWAT metabolome in floxed and H3atKO mice fed HFD (n = 6 per group); e Hematoxylin–eosin staining of IngWAT from floxed and H3atKO mice fed HFD, scale bar is 100 μm; f Body temperature of HFD floxed and H3atKO mice exposed to 4 °C for 100 min (n = 8 per group); g, h Gene expression analysis of browning genes and lipolytic/lipogenic genes in IngWAT of floxed and H3atKO mice (n = 5 per group); i, j H3K27ac ChIP analysis showing lack of enrichment on Pparg and Ucp1 enhancers and on putative regulatory regions of Ppara gene in IngWAT from H3atKO HFD mice (n = 3 per group). Data are presented as mean ± s.e.m. Statistical analysis: Student’s t-test, *p < 0.05.
observed in Pparg and Ucp1 enhancers and in Ppara putative regulatory regions. To demonstrate that acetylation of these regions in adipocytes lacking HDAC3 was dependent on the increased acetyl-CoA arising from ACLY-mediated cleavage of citrate, we co-silenced both ACLY and HDAC3 in C3H/10T1/2 cells with shRNAs. Efficiency of ACLY knock down was confirmed by western blot (Supplementary Fig. 12a) and by qPCR (Fig. 6d). Interestingly, we found that ACLY silencing completely blocked adipogenesis as shown by dramatically decreased number of differentiated adipocytes (Fig. 6c, third panel) and by reduced expression of adipose markers Pparg and Glut4 (Fig. 6d, black bars). Most importantly, co-silencing of ACLY and HDAC3 completely abrogated the effects of HDAC3 inactivation, since few cells appeared fully differentiated to adipocytes (Fig. 6c, fourth panel). Expression analysis showed that genes that were strongly induced upon HDAC3 silencing (Ucp1, Ppara, Pparg, Glut4, Pdk4, Pcx, Atgl, Acadl, and Fasn), were significantly reduced in cells lacking ACLY as well as in cells lacking both HDAC3 and ACLY (Fig. 6d). ChIP analysis showed significant reduction of H3K27 acetylation on −5 kb Ucp1 enhancer and on...
Ppara putative regions in co-silenced cells compared to shHDAC3 treated cells, while a 50% reduction, though not statistically significant, was observed for Pparγ enhancer (Fig. 6e). Since these results clearly demonstrate the pivotal role of ACLY in determining the phenotype of HDAC3 knocked down cells, we infer that ACLY is a central player in the transcriptional and metabolic outcome of Hdac3 ablation in mice. Another potential source of acetyl groups for de novo FA synthesis is acetate that can be converted to acetyl-CoA by cytosolic acetyl-CoA synthetase (ACS5). Interestingly, Acss2 gene was strongly induced in IngWAT of H3atKO mice (Supplementary Fig. 12b). However, knock down of ACS2 (Supplementary Fig. 12c) did not affect adipocyte morphology (Supplementary Fig. 12d) and gene expression (Supplementary Fig. 12e) when compared to scrambled shRNA. Also, as opposed to ACLY siRNA, co-silencing of ACS2 and HDAC3 by shRNA did not result in any morphological or metabolic changes (Supplementary Fig. 12d). Our results mirror the observations of Welten et al.13 who showed that ACLY is the key player in coordinating nuclear activity (that is, chromatin acetylation) and cellular metabolism, whereas ACS2, the other enzyme potentially providing extra mitochondrial acetyl-CoA, plays a minor or an ancillary role in these processes.

No effect of Hdac3 KO on lipid metabolism with high fat diet. Having observed profound remodeling of WAT metabolism in H3atKO mice, we investigated whether adipose ablation of HDAC3 was able to prevent or attenuate diet-induced obesity. To this end, Hdac3 floxed and knock out mice were fed for 24 weeks with an obesogenic high fat diet (HFD). Intriguingly, we found no differences in body weight gain (Fig. 7a) and in glucose tolerance (Fig. 7b). Metabolic analysis (Fig. 7c, d, Supplementary Table 2) of IngWAT showed that effects on glycolysis, TCA cycle metabolites (Supplementary Fig. 13a–c), intermediates of the pentose phosphate pathway (Supplementary Fig. 13d), FAs (Supplementary Fig. 13e), and acyl carnitines (Supplementary Fig. 13f), observed in H3atKO fed LFD, were lost in mice fed HFD. Accordingly, expression analysis of genes related to glucose and citrate metabolism (Supplementary Fig. 14a, b) showed no change. Genes involved in lipolysis and FA β-oxidation were not significantly upregulated in knock out mice (Fig. 7h, Supplementary Fig. 14c), while among genes of FA synthesis only Fasn was significantly increased, though to a lesser extent (6-fold increase in H3atKO, Fig. 7h) as compared to mice fed low fat diet (LFD, 43-fold increase in H3atKO, Fig. 4e). Moreover, no morphological differences in inguinal adipocytes from floxed and H3atKO mice were highlighted by histological analysis (Fig. 7c). As a consequence of the lack of metabolic remodeling, IngWAT of H3atKO mice fed HFD did not undergo browning, as cold challenge experiment showed no differences in maintenance of body temperature in floxed and H3atKO mice exposed to 4°C (Fig. 7f), and browning genes were not upregulated in knock out mice (Fig. 7g). Also, upregulation of adipocyte functionality genes (including Pparγ) and of oxidative metabolism genes was lost upon HFD feeding (Supplementary Fig. 14c). No difference in global pan- and lysine 27 acetylation of histone H3 was detected upon Hdac3 ablation (Supplementary Fig. 15d, e), and a statistically significant reduction of ACLY positive nuclei compared to floxed and H3atKO fed LFD (Supplementary Fig. 15d, e). Accordingly, ChIP analysis demonstrated that ablation of Hdac3 did not affect H3K27 acetylation of Pparγ and Ucp1 enhancers, and of Ppara putative regulatory regions in presence of a high fat feeding (Figs. 7i, j).

As shown above, the peculiar phenotype observed in H3atKO mice is sustained by rewiring of lipid metabolism, with concomitant activation of FA synthesis and β-oxidation genetic networks resulting in the onset of a futile cycle. To gain insight on how HFD overcomes the metabolic and transcriptional effects of adipose ablation of Hdac3, we focused on lipid metabolic pathways that can be modulated in adipose tissue upon high fat feeding. It has been demonstrated that HFD reduces lipolysis mediated by catecholamines in adipose tissue, and that high fat feeding reduces levels of lipases. Among these, particularly relevant is ATGL, since its activity is fundamental to feed the futile cycle of FAs in adipose tissues during β-adrenergic activation and to maintain brown-like phenotype. We measured ATGL expression in IngWAT of floxed and H3atKO mice fed LFD or HFD and we found that ablation of Hdac3 increased expression of ATGL with both diets. However, when fed HFD floxed and knock out mice showed significantly lower levels of ATGL as compared to H3atKO LFD fed mice (Fig. 8a). This observation suggests that the lipolytic activity of IngWAT releasing free FAs (FFAs) is reduced upon HFD feeding. Notably, knock down of ATGL in brown adipocytes prevented induction of genes involved in FA β-oxidation, owing to lack of lipolytic products (FFAs) that act as ligands for PPARs. In our model a key gene regulated upon Hdac3 ablation in IngWAT is Pdk4, whose protein product phosphorylates and inactivates pyruvate dehydrogenase (PDHE1α), thus allowing the entrance of oxaloacetate in the TCA cycle to form citrate. Importantly, Pdk4 is transcriptionally activated by PPARα. As previously shown (Fig. 7j), H3K27 hyperacetylation on Ppara putative

Fig. 8 Role of PPARα and ChREBPβ in lipolysis and lipogenesis in WAT and in adipocytes. a Western blot analysis and quantification of ATGL in IngWAT from floxed and H3atKO mice fed LFD or HFD (n = 2, where each replicate represent a pool of samples from 3 mice); b, c mRNA expression of Ppara and Pdk4 in IngWAT from floxed and H3atKO mice fed LFD or HFD (n = 9–11 for LFD mice, n = 5 per group for HFD samples); d Western blot and quantification of p-ser-293 PDHE1α, p-ser-300 PDHE1α, PDHE1α in IngWAT of H3atKO 3-week and knock out mice fed LFD or HFD (n = 2, where each replicate represent a pool of samples from 3 mice); Data are presented as mean ± s.e.m. Statistical analysis: two-way ANOVA, Tukey as post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Floxed LFD, †p < 0.05, ††p < 0.01, †††p < 0.001 vs. HFD. e Western blot and quantification of p-ser-293 PDHE1α, p-ser-300 PDHE1α, PDHE1α in cells infected with adenovirus expressing shRNA targeted to Hdac3 or to a scrambled control shRNA (scramble) and differentiated in presence of vehicle or 10 μM GW6471 (n = 2, where each replicate represent a pool of 3 samples); f Morphology of cells infected with adenovirus expressing shRNA targeted to Hdac3 or to a scrambled control shRNA (scramble) and differentiated in presence of vehicle or 10 μM GW6471, scale bar is 50 μm; g Gene expression analysis on mRNA from cells infected with adenovirus expressing shRNA targeted to Hdac3 or to a scrambled control shRNA (scramble) and differentiated in presence of vehicle or 10 μM GW6471 (n = 3 per group). Data are presented as mean ± s.e.m. Statistical analysis: two-way ANOVA, Tukey as post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001 vs. scramble + Vehicle, †p < 0.05, ††p < 0.01, and †††p < 0.001 vs. scramble + HFD. h, i mRNA expression of ChREBPβ, Glut4, Pparγ in IngWAT of H3atKO 3-week and knock out mice fed LFD or HFD (n = 9–11 for LFD mice, n = 5 per group for HFD samples); Two-way ANOVA, Tukey as post hoc test, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. Floxed LFD, †p < 0.05, ††p < 0.01, and †††p < 0.001 vs. H3atKO LFD.
regulatory regions in response to Hdac3 ablation was blunted in IngWAT of HFD fed mice. Consequently, Ppara expression was significantly reduced in floxed and H3atKO mice fed HFD when compared to H3atKO mice fed LFD (Fig. 8b). Moreover, HFD feeding, by blocking ATGL reduced the availability of FFAs for PPARα activation. As a result of lower transcription and activation of PPARα, we detected lower expression of Pdk4 in floxed and H3atKO mice fed HFD when compared to H3atKO mice fed LFD (Fig. 8c). Moreover, HFD reduces phosphorylation of PDHE1α at Ser293,30. In fact, both Ser293 and Ser300 resulted significantly less phosphorylated in floxed and H3atKO mice fed HFD (Fig. 8d), suggesting that the PDHE1α kinase activity of PDK4 in WAT from these mice was reduced. To elucidate the role of PPARα as mediator of effects of metabolic remodeling induced by Hdac3 ablation, cells infected with scramble or HDAC3 shRNAs were treated with vehicle or with the PPARα antagonist GW6471, to mimic in vitro the lack of activation of PPARα that occurs in HFD regimen. Hdac3 knockdown down in C3H/10T1/2 adipocytes tended to increase phosphorylation status of PDHE1α, while no increase was found upon treatment with GW6471 (Fig. 8e). Consistent with published literature,31, treatment with PPARα antagonist decreased the rate of differentiation (Fig. 8f), reducing expression of adipocyte markers Pparγ and Glut4 (Fig. 8g). Also, the expression of Pdk4 and other key genes upregulated by shHDAC3 (Ucp1, Acadl, Acly, and Fasn) was significantly reduced in presence of GW6471 (Fig. 8g), implying that ablation of Hdac3 induced expression of these genes, at least in part, via PPARα.

Another key point in the metabolic remodeling in WAT of H3atKO mice was the strong activation of a lipogenic program, that leads to higher level of ACLY, allowing a concomitant chromatin rearrangement that leads to increased expression of genes involved in higher metabolic requirements of adipocytes. As shown above, de novo FA synthesis as well as chromatin remodeling was abrogated in H3atKO mice fed HFD. It has been elegantly demonstrated that adipose tissue lipogenesis is primarily regulated by ChREBP, and that, among ChREBP isoforms, the most affected by HFD is ChREBPβ isoform.32 Interestingly, the expression of this gene was significantly increased in H3atKO mice fed LFD, but, consistent with published literature, we found reduction of ChREBPβ upon HFD feeding in both floxed and knock out mice (Fig. 8h). Analyzing published H3K27ac ChIP-Seq data (GSE63964), we noticed several regions located upstream and downstream the TSS for Exon1b of ChREBP gene (the alternative first exon for ChREBPβ isoform) showing higher H3K27 acetylation profile in BAT vs. WAT samples (Supplementary Fig. 16a, b). However, H3K27ac ChIP analysis showed no increase in acetylation status of these putative regulatory regions in IngWAT of H3atKO compared to floxed mice fed LFD (Supplementary Fig. 16c), ruling out that ablation of HDAC3 drives ChREBPβ expression by directly modulating histone acetylation levels. Importantly, it is known that ChREBPβ gene transcription is regulated in response to glucose levels through ChREBPα and it has been demonstrated that increased expression of the glucose transporter Glut4 correlates with higher ChREBPβ expression.32 Glut4 in adipose tissue is
Discussion

In this paper we disentangled the role of the histone modifier HDAC3 in adipose tissue physiology. The main finding we report is that adipose specific ablation of Hdac3 affects FA metabolism and induces browning of white fat, promoting the global thermogenic capacity, without affecting BAT phenotype. Transcrpotype analysis of IngWAT proved that ablation of HDAC3 did not de-repress globally gene transcription, demonstrating that the lack of HDAC3 does not lead to general reactivation of silenced genes. We demonstrated that remodeling of WAT phenotype is sustained by the metabolic rewiring of glucose- and lipid metabolism (Fig. 9). Our results highlight the anaplerotic function of glucose/glycolysis to provide oxaloacetate to TCA cycle in our knock out model. In this frame, FAs represent an alternative source of acetyl-CoA thus explaining the exceptional activation of FA lipolysis and β-oxidation in WAT of mice lacking HDAC3. Concomitantly, mitochondrial citrate is exported to cytosol, where it is required for de novo lipogenesis. Thus, by exploiting this animal model, we learnt that WAT browning induced by HDAC3 ablation activates a “futile cycle” to meet the high metabolic demand of brown-like fat, whereby FFAs activate UCP1 and their oxidation provides reduced coenzymes and further reducing the transcription of their target genes.

Other authors described a different futile cycle, operating in brown and beige adipocytes, involving creatine metabolism to sustain adipose tissue energy expenditure and thermogenic capacity, demonstrating that creatine enhances respiration in beige-fat mitochondria when ADP is limiting. These evidences, along with our experiments, suggest that beige/brown adipocytes can trigger paradoxical metabolic pathways to sustain the incredibly high metabolic demand during thermogenic activation. We are tempted to speculate that such futile cycle of FA metabolism could be operative in other tissues in conditions of high energy requirements, such as skeletal muscle during sustained physical exercise, or heart. Future investigations will be required to test this fascinating hypothesis and to verify whether HDAC3 is involved in the regulation of the metabolic phenotype in these organs.

Intriguingly, WAT from mice exposed to cold (4°C) showed similar profile of Pdk4 expression as well as of genes of FA futile cycle. Based on these results it is tempting to speculate that Hdac3 ablation phenocopies, at least in part, the transcriptional program elicited by cold exposure. Future experiments will focus on whether and how HDAC3 is involved in the adaptive response to cold exposure.

PDK4 and ALCY, which are upregulated upon Hdac3 ablation, are key actors in establishing the phenotype of Hdac3 knockout mice. PDK4 blocks the conversion of pyruvate to acetyl-CoA, thus making FA β-oxidation the alternative source of acetyl-CoA. On the other hand, ALCY is a key enzyme in lipogenesis and also provides acetyl-CoA for histone acetylation. Selective hyperacetylation of Ucp1 and Pparg enhancers and Ppara putative regulatory regions in adipocytes lacking HDAC3 are lost when ALCY is also knocked down concomitantly with HDAC3, highlighting the crucial role of this lipogenic enzyme in establishing the phenotype induced upon HDAC3 ablation. Our results demonstrate that HDAC3 directly regulates Ucp1 transcription, but it also plays a role in the regulation of PPARα and PPARγ, which are known to drive UCP1 expression.

PPARα stimulates FA β-oxidation, but it is also essential in switching off the flow of glucose to acetyl-CoA, which is channeled to oxaloacetate, by increasing PDK4 expression. PPARα mediates, at in least in part, the effects of Hdac3 knock down, as confirmed in the experiment with the antagonist GW6471. On the other hand, PPARγ may be important for FA uptake and usage by promoting triglycerides hydrolysis via LPL and ATGL (PPARγ target genes) that provide FAs as fuel available for high energy expenditure. Furthermore, PPARγ regulates Glut4 expression, favoring glucose internalization and expression of ChREBPα, one of the main regulators of lipogenesis in adipose tissue. Thus, PPARα and γ emerged as key targets of HDAC3, whose de-repression upon Hdac3 ablation induces WAT browning. A key question is how HFD completely offsets the effects of HDAC3 genetic inactivation. It should be noted that PPARα are activated by FFAs primarily resulting from lipolytic action of ATGL. It has been shown that high fat feeding reduces protein expression of ATGL, therefore lower rate of lipolysis and reduced availability of FFAs are expected to strongly blunt PPARα activation in both floxed and Hdac3 knock out mice fed HFD. The lack of PPARα activation determines lower Pdk4 transcription, resulting in loss of pyruvate dehydrogenase phosphorylation in HFD fed mice, that allows a different glucose utilization and reduces the rate of FA β-oxidation. Intriguingly, high fat feeding also reduced expression of Pparg and Ppara genes, further reducing the transcription of their target genes. These effects, paralleled by lower expression of ChREBPα and of its lipogenic target genes in response to HFD, disrupt the epigenic and metabolic remodeling induced by adipose ablation of Hdac3. We also demonstrate that when H3atKO mice are fed HFD the FA futile cycle is less active. Our observations underline a possible mechanism operative under normal physiological conditions, which is impaired with an obesogenic diet.

In conclusion, an intrinsic crosstalk between metabolic pathways and chromatin remodeling (for example, histone acetylation) emerged by disentangling the complex phenotype of H3atKO mice and we identified HDAC3 as a molecular brake of the flux of acetyl groups from mitochondria to selective regions of chromatin in white fat. Consequently, genetic ablation of HDAC3 drives transcriptional remodeling of “hot regions” on chromatin, resulting in a different adipose phenotype, with higher FA utilization. Any cues (for example, HFD) that alter this equilibrium between chromatin remodeling and cellular metabolism may lead to a different phenotypic outcome (for example, reduced FA utilization).

Altogether, our results shed light on new facets of adipose tissue physiology, unlocking mechanisms that could be explored for new approaches in the treatment of adipose tissue dysfunction related to metabolic disorders.

Methods

Differenniation and infection of C3H/10T1/2 Cells. C3H/10T1/2, Clone 8 (ATCC CCL-226) were used as a cellular model of pre-adipocytes to investigate the molecular mechanism underlying the effects observed in vivo. Cells were maintained in Dulbecco’s modified Eagle’s medium–10% FBS. To induce differentiation cells were maintained for 3 days in medium supplemented with 5 μg/ml insulin, 0.5 mM 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 days. At the induction of differentiation cell were infected with 100 MOI scramble or shHdac3 adenovectors (Vector BioLabs) to knockdown HDAC3. To silence ALCY and Acss2, cells were infected with 50 MOI shALCY/shACSS2 adenovectors (Vector BioLabs). To perform co-silencing of Hdac3 and ALCY/Acss2,
cells at the induction of differentiation were infected with 100 MOI shHdac3 + 50 MOI shAcly or 100 MOI shHdac3 + 50 MOI shCacs2. In this case, cells infected with 150 MOI Scramble were used as negative controls.

To evaluate the role of PPAR in the metabolic rewiring consequent to Hdac3 ablation, cells were co-treated with 100 MOI Scramble or shHdac3 and vehicle (DMSO) or 10 μM GW6471 (Sigma Aldrich) at the induction of differentiation for 3 days, and then maintained with 5 μg/ml insulin and vehicle/GW6471 for the following 6 days.

**EC tracing analysis.** At day 9 of differentiation C3H/10T1/2 cells previously infected with Scramble or shHDAC3 adenoviral vectors were treated with 100 μM [U-13C]Pamitate (Sigma Aldrich) and 1 mM Carnitine (Sigma Aldrich) for 30 min. [U-13C]Pamitate is prepared as described previously.

Methanolic extracts of cells were analyzed by electrospray ionization flow injection analysis tandem mass spectrometry (FIA-MS/MS). For citrate, acetylCoA, palmitate, and malonylCoA each isomer was resolved by m/z ratios and quantified by Mass Distribution Vector (MDV), as described previously.

Then, the percentage of MDV for each isomer was normalized by protein content, measured by BCA Assay (Euroclone).

**Acidic extraction of histones and mass spectrometry analysis.** At day 3 of differentiation, C3H/10T1/2 cells previously infected with Scramble or shHdac3 adenoviral vectors were treated with 500 μM [U-13C]Pamitate (Sigma Aldrich), 25 μM CoA (Sigma Aldrich), and 1 mM Carnitine (Sigma Aldrich) for 3 h and then pelleted. Each cellular pellet (three biological replicates for each condition) was re-suspended carefully in 7.5 ml of N-Buffer (10% Sucrose, 0.5 mM EGTA, pH 8.0, 60 mM KCl, 15 mM HEPES, pH 7.5, 30 μg/ml Spermidine, protease inhibitors cocktail, phosphatase inhibitors cocktail, 1 mM DTT) and 500 μl of Triton X-100 solution 8% (460 μl of N-Buffer + 40 μl Triton X 100). The samples were rolled for 10' at 4°C. Then each lysate was put onto a sucrose cushion (2 g of sucrose in 20 ml of N-buffer) and centrifuged at 4,000 rpm for 40'. The supernatant was removed while the nuclear pellet was washed twice with 2 ml of ice-cold PBS. Upon the addition of an equal volume of HCl 0.8 N, the nuclei were extracted overnight at 4°C rolling. The next day each sample was centrifuged at 13,000 rpm for 10'. The pellets were re-extracted in 0.4 N HCl for 4h. All the supernatants were collected and pooled. In order to change the supernatant buffer 0.4 N HCl buffer to 0.1 M CH3COOH and finally to water, AMICON filters (MWCO 3 kDa, Millipore) were used. Protein concentration was determined using Direct Detect IR Spectrophotometer (Millipore).

For the analysis of peptides, the bands corresponding to H3 histone were excised upon Coomassie staining, dediroluted with tritioethanol, alkylated with iodoacetamide and digested with sequencing grade endoproteasine Arg-C (Roche Diagnostics). For selective analysis of acetylation of Lysine 27, the bands corresponding to H3 histone were excised, modified with acetic anhydride-d6 (SIGMA) and digested with sequencing grade trypsin (Roche Diagnostics). The peptides were extracted from gel, desalted on SCX and C18 home-made stage (c), using a home-made 12.5 cm column (360 μm OD, 75 μm I.D.) Reproil C18 AQ 120 Å resin, Dr Maisch GmbH), 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A gradient from 0 to 45% B in 45' was applied in order to achieve peptide separation at 300 nl/min. Full scan mass spectra were acquired on the Q-Exactive mass spectrometer in the mass range m/z 300–2000 Da with the resolution set to 70,000 at m/z 400 and a target value (AGC) of 3 × 106. The 'lock-mass' option was used for accurate mass measurements. The ten most intense doubly and triply charged ions were automatically selected and fragmented by HCD with a normalized collision energy setting of 27, AGC of 1 × 105 for MS/MS. Target ions already selected for the MS/MS were dynamically excluded for 15 s. To quantify histone modifications, the area under the curve (AUC) of the extracted ion chromatograms for the modified forms relative to H3 histone (and selectively relative to acetylated lysine 27 in H3(27 ac)) was validated by visual inspection of the mass spectra.

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

**Gene expression.** RNA from cell cultures was isolated with TRIzol (Invitrogen) and purified using the Nucleospin RNA I kit (Macherey-Nagel). RNA from adipose tissues was isolated with Qiazol and purified using Qiazol Reagent (Qiagen), purified with commercial kit (RNeasy Lipid Tissue Mini kit, Qiagen), and fragmented with Dnase I (Thermo Scientific, Wilmington, DE). Specific mRNA was amplified and quantitated by real time PCR, using iScriptTM One Step RT PCR for Probes (cat. # 170-8894, Bio-Rad Laboratories), following the manufacturer’s instructions. Data were normalized to 36B4 mRNA and quantitated setting up a standard curve. Probes were quantified by real-time PCR using SYBR Green or TaqMan probes on a CFX984 real-time system instrument in 384 well format (Bio-Rad Laboratories) using the iScriptTM One-step qRT-PCR kit for SYBR Green or for probes (Bio-Rad Laboratories). Primers and probes were obtained from Eurofins MWG Operon. For gene expression analysis in C3H/10T1/2 cells differentiated with Scramble or shHdac3, data from four independent experiments were included (n = 4). Complete list of primers is reported in Supplementary Table 3.

**RNA-seq.** RNA-seq was performed in triplicate. Each sequenced sample was a pool of 500 ng of total RNA from IngWAT of 2/3 individual mice. RNA-seq libraries were prepared with PolyA selection using 500 ng of total RNA and the Illumina TruSeq Stranded RNA reagents (Illumina; San Diego, CA, USA) on a Sciclone liquid handling robot (PerkinElmer; Waltham, MA, USA) using a PerkinElmer developed and optimized script. Cluster generation was performed with the Illumina TruSeq SR Cluster Kit v3 reagents and sequenced on the Illumina HiSeq 2500 using TruSeq SBS KIT v3 reagents. Sequencing data were processed using the Illumina Pipeline Casava 1.82. Purity-filtered reads were adapter and quality trimmed with Cutadapt (v. 1.15) and filtered for low abundance complexity with seq_crumbs (v. 0.1.8). Reads were aligned against Mus musculus version (GRCm38) genome using STAR (v. 2.4.2a). The number of read counts per gene locus was summarized with htsseq-count (v. 0.6.1.4) using Mus musculus (Ensembl v65 GRCm38.s82) gene annotation. Statistical analysis was performed for genes in R (R version 3.2.3). Genes with low counts were filtered out according to the rule of 1 count per million (rpm) in at least 1 sample. Library sizes were scaled using TMM normalization (DESeq package version 1.32.14), and log-transformed with limma voom function (Limma package version 3.26.9). Differential expression was computed with limma (46). We used DAVID (47, 48) to highlight the biological functions enriched in differentially expressed genes. The annotated terms were grouped according to the degree of their co-association genes with the functional annotation clustering feature (Supplementary Table 3). The Group Enrichment Score, namely the geometric mean (in-log scale) of member’s p-values in a corresponding annotation cluster, is used to rank their biological significance.

**Protein analysis.** C3H/10T1/2 cells cultured in 6-well plate and adipose tissues were lysed with SDS 2X sample buffer (20% glycerol, 4% SDS, 100 mM Tris-HCl, pH 6.8) and 100 mM dithiothreitol, 0.02% bromophenol blue, 1 mM protease and phosphatase 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 1X TBS. The primary antibodies are listed in Supplementary Table 6.

HRP-conjugated goat anti-mouse (Sigma-Aldrich) and goat anti-rabbit (Cell Signaling) secondary antibodies were used for detection with chemiluminescence (ECL, Pierce).

**Chromatin immunoprecipitation.** IngWAT or C3H/10T1/2 cells infected with 100 MOI scramble or shHdac3 adenovectors and differentiated for 9 days were cross-linked for 10 min with 1% formaldehyde. Cross-linking was stopped by the addition of 100 mM glycine (final concentration 125 mM). Cells were pelleted and lysed, and DNA was sheared by sonication: 8–10 pulses for 10 s for 12 differentiated cells and 15–20 pulses for 10 s for 9 days-differentiated cells (SLPe sonicator, Branson). H3K27ac antibody (1 μg, ab74729, Abcam) was pre-incubated with 20 μl G protein DynaBeads (Life technologies) for 1 h at room temperature, to allow the binding of the antibody to DynaBeads. Cross-linked chromatin was incubated with DynaBeads solution overnight at 4°C. After reversing the crosslinking by incubating overnight at 65 °C, the DNA was cleaned on QIAquick gel extraction kit columns (QIAGEN). Inputs and immunoprecipitated samples were analyzed by qRT-PCR using gene-specific primers, listed in Supplementary Table 4. Data are expressed as 10% of input.

**Animal studies.** To obtain specific deletion of Hdac3 in adipose tissues, we crossed Hdac3 floxed mice in C57Bl/6J background (provided by Dr. Scott Hiebert, Vanderbilt University) with B6FVB-Tg(Adipoq-cre) 1Evr/J (stock # 010803, The Jackson Laboratory).
were stained with hematoxylin and eosin. Images were taken at 20× magnification. Retrieval was performed with 0.05 mol/l NH₄Cl for 30 min, room temperature. Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).



Results

1. Fisher, M. et al. FGFR2 regulates PGC-1α and brown fat development in adipose tissue. Genes Dev. 26, 271–281 (2012).

2. Ohno, H., Shinoda, K., Spiegelman Bruce, M. & Kajimura, S. PPARγ agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell Metab. 15, 395–404 (2012).

3. Bostrom, P. et al. A PGC-1α-dependent myokine that drives brown fat-like development of white fat and thermogenesis. Nature 483, 463–468 (2012).

4. Knutson, S. K. et al. Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. EMBO J. 27, 1017–1028 (2008).

5. Sun, Z. et. al. Hepatic Hdc3 promotes glucose uncoupling by repressing lipogenesis and sequestration. Nat. Met. 18, 934–942 (2012).

6. Sun, Z. et. al. Diet-induced lethality due to deletion of the Hdc3 gene in heart and skeletal muscle. J. Biol. Chem. 286, 33301–33309 (2011).

7. Montgomery, R. L. et al. Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice. J. Clin. Invest. 118, 3588–3597 (2008).

8. Galmozzii, A. et al. Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue. Diabetologia 62, 732–742 (2013).

9. Ferrari, A. et al. Attenuation of diet-induced obesity and insulin resistance with a chemical inhibitor of histone deacetylase 3. J Int Obes 41, 289–298 (2016).

10. Shinoda, K. et al. Phosphoproteomics identifies CK2α as a negative regulator of beige adipocyte thermogenesis and energy expenditure. Cell Metab. 22, 997–1008 (2015).

11. Li, P. et al. Adipocyte NCoR knockout decreases PPARγ phosphorylation and enhances PPARγ activity and insulin sensitivity. Cell 147, 815–826 (2011).

12. Li, J. et al. Both coactivators kinesin SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J. 19, 4342–4350 (2000).

13. Welten, K. E. et al. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324, 1076–1080 (2009).

14. McDonnell, E. et al. Lipids reprogram metabolism to become a major carbon source for histone acetylation. Cell Rep. 17, 1463–1472 (2016).

15. Abe, Y. et al. JMJD1A is a signal-sensing scaffold that regulates acute chromatin dynamics via SMU/SNF association for thermogenesis. Nat. Commun. 6, 7052 (2015).

16. Feng, D. et al. A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. Science 331, 1315–1319 (2011).

17. Yu, X. X., Lewin, D. A., Forrest, W. & Adams, S. H. Cold elicits the simultaneous induction of fatty acid synthesis and beta-oxidation in murine brown adipose tissue: prediction from differential gene expression and confirmation in vivo. FASEB J. 16, 155–168 (2002).

18. Brayburn, P. Fatty acid synthesis in mouse brown adipose tissue influence the temperature in the proportion of whole-body fatty acid synthesis. J. Biol. Chem. 281, 17387–17394 (2006).

19. Festuccia, W. T., Blanchard, P.-G. & Deshaies, Y. Control of brown adipose tissue glucose and lipid metabolism by PPARy. Front. Endocrinol. 2, 84 (2011).

20. Bardin, M. J., Waley, S. E., Naviaux, R. K., Murphy, A. N. & Dixon, J. E. Monitoring phosphorylation of the pyruvate dehydrogenase complex. Anal. Biochem. 389, 157–164 (2009).

21. Hahn, P. & Novak, M. Development of brown and white adipose tissue. J. Lipid Res. 16, 79–91 (1975).

22. Mottillo, E. P. et al. Coupling of lipolysis and de novo lipogenesis in brown, beige, and white adipose tissues during chronic β3-adrenergic receptor activation. J. Lipid Res. 55, 2276–2286 (2014).

23. Koves, T. R. et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 7, 45–56 (2008).

24. Sun, Z. et al. Deacetylase-independent function of HDAC2 in transcription and metabolism requires nuclear receptor corepressor. Mol. Cell 52, 769–782 (2013).

25. Ramlee, M. K. et al. Histone H3 K27 acetylation marks a potent enhancer element on the adipogenic master regulator gene Pparg2. Cell Cycle 13, 3414–3422 (2014).

26. Contreras, A. V., Torres, N. & Tovar, A. R. PPAR-α as a key nutritional and environmental sensor for metabolic adaptation. Adv. Nutr. 4, 439–452 (2013).

27. Serre, P. A. The citrate cleavage enzyme: I. Distribution and purification. J. Biol. Chem. 234, 2544–2547 (1959).
29. Mottillo, E. P., Bloch, A. E., Leff, T. & Granneman, J. G. Lipolytic products in brown adipocytes to match fatty acid oxidation with supply. J. Biol. Chem. 287, 25038–25048 (2012).

30. Ali Shaik, A. et al. Phosphoprotein network analysis of white adipose tissues unveils deregulated pathways in response to high-fat diet. Sci. Rep. 6, 25844 (2016).

31. Goto, T. et al. Activation of peroxisome proliferator-activated receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. J. Lipid Res. 52, 873–884 (2011).

32. Herman, M. A. et al. A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. Nature 484, 333–338 (2012).

33. Harms, M. J. et al. PRDM16 binds MED1 and controls chromatin architecture to determine a brown fat transcriptional program. Genes Dev. 29, 298–307 (2015).

34. Armoni, M. et al. Peroxisome proliferator-activated receptor-γ represses GLUT4 promoter activity in primary adipocytes, and rosiglitazone alleviates this effect. J. Biol. Chem. 278, 30614–30623 (2003).

35. Kazak, L. et al. A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. Cell 163, 643–655 (2015).

36. Villarroya, F., Iglesias, R. & Giralt, M. PPARs in the control of uncoupling proteins gene expression. PPAR Res. 2007, 74364 (2007).

37. Huynh F. K., Green M. F., Koves T. R. & Hirschey M. D. A roadmap for interpreting 13C metabolite labeling patterns from cells. Curr. Opin. Biotechnol. 34, 189–201 (2015).

38. Buescher, J. M. et al. Improved bottom-up strategy to efficiently separate hypermodified histone peptides through ultra-HPLC separation on a bench top Orbitrap instrument. Proteomics 14, 2212–2225 (2014).

39. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10–12 (2011).

40. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Biostatistics 11, 34–51 (2010).

41. Law, C. W., Chen, Y., Shi, W. & Smyth, G. K. voom: precision weights unlock linear model analysis tools for RNA-seq count data. Genome Biol. 15, R29 (2014).

42. Ritchie, M. E. et al. Limma powers differential expression analyses for RNA-seq and microarray studies. Nucleic Acids Res. 42, e47 (2015).

43. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–59 (2009).

44. Cummins, T. D. et al. Metabolic remodeling of white adipose tissue in obesity. Am. J. Physiol. Endocrinol. Metab. 307, E262–E277 (2014).

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Author contributions
A.F. conceived, planned and executed experiments, and wrote the manuscript with M.C.; R.L. executed immunofluorescence and immunohistochemistry staining on adipose tissues and performed in vivo studies with mice; E.F. contributed to the generation, maintenance of Hdac3 KO mice and to in vivo tests; R.S. performed ChIP assays from IngWAT of mice; N.M. contributed to metabolomic analyses; C.G. executed metabolic analyses; F.G. processed samples for RNA sequencing; B.D. supervised RNA sequencing experiments and helped manuscript corrections; A.A. and C.M. performed proteomic analysis of histones; D.C. supervised RNA sequencing experiments and helped manuscript corrections; A.A. and C.M. performed proteomic analysis of histones; D.C. supervised data of metabolomic analysis; E.D.F. contributed in the discussion of manuscript data; S.W.H. provided Hac1 KO mice and contributed to the manuscript correction; M.C. planned the overall experimental design, supervised experiments, discussed data and wrote the manuscript with A.F.

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