HDAC9 knockout mice are protected from adipose tissue
dysfunction and systemic metabolic disease during high fat feeding

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Abstract

During chronic caloric excess, adipose tissue expands primarily by enlargement of individual adipocytes, which become stressed with lipid overloading, thereby contributing to obesity-related disease. Although adipose tissue contains numerous preadipocytes, differentiation into functionally competent adipocytes is insufficient to accommodate the chronic caloric excess and prevent adipocyte overloading. We report for the first time that chronic high fat diet (HFD) impairs adipogenic differentiation, leading to accumulation of inefficiently differentiated adipocytes with blunted expression of adipogenic differentiation-specific genes. Preadipocytes from these mice likewise exhibit impaired adipogenic differentiation, and this phenotype persists during in vitro cell culture. HFD-induced impaired adipogenic differentiation is associated with elevated expression of histone deacetylase 9 (HDAC9), an endogenous negative regulator of adipogenic differentiation. Genetic ablation of HDAC9 improves adipogenic differentiation and systemic metabolic state during HFD, resulting in diminished weight gain, improved glucose tolerance and insulin sensitivity, and reduced hepatosteatosis. Moreover, compared to wild-type mice, HDAC9 knockout mice exhibit upregulated expression of beige adipocyte marker genes, particularly during HFD, in association with increased energy expenditure and adaptive thermogenesis. These results suggest that targeting HDAC9 may be an effective strategy for combating obesity-related metabolic disease.
Adipose tissue plays a vital homeostatic role in storing excess calories as triglyceride lipid within adipocytes. Adipocytes also function as endocrine cells, secreting hormones and biologically active molecules that regulate cellular lipid storage capacity, tissue and systemic insulin sensitivity, and metabolic energy balance. During chronic caloric excess, adipose tissue expands primarily by enlargement of individual adipocytes. Over time, these enlarged adipocytes become mechanically stressed with lipid overloading and fail to exhibit proper endocrine function. This contributes to adipose tissue inflammation, ectopic lipid accumulation, decreased circulating adiponectin levels, glucose intolerance and insulin resistance. Adipose tissues contain a huge reserve of precursor cells—adipose derived stem cells and committed preadipocytes—representing approximately 20-40% of the adipose tissue cellularity (1). It is estimated that daily turn-over of adipocytes could be as high as 1-5% under resting conditions, and obesity markedly stimulates preadipocyte replication, particularly in subcutaneous adipose tissues (2,3). However, differentiation of preadipocytes into functionally competent adipocytes is nevertheless insufficient to accommodate the chronic caloric excess and prevent adipocyte overloading.

Adipogenic differentiation is a tightly controlled process that involves activation of key transcription factors (PPARγ and CEBPα) that induce expression of adipogenic genes (e.g., FABP4 and adiponectin) leading to acquisition of the mature adipocyte phenotype. In addition, epigenetic processes, governed by the actions of chromatin modifying enzymes, including the histone acetyltransferase (HAT) and histone deacetylase (HDAC) family of proteins, play an important role in adipogenic differentiation (4). We recently reported that HDAC9, a class II HDAC, is an endogenous negative regulator of adipogenic differentiation, and that down-
regulation of HDAC9 is necessary for adipogenic differentiation of preadipocytes (5), pointing to a key role of HDAC9 in adipogenic differentiation.

In the present study, we report that chronic HFD impairs adipogenic differentiation, promoting accumulation of inefficiently differentiated adipocytes that exhibit diminished expression of adipogenic differentiation-specific genes. We additionally observed that HFD markedly down-regulates beige adipocyte-specific gene expression in white adipose tissues, in conjunction with decreased FGF21 expression in this tissue, a known autocrine/paracrine inducer of beige phenotype in white adipose tissues (6). Preadipocytes from HFD mice exhibit impaired adipogenic differentiation in vitro, a phenotype that persists despite passage of the cells in culture. This impaired in vitro differentiation is associated with blunted down-regulation of HDAC9 expression. Genetic ablation of HDAC9 blocked the deleterious effects of HFD on adipogenic differentiation, improved insulin sensitivity and glucose tolerance, and prevented ectopic lipid accumulation in the liver. Compared with wild-type mice, HDAC9 knockout mice also exhibit elevated expression of beige adipocyte marker genes in white adipose tissues, concomitant with increased energy expenditure and adaptive thermogenesis. Our study provides evidence that HDAC9 plays a critical role in HDF-induced adipose tissue and metabolic dysfunction, suggesting that targeting HDAC9 could be an effective strategy for combating obesity-related disease.
RESEARCH DESIGN AND METHODS

Mice. HDAC9 knockout mice in the mixed C57BL/6J and 129 background were obtained from Dr. Eric Olson (7). These mice were backcrossed with C57 BL/6J mice for seven generations in our laboratories. Subsequently, HDAC9 heterozygous mice were bred to obtain HDAC9 knockout (−/−) and their wild-type (+/+) littermate controls for use in our experiments.

Male HDAC9 knockout mice and their wild-type littermate controls were housed individually and maintained on chow diet after weaning. At 8 weeks of their age, these mice were either maintained on chow (Harlan Teklad, LM-485) or switched to HFD (Research Diet, D12492, with 60% calories from fat) for 12 more weeks. Thereafter, mice were euthanized, blood collected via cardiocentesis, and adipose tissues and liver were collected following tissue perfusion with ice-cold saline. All animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the University of Cincinnati, following appropriate guidelines.

Isolation of preadipocytes, adipocytes, and in vitro adipogenic differentiation. Subcutaneous (inguinal) and epididymal adipose tissues were dissected out, and stromovascular cells and adipocytes were isolated as we described previously by collagenase digestion (5). Preadipocytes were cultured and in vitro differentiated according to the methods as we described previously (5,8).

RNA isolation, qPCR, western blot and lipid droplets measurement. RNA was isolated utilizing RNeasy lipid mini kit (Qiagen) and qPCR quantification of mRNA levels was performed as we described previously (5,8) using Syber-green qPCR kit (Agilent). We selected Arbp (acidic ribosomal phosphoprotein P0) mRNA as a reference for normalization of transcripts under investigation. The choice of Arbp is based on our observation that the levels of this gene in
adipose tissues and adipocytes do not change with adipogenic differentiation or with obesity, a finding similar to that reported by Romero et. al. (9). The primer sequences used in the qPCR assay are provided in Supplementary Table 1.

Western blot analysis and lipid droplet measurement were performed as described previously (5). Mean daily food consumption was determined in chow diet and HFD groups by calculating the amount of consumed food at 24 hr intervals for 5 days. These experiments were repeated twice and the mean daily food consumption was estimated.

**Body fat measurements.** To assess weight gain, body weights were obtained weekly for mice fed chow or HFD. Body fat mass was measured in conscious mice fed chow or HFD for 12 weeks, using $^1$H magnetic resonance spectroscopy (EchoMRI-100; Echomedical Systems) as described previously (10).

**Indirect calorimetry.** Mice were allowed to acclimate to respiratory chambers for a period of 2 days. Subsequently, energy expenditure (EE), oxygen consumption, carbon dioxide production and respiratory quotient (RQ) were measured for 24 hr during 12 hr light/12 hr dark cycles. The volume of oxygen consumed ($V_O_2$; [ml/(kg × h)]) and carbon dioxide produced ($V_CO_2$; [ml/(kg × h)]) were measured to calculate energy expenditure (10). Locomotor activity was similarly measured over a 24 hr period. Core body temperatures during cold challenge were collected from single housed 12-hour fasted mice and were measured rectally with a rodent microprobe and thermometer (Thermalert) at 1 hour intervals after placing the mice at 4°C at the beginning of the light cycle. Additional core body temperatures were collected in fed mice at the beginning of the light cycle.

**Examination of insulin levels and glucose tolerance.** For glucose tolerance, glucose levels were measured from tail vein collections immediately prior and at 7.5, 15, 30, 60, 120 and 180
min after an intraperitoneal (IP) injection of glucose at 2 g/kg body weight into mice fasted for 12 hours (n = 12 or 8) using glucose strips and repeated measurements. Assessment of pancreatic insulin response to an IP glucose bolus at 2 g/kg body weight involved measuring plasma insulin (Ultra Sensitive Rat Insulin ELISA kit, Crystal Chem, Chicago) in samples collected from tail veins at 0, 7.5, 15, 30 and 60 min after glucose injection in mice fasted for 12 hr. Insulin sensitivity was assessed by IP delivery if 0.75 U/kg body weight of porcine insulin and measurement of plasma glucose from tail vein collections at 0, 15, 30, 60 and 90 min after injection in 6 hr-fasted mice. Plasma levels of adiponectin, leptin, and resistin were measured in mice after overnight fasting, utilizing commercially available ELISA kits (R&D).

**Histological measurement of adipose tissue and liver.** Tissues were fixed by immersion in neutral buffered formalin (10%), dehydrated in ethanol and then transferred to xylene solution for embedding in paraffin. Five µm sections were stained with hematoxylin-eosin and images were analyzed with light microscopy. Brown adipose and hepatic lipid accumulation was examined microscopically after H&E stain. Adipocyte area calculations were determined by measuring the area of greater than 120 cells per 200x magnification tissue sections using Image J v 1.46. Median adipocyte area was presented as mean ± SEM.

**Statistical analysis.** Data are expressed as mean ± SEM. Analysis of variance was accomplished with a one-way ANOVA followed by an unpaired, two-tailed student’s t test for P value using Sigma Plot v11. P values less than 0.05 were considered statistically significant. Linear regression analysis included R² and analysis of covariance (ANCOVA) for the analyses of body weight gain and cumulative energy expenditure data.

**RESULTS**
Impaired adipogenic differentiation of preadipocytes from high fat fed mice. The influence of HFD on adipogenic differentiation was assessed by comparing the ability of preadipocytes from mice fed a chow or a HFD to differentiate into mature adipocytes in vitro. Although the number of adipogenic stem/progenitor cells is not reduced by high fat feeding (3), the potential of these preadipocytes to differentiate into mature adipocytes was markedly blunted. Preadipocytes from chow fed mice promptly differentiated into mature adipocytes when incubated with adipocyte differentiation medium as expected, with nearly 90% of the cells accumulating lipid droplets in the cytoplasmic compartment (Fig. 1A), accompanied by up-regulated expression of adipogenic differentiation-specific genes such as C/EBPα, PPARγ, FABP4, and adiponectin (Fig. 1B). In contrast, preadipocytes from HFD mice exhibited blunted adipogenic differentiation as evidenced by diminished cytoplasmic lipid droplet accumulation (Fig. 1A) and reduced expression of adipogenic differentiation-specific genes under an identical differentiation protocol (Fig. 1C). The data reported in the present study were obtained using freshly isolated preadipocytes, expanded in culture, and examined after the third passage. Cells at higher passage numbers (6-8) began to show a noticeable decline in their potential for adipogenic differentiation and were thus not extensively studied. Nevertheless, compared with control (chow fed) mice, preadipocytes from HFD mice continued to exhibit reduced adipogenic differentiation despite repeated passaging (5-6) of these cells (data not shown).

High fat diet perturbs adipogenic differentiation via HDAC9. We previously demonstrated that HDAC9 down-regulation precedes adipogenic differentiation of preadipocytes both in vitro and in vivo, and that over-expression of HDAC9 prevents, while HDAC9 gene deletion accelerates, adipogenic differentiation (5). Together, these data imply that HDAC9 is a negative regulator of adipogenic differentiation, and its down-regulation is necessary for activation of the
adipogenic differentiation program in preadipocytes (5). This prompted us to determine whether HFD perturbs adipogenic differentiation through a mechanism involving HDAC9. Basal HDAC9 mRNA and protein expression were similar in preadipocytes isolated from chow and HFD mice prior to the onset of differentiation (Fig. 2, day 0). As we previously reported, preadipocytes from chow-fed mice exhibited dramatic down-regulation of HDAC9 mRNA and protein levels upon induction of adipogenic differentiation (Fig. 2). Under identical conditions, preadipocytes from HFD mice exhibited blunted down-regulation of HDAC9 mRNA and protein levels (Fig. 2). HDAC9 is constitutively a nuclear protein in preadipocytes from both CD-fed and HFD-fed mice, and the induction of adipogenic differentiation down-regulates nuclear HDAC9 protein levels only in preadipocytes from the chow-fed mice (data not shown), a finding consistent with our earlier report (5). These findings suggest that aberrant HDAC9 expression in preadipocytes from HFD mice may contribute to blunted adipogenic differentiation of these cells.

**HDAC9 gene deletion improves adipogenic differentiation in high fat diet-fed mice.** The relationship between HFD, impaired adipogenic differentiation and HDAC9 was explored by comparing the differentiation potential of preadipocytes isolated from wild-type mice versus HDAC9 knockout mice after feeding HFD for 12 weeks. The preadipocytes isolated from HFD wild-type and HDAC9 knockout mice exhibited similar growth rates, morphologic appearance, and preadipocyte-specific Pref1 gene expression (data not shown). However, when induced to undergo adipogenic differentiation, preadipocytes from HDAC9 knockout mice fed HFD showed significant lipid accumulation and elevated expression of adipogenic differentiation-specific genes (C/EBPα, PPARγ, FABP4, and adiponectin), at levels similar to those observed during differentiation of preadipocytes from chow-fed mice, and in marked contrast to the results
observed in preadipocytes from HFD wild-type mice (Fig. 3). To verify that these in vitro findings are relevant to adipogenic differentiation in vivo, we examined adipocytes freshly isolated from adipose tissues by collagenase digestion. HDAC9 mRNA and protein levels were significantly higher in freshly isolated adipocytes and adipose tissues from HFD wild-type mice compared to chow-fed mice (Fig. 4A). Importantly, freshly isolated adipocytes from HFD wild-type mice exhibited blunted expression of adipocyte differentiation-specific genes C/EBPα, PPARγ, and adiponectin, which was abrogated by HDAC9 gene deletion (Fig. 4B). HDAC9 gene deletion similarly prevented blunted adipogenic gene expression in adipose tissues from both subcutaneous and visceral (epididymal) depots of HFD mice (Supplementary Fig. 1). Taken together, these findings suggest that HDAC9 expression contributes to HFD-induced impaired adipogenic differentiation.

**HDAC9 gene deletion prevents HFD-induced obesity.** We next examined the effects of HDAC9 gene deletion on HFD-induced obesity. Our initial survey demonstrated that chow-fed HDAC9 knockout mice displayed a slight but statistically insignificant trend of lower body weight (22.6 ± 0.9 gm) compared to chow-fed wild-type mice (23.8 ± 1.5 gm). Upon high fat feeding, HDAC9 knockout mice gained significantly less weight compared to wild-type control mice (Figs. 5A, B). However, HDAC9 knockout and wild-type control mice exhibited similar locomotor activities (Fig. 5C) and consumed comparable amounts of food (Fig. 5D). NMR evaluation of whole body adiposity demonstrated reduced fat mass both in CD- and HFD-fed HDAC9 knockout mice (Fig. 5E), and subcutaneous and epididymal adipose tissue weights were lower in HDAC9 knockout mice compared to wild-type mice (Figs. 5F, G). Histological analysis of the subcutaneous adipose tissues showed that adipocytes in HDAC9 knockout mice were smaller under HFD-fed conditions compared to their wild-type littermate controls (Fig. 5H,
I) and that multilocular adipocytes were abundant in CD-fed HDAC9 knockout mice (Fig. 5I), a finding reminiscent of beige phenotypic changes of white adipose tissue (11).

**HDAC9 gene deletion improves glucose tolerance, insulin sensitivity, and lipid accumulation in liver.** Comparison of glucose tolerance between wild-type and HDAC9 knockout mice demonstrated that HDAC9 knockout mice were significantly more glucose tolerant following both CD (Fig. 6A) and HFD (Fig. 6B). The improved glucose tolerance in HDAC9 knockout mice was not due to enhanced insulin secretion, as similar plasma insulin levels were observed in both groups of CD-fed mice (Fig. 6C), while plasma insulin levels were significantly lower in HFD-fed HDAC9 knockout mice as compared with wild-type mice (Fig. 6D). Insulin injection into CD-fed wild-type and HDAC9 knockout mice induced a rapid decrease in plasma glucose levels, consistent with preserved insulin sensitivity (Fig. 6E). Importantly, after HFD, insulin sensitivity was improved in HDAC9 knockout mice as compared with wild-type mice (Fig. 6F). Future studies are needed to determine the relative effects of HDAC9 gene deletion on insulin sensitivity of specific target cells, including adipocytes. Under these experimental conditions, HFD did not significantly affect plasma adiponectin (data not shown) or lipid profile, except for total cholesterol levels (Supplementary Table 2). However, HFD upregulated plasma pro-inflammatory adipokines leptin (Fig. 6G) and resistin (Fig. 6H) in wild-type mice, which was attenuated in HDAC9 knockout mice.

Because adipose tissue dysfunction promotes ectopic lipid deposition and accumulation in non-adipose tissues, we assessed lipid accumulation in the liver following HFD. HFD precipitated dramatic lipid accumulation in the livers of wild-type mice, which was completely prevented by HDAC9 gene deletion (Fig. 6I). Additionally, liver mass was significantly lower in
HDAC9 knockout mice compared to wild-type mice (Fig. 6J). These results provide evidence that HDAC9 gene deletion is also protective against HFD-induced hepatosteatosis.

**HDAC9 regulates energy expenditure and adaptive thermogenesis: effects on brown adipose tissue.** Our data indicated that HDAC9 gene deletion results in reduced fat mass and improved metabolic state despite no differences in locomotor activity or food consumption. Subsequently, we determined that HDAC9 knockout mice exhibited elevated energy expenditure (Fig. 7A) and oxygen consumption (Fig. 7B) compared to their wild-type littermates under chow-fed conditions. No differences were detected in RQ between genotypes (data not shown). In addition, as compared to wild-type mice, HDAC9 knockout mice exhibited a higher basal body temperature and were protected against hypothermia following cold challenge (Fig. 7C), implying improved adaptive thermogenic capacity.

The above observations raised the possibility that HDAC9 gene deletion might affect the amount and/or quality of thermogenic brown adipose tissue. HDAC9 is expressed in brown adipose tissue and HDAC9 knockout mice surprisingly exhibited nearly 50% less brown adipose tissue mass compared to similarly maintained wild-type mice (Fig. 7D). Interestingly, histology demonstrated reduced lipid accumulation in brown adipose tissue of HDAC9 knockout mice fed a HFD as compared with wild-type mice (Fig. 7E). Thus, we next investigated the impact of HFD on the phenotype of brown adipose tissue. Unlike the dramatic changes observed in white adipose tissues (Fig. 1C), brown adipose tissue was largely unaffected by HFD, as demonstrated by qualitatively minor changes in the expression of adipogenic differentiation-specific or thermogenic genes (Fig. 7F), findings similar to those of Fitzgibbons et. al. (12). Moreover, expression of UCP1 in brown adipose tissue was not upregulated in HDAC9 knockout mice as compared to wild-type mice in the setting of HFD (Fig. 7F). Taken together, these findings
suggest that alterations in the phenotype of brown adipose tissue consequent to HDAC9 gene deletion are unlikely to account for the marked differences in lean body mass, energy expenditure, adaptive thermogenesis, and metabolic profile following HFD.

**Effects of HFD on beige adipocyte gene expression: impact of HDAC9 gene deletion.** Emerging evidence indicates that a specific class of brown-like adipocytes (beige adipocytes), with thermogenic characteristics, accumulate in the white adipose depots of mice (13). Beige adipocytes are reported to be involved in regulation of adaptive thermogenesis and energy expenditure, body fat accumulation, and metabolic homeostasis (14-18). To investigate the potential role of HDAC9 in regulating beige adipocytes, we first compared the levels of beige-specific genes in adipocytes from subcutaneous adipose tissues of CD-fed wild-type and HDAC9 knockout mice. Expression of beige adipocyte marker genes PRDM16, UCP1, CIDEA, and PGC1α was significantly higher (Fig. 8A), and clusters of multiloculated beige adipocytes were more readily apparent (Fig. 5I), in HDAC9 knockout mice as compared with wild-type mice. Importantly, HFD dramatically decreased expression of beige adipocyte marker genes in wild-type mice, which was strongly abrogated by HDAC9 gene deletion (Fig. 8A). By comparison, the effects of HFD and HDAC9 gene deletion on beige adipocyte marker genes in adipocytes from epididymal adipose tissue were not significant (Fig. 8B). These findings pertaining to differential expression of beige adipocyte-specific genes in subcutaneous adipose tissues are consistent with the leaner adipose tissue mass, increased energy expenditure, and enhanced adaptive thermogenesis observed in HDAC9 knockout mice.

Because adipose tissue-derived fibroblast growth factor 21 (FGF21) was shown previously to function as an autocrine/paracrine inducer of thermogenic gene expression and beige phenotype in white adipose tissues (6), we examined FGF21 expression in subcutaneous
adipocytes of CD-fed versus HFD-fed mice. HFD profoundly reduced FGF21 levels in subcutaneous adipocytes of wild-type, but not HDAC9 knockout mice (Fig. 8C), suggesting that preserved FGF21 expression, leading to increase in beige adipocytes, may in part mediate the beneficial effects of HDAC9 gene deletion on HFD-induced adipose tissue dysfunction. HFD however did not influence FGF21 levels in epididymal adipocytes.

**DISCUSSION**

We previously demonstrated that HDAC9 is a negative regulator of adipogenic differentiation; expression of HDAC9 falls abruptly at the onset of differentiation of preadipocytes, thereby permitting activation of the adipogenic differentiation program (5). We now report that this regulatory mechanism is disrupted by HFD, leading to blunted HDAC9 down-regulation and impaired adipogenic differentiation. HDAC9 gene deletion prevents impaired adipogenesis in vitro, and accumulation of improperly differentiated adipocytes in vivo, while reducing ectopic lipid accumulation in the liver, and improving systemic insulin sensitivity and glucose tolerance. Additionally, HDAC9 knockout mice exhibit increased energy expenditure and are protected from the hypothermic effects of fasting and cold challenge. Moreover, HDAC9 gene deletion promotes elevated basal expression of beige adipocyte marker genes in white adipose tissue, and prevents their down-regulation in response to HFD. HDAC9 gene deletion also prevents HFD-induced down-regulation of FGF21, a known autocrine/paracrine inducer of beige phenotype, in white adipose tissues (6). Our findings thus provide novel insight into the role of HDAC9 in regulating adipose tissue function during diet-induced obesity.

The mechanisms whereby HFD disrupts HDAC9 down-regulation and adipogenic differentiation of preadipocytes remain to be determined. HFD does not negatively influence
adipogenic precursor cell abundance in adipose tissues (3), implying that sufficient numbers of precursor cells are available to support adipogenic differentiation. Moreover, HDAC9 levels in preadipocytes from chow and HFD mice were similar prior to the onset of adipogenic differentiation. However, unlike preadipocytes from chow fed mice, preadipocytes from HFD mice failed to sufficiently down-regulate HDAC9 expression, which appears necessary for adipogenic differentiation (5). Considering that the cells in our study were isolated and propagated through in vitro culture with multiple rounds of cell division, the impaired adipocyte differentiation is independent of the impact of HFD on the in vivo milieu of adipose tissues (e.g., hypoxia, neuronal input, hormonal influences, etc.). Our results, therefore, suggest that HFD impairs adipocyte differentiation through cell autonomous and heritable (epigenetic) mechanisms involving dysregulated HDAC9 expression in these cells. The precise nature of these epigenetic regulatory mechanisms, and whether they are reversible with prolonged discontinuation of HFD, remains to be determined.

Our data also suggest that endogenous HDAC9 directly contributes to the adverse systemic metabolic consequences of HFD. Thus, HDAC9 knockout mice were leaner, less glucose intolerant, and more insulin sensitive than their wild-type littermates despite similar food consumption and locomotor activity. HDAC9 is expressed in islet β-cells, and HDAC9 gene deletion in pancreas was reported to enhance β-cell mass (19). Although these observations suggested that HDAC9 inactivation potentially could confer metabolic benefits by increasing insulin secretion, our data indicate that differences in insulin sensitivity, and not secretion, are responsible for improved glucose tolerance in HDAC9 knockout mice (Figure 6). Rather, our data suggest that the metabolic benefits of HDAC9 gene deletion in chronic HFD are potentially the result of increased thermogenic energy expenditure related to up-regulated beige adipocyte
gene expression. Thus, beige-specific genes in subcutaneous adipose tissues are down-regulated during HFD in wild-type mice, in parallel with increased body mass, expanded white adipose tissue depots, systemic insulin resistance, and glucose intolerance, all of which were markedly attenuated in HDAC9 knockout mice. By contrast, while HDAC9 gene deletion led to reduced brown tissue adipose mass during HFD, it had little impact on thermogenic brown adipose gene expression. Whether the reduced brown adipose tissue mass is a compensatory response to increased beige adipocytes, or vice versa, is presently unclear (15). Further investigations will be required to establish with certainty whether increased beige adipocyte gene expression mediates the improved metabolic phenotype of HDAC9 knockout mice. Nevertheless, these findings raise the intriguing possibility that down-regulation of beige adipocytes contributes to the deleterious metabolic effects of HFD, a process that could potentially be ablated by therapeutically targeting HDAC9.

Precisely how HDAC9 regulates beige adipocytes remains to be determined; however, we observed that FGF21 expression was dramatically reduced in subcutaneous adipocytes of HFD-fed wild-type, but not of HDAC9 knockout, mice. FGF21 plays a major role in inducing beige adipocyte gene expression, including UCP1, leading to upregulation of thermogenesis (6, 20). The fact that HDAC9 gene deletion prevents HFD-induced FGF21 down-regulation implies that endogenous HDAC9 could be a negative regulator of FGF21. Further studies will be required to determine the potentially complex underlying mechanisms of regulation of FGF21 by HDAC9 during HFD. Since beige adipocytes are inversely related to obesity in humans (14, 21, 22), our findings support the notion that targeting HDAC9 could be a fruitful approach to treating obesity-related disease.
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Authors declare no conflict of interest.

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REFERENCES


FIGURE LEGENDS

Fig. 1. High fat feeding impairs *in vitro* adipogenic differentiation of preadipocytes from mouse subcutaneous adipose tissue. Lipid droplet accumulation (A) during *in vitro* adipogenic differentiation of preadipocytes from subcutaneous adipose depot of chow diet (CD) and high fat diet (HFD) fed mice. Neutral lipids were labeled with oil-red-O and visualized by light microscopy following 7 days of differentiation. Oil red-O stained neutral lipids were extracted from cells following 3 and 7 days of differentiation, quantified spectrophotometrically, and normalized against cellular protein levels (right panel). Values are mean ± SEM of three experiments; *P*<0.05. Induction of adipogenic differentiation-specific genes *C/EBP*α, *PPAR*γ, *FABP*4, and *adiponectin* following 7-day in vitro differentiation of subcutaneous preadipocytes from CD fed mice (B). Relative mRNA levels of adipogenic differentiation-specific genes in 7-day in vitro differentiated preadipocytes from CD and HFD fed mice (C). mRNA levels were quantified by RT-qPCR and normalized to *Arbp* (acidic ribosomal phosphoprotein P0). Values represent mean ± SEM of fold increase from CD using preadipocytes from three different isolates; *P*<0.005.

Fig. 2. Impaired *in vitro* adipogenic differentiation parallels elevated HDAC9 expression. HDAC9 mRNA (A) and protein (B) expression were quantified by RT-qPCR and western blot analyses, respectively on the indicated days of differentiation. Values represent percent change from preadipocytes (0 day) and are expressed as mean ± SEM of three different experiments. Western blot images were analyzed utilizing ImageJ software and expressed as percent of 0 day levels in each condition after normalization to corresponding GAPDH values; *P*<0.05.
Fig. 3. HDAC9 gene deletion improves adipogenic differentiation of preadipocytes from subcutaneous adipose tissues of HFD mice.  

Adipogenic gene expression (A) and lipid droplet accumulation (B) were determined as described in Figure 1. Values represent mean ± SEM from 4 experiments; *P<0.05.

Fig. 4. HDAC9 gene deletion prevents accumulation of inefficiently differentiated adipocytes in subcutaneous adipose tissue of HFD mice.  

Adipocytes (in vivo differentiated) were isolated from subcutaneous adipose depots of CD and HFD fed mice; HDAC9 mRNA levels in these cells were analyzed by qRT-PCR. HDAC9 protein levels were determined in epididymal adipose tissue of CD and HFD fed mice by western blot analysis. For mRNA expression, values represent mean ± SEM from 4 groups of mice; *P<0.001 while Image J analysis of western blot data were normalized to corresponding GAPDH values and represent mean ± SEM from 3 groups of mice, *P<0.05. Effects of HFD on mRNA expression of adipogenic differentiation-specific genes, C/EBPα, PPARγ, and adiponectin, in freshly isolated adipocytes (in vivo differentiated) from subcutaneous adipose tissues of wild-type (+/+) and HDAC9 knockout (-/-) mice (B). Values are mean ± SEM of 4 experiments; *P<0.05.

Fig. 5. HDAC9 gene deletion attenuates the gain in body weight, adiposity, and adipocyte enlargement induced by HFD.  

Growth curves of male wild-type (filled circles) and HDAC9 knockout mice (open circles) fed HFD (A); *P<0.0001, slope of wild-type versus HDAC9 knockout growth curves. Mean weekly body weight gain of wild-type (+/+) and HDAC9 knockout (-/-) mice fed HFD (B); *P< 0.001. Cumulative locomotor activities over a 24 h period of wild-type (+/+) and knockout mice (-/-) on CD (C). Mean daily food consumption in wild-type mice fed CD (open circles) and HFD (closed circles) versus comparably-fed HDAC9 knockout mice (open and closed triangles, respectively) (D). Whole body adiposity measured by
NMR in CD and HFD mice (E). Black bars represent wild-type while gray bars represent HDAC9 knockout mice; *p<0.05. Subcutaneous (F) and epididymal (G) adipose tissue weights of wild-type (black bars) and HDAC9 knockout (gray bars) mice maintained on CD or HFD for 12 weeks. Values are mean ± SEM from 6 mice; #P, <0.05 compared to wild-type CD mice; *, P<0.05 compared to wild-type HFD mice. Median adipocyte cross-sectional areas (H) were determined from adipose tissues collected from mice fed CD or HFD for 12 weeks. Data represent mean ± SEM of median adipocyte areas from n = 6 per group; *P<0.05. Microscopic images of H&E stained subcutaneous adipose tissues (I) from wild-type (+/+) and HDAC9 knockout (-/-) mice maintained on CD or HFD for 12 weeks. Note the presence of multilocular adipocytes (arrows) within the inset.

**Fig. 6.** HDAC9 gene deletion improves glucose tolerance, insulin sensitivity, and lipid accumulation in liver. Glucose tolerance of mice fed CD (A) or HFD (B) for 12 weeks, and glucose-stimulated insulin secretion (C, D), were determined following an intraperitoneal (IP) injection of a glucose solution (2 g/kg body weight). Closed symbols represent wild-type (+/+) and open symbols represent HDAC9 knockout (-/-) mice. Values represent mean ± SEM from 6-10 mice in each group (note that baseline insulin levels were subtracted in C); *P<0.05 compared to comparably fed wild-type mice. Systemic insulin sensitivity was assessed measuring plasma glucose concentrations after an IP injection of insulin (0.75 U/kg) in CD (E) and HFD (F) fed mice following a 6 hour fast. Values represent mean ± SEM from 6 mice in each group; * P<0.05 compared to wild-type HFD. Plasma leptin (G) and resistin (H) levels were assayed in wild-type and HDAC9 knockout mice maintained on CD and HFD. Values are mean ± SEM from 6 mice in each group; * P<0.05 compared to CD, #P<0.05 compared to HFD fed wild-type mice. Representative histology of hemotoxylin and eosin-stained paraffin-
embedded liver tissue sections from HFD fed wild-type and HDAC9 knockout mice (I), and liver mass of wild-type and HDAC9 knockout mice fed CD and HFD; *P<0.05. Values are mean ± SEM from 4-6 mice in each group.

**Fig. 7. HDAC9 knockout mice exhibit increased energy expenditure, oxygen consumption, and resistance to cold challenge.** Energy expenditure (24 hour) was determined in wild-type (black line) and HDAC9 knockout (red line) mice maintained on CD (A). Thick black region in the x-axis represents dark period (12 hrs); *P<0.05. O$_2$ consumption was quantified in wild-type (+/-) and HDAC9 knockout (-/-) mice maintained on CD (B); *P<0.05. Body temperature was measured (C) under basal conditions and following cold challenge. Brown adipose tissue (interscapular) depots were dissected and weighed (D) in wild-type and HDAC9 knockout mice fed a CD; *P<0.05. Histology (H & E staining) shows lipid accumulation in brown adipose tissue following HFD in wild-type and HDAC9 knockout mice (E). Expression of adipogenic (PPAR$_\gamma$ and FABP4) and brown adipose tissue (PRDM16, CIDEA, PGC1$\alpha$, and UCP1) marker genes (F) in brown (interscapular) adipose tissue following HFD and CD was quantified in wild-type and HDAC9 knockout mice; *P<0.05.

**Fig. 8. HDAC9 gene deletion prevents beige adipocyte marker genes down-regulation in subcutaneous adipocytes of HFD fed mice.** Expression of beige adipocyte marker genes in subcutaneous (A) and epididymal (B) adipocytes of wild-type and HDAC9 knockout mice fed CD and HFD was determined by RT-qPCR as described in Figure 1; *P<0.05. Compared to subcutaneous, epididymal adipocytes express nearly 100 fold lower UCP1 mRNA levels in wild-type mice under CD fed condition. FGF21 mRNA levels (C) in subcutaneous and epididymal adipocytes of wild-type and HDAC9 knockout mice in CD and HFD; *P<0.001. Values are mean ± SEM of 4-6 mice in each experimental condition. Compared to subcutaneous,
epididymal adipocytes express nearly 10 fold lower FGF21 mRNA levels in wild-type mice under CD fed condition.
**Fig. 1**

**A**

CD vs HFD

**B**

- **C/EBPα**
  - Relative mRNA levels (Fold induction)
  - 0, 5, 10
- **PPARγ**
  - Relative mRNA levels (Fold induction)
  - 0, 5, 10
- **FABP4**
  - Relative mRNA levels (Fold induction)
  - 0, 5, 10
- **Adiponectin**
  - Relative mRNA levels (Fold induction)
  - 0, 50, 100

**C**

CD vs HFD

- **C/EBPα**
  - Relative mRNA levels (% CD)
  - 0, 25, 50, 75, 100
- **PPARγ**
  - Relative mRNA levels (% CD)
  - 0, 25, 50, 75, 100
- **FABP4**
  - Relative mRNA levels (% CD)
  - 0, 25, 50, 75, 100
- **Adiponectin**
  - Relative mRNA levels (% CD)
  - 0, 25, 50, 75, 100
Fig. 2
**Fig. 3**

Panel A: Relative mRNA levels of C/EBP, PPAR, FABP4, and Adiponectin in wild-type (+/+) and knockout (-/-) mice. Bars are presented as mean ± SEM, and statistical significance is indicated by asterisks. 

Panel B: Images of wild-type (+/+) and knockout (-/-) mice with differences in tissue appearance and biochemical analysis of OD_{520} mg protein (arbitrary unit).
**Fig. 4**

**A**

- **HDAC9 mRNA levels** (Fold change over CD)
- **PPAR mRNA levels** (Fold change over +/- CD)
- **Adiponectin mRNA levels** (Fold change over +/- CD)

**B**

- **C/EBPβ mRNA levels** (Folds change over +/- CD)
- **PPAR mRNA levels** (Fold change over +/- CD)
- **Adiponectin mRNA levels** (Fold change over +/- CD)

The figures show the expression levels of various genes under different conditions (CD vs. HFD) and genotypes (+/+ vs. +/-). The asterisk (*) indicates statistically significant differences.
Fig. 5
Fig. 6

A. Blood glucose (mg/dL) over time after ip glucose.

B. Blood glucose (mg/dL) over time after ip glucose.

C. Plasma insulin (ng/ml) over time.

D. Plasma insulin (ng/ml) over time.

E. Plasma Glucose (mg/dL) over time.

F. Plasma Glucose (mg/dL) over time.

G. Plasma Leptin (ng/ml).

H. Plasma Resistin (ng/ml).

I. Tissue weight (gm).

J. CD vs HFD.
**Fig. 7**

- **A**
  - Graph showing Energy Expenditure (cal/hr/kg) over Time.
  - The red line indicates a linear increase with symbols indicating statistical significance.

- **B**
  - Bar graph showing VO2 Consumption (mL/kg/min).
  - The bars are labeled with significance markers (*).

- **C**
  - Graph showing Core Body Temp (°C) over Hours at 4°C.
  - The graph includes symbols indicating statistical significance (*).

- **D**
  - Bar graph showing Brown Adipose Tissue weight.
  - Symbols indicate statistical significance (*).

- **E**
  - Images showing tissue samples labeled as +/- and -/-.

- **F**
  - Bar graphs showing mRNA levels (%CD) for PPAR, FABP4, PROM16, CIDEA, and PGC1a.
  - The graphs compare CD and HFD conditions.

**Diabetes**

- The page content is related to diabetes with graphs and bar charts showing energy expenditure, VO2 consumption, core body temperature, brown adipose tissue weight, and mRNA levels under different conditions (CD vs. HFD).
Fig. 8

PRDM16

UCP1

CIDEA

PGC1α

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

mRNA levels

(Fold change over +/+ CD)

Diabetes
Supplementary Table 1. Primer sequences utilized for qPCR evaluation of gene expression

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<thead>
<tr>
<th>Gene</th>
<th>Gene Bank (Accession Number)</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>C/EBPα</td>
<td>NM_007678.3</td>
<td>AGAAGTCGGTGGAAGAAGACGA</td>
<td>GCGTTGTTGGGTTATCTCGGCT</td>
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<td>Adiponectin</td>
<td>NM_009605.4</td>
<td>GCACTGGCAAGTTCTACTGCAACA</td>
<td>AGAGAACGGGCTTTGTCTTCTTGA</td>
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<td>PPARγ</td>
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<td>ACATAAAGTCTCTCCCGCTGACCA</td>
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<td>FABP4</td>
<td>NM_024406.2</td>
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<td>PRDM16</td>
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<td>PGC1α</td>
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Supplementary Table 2. Fasting Serum Lipids in HDAC9\(^{+/+}\) and HDAC9\(^{-/-}\) mice

<table>
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<tr>
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<th>High Fat Diet</th>
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<tr>
<td></td>
<td>+/+ (n = 6)</td>
<td>-/- (n = 6)</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>105.5 ± 13.5</td>
<td>105.5 ± 13.4</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>59.5 ± 3.2</td>
<td>59.7 ± 6.5</td>
</tr>
<tr>
<td>NEFA (moles/L)</td>
<td>0.578 ± 0.20</td>
<td>0.563 ± 0.13</td>
</tr>
</tbody>
</table>

Data are means ± SEM; *P<0.05 differences between diets, no significant differences between genotypes.
Supplementary Fig. 1  Adiponectin (A) and PPARγ (B) mRNA levels in subcutaneous (SQ) and epididymal (Epi) adipose tissues of wild-type (+/+), and HDAC9 knockout (-/-) mice maintained on CD and HFD; *P<0.05 compared to +/+ CD, #P<0.05 compared to +/+ HFD.