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HDAC9 Knockout Mice Are Protected From Adipose Tissue Dysfunction and Systemic Metabolic Disease During High-Fat Feeding

OBESITY STUDIES

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 a 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 an HFD, resulting in diminished weight gain, improved glucose tolerance and insulin sensitivity, and reduced hepatosteatosis. Moreover, compared with wild-type mice, HDAC9 knockout mice exhibit upregulated expression of beige adipocyte marker genes, particularly during an 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.

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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

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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 turnover 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 and histone deacetylase (HDAC) family of proteins, play an important role in adipogenic differentiation (4). We recently reported that histone deacetylase 9 (HDAC9), a class II HDAC, is an endogenous negative regulator of adipogenic differentiation and that downregulation of HDAC9 is necessary for adipogenic differentiation of preadipocytes (5), pointing to a key role of HDAC9 in adipogenic differentiation.

In the current study, we report that a chronic high-fat diet (HFD) impairs adipogenic differentiation, promoting accumulation of inefficiently differentiated adipocytes that exhibit diminished expression of adipogenic differentiation-specific genes. We additionally observed that an HFD markedly downregulates 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 downregulation of HDAC9 expression. Genetic ablation of HDAC9 blocked the deleterious effects of an 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 HFD-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 C57BL/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 age, these mice were either maintained on chow (Harlan Teklad, LM-485) or switched to an HFD (Research Diet, D12492, with 60% calories from fat) for 12 more weeks. Thereafter, mice were killed, blood was 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, Quantitative PCR, Western Blot, and Lipid Droplets Measurement

RNA was isolated utilizing a RNeasy lipid mini kit (Qiagen), and quantitative PCR (qPCR) quantification of mRNA levels was performed as we described previously (5,8) using a Syber green qPCR kit (Agilent). We selected acidic ribosomal phosphoprotein P0 (Arbp) 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-h 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 an HFD. Body fat mass was measured in conscious mice fed chow or an 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, oxygen consumption, carbon dioxide production, and respiratory quotient were measured for 24 h during 12-h light/12-h dark cycles. The volume of oxygen consumed (Vo_2 [ml/(kg × h)]) and carbon dioxide produced (Vco_2 [ml/(kg × h)]) were measured to calculate energy expenditure (10). Locomotor activity was similarly measured over a 24-h period. Core body temperatures during cold challenge were collected from single housed 12-h–fasted mice and were measured rectally with a rodent microprobe and thermometer (Thermalert) at 1 h 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 to and 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 h (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, IL) in samples collected from tail veins at 0, 7.5, 15, 30, and 60 min after glucose injection in mice fasted for 12 h. Insulin sensitivity was assessed by IP delivery of 0.75 units/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-h-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-micrometer 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 200× magnification tissue sections using ImageJ version 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 t test for P value using Sigma Plot version 11. P values <0.05 were considered statistically significant. Linear regression analysis included R^2 and 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 an HFD on adipogenic differentiation was assessed by comparing the ability of preadipocytes from mice fed chow or an 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. 1*A*), accompanied by upregulated expression of adipogenic differentiation-specific genes such as C/EBPα, PPARγ, FABP4, and adiponectin (Fig. 1*B*). In contrast, preadipocytes from HFD mice exhibited blunted adipogenic differentiation as evidenced by diminished cytoplasmic lipid droplet accumulation (Fig. 1*A*) and reduced expression of adipogenic differentiation-specific genes under an identical differentiation protocol (Fig. 1*C*). The data reported in the current 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).

HFD Perturbs Adipogenic Differentiation via HDAC9

We previously demonstrated that HDAC9 downregulation precedes adipogenic differentiation of preadipocytes both in vitro and in vivo and that overexpression 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 downregulation is necessary for activation of the adipogenic differentiation program in preadipocytes (5). This prompted us to determine whether an 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 downregulation of HDAC9 mRNA and protein levels upon induction of adipogenic

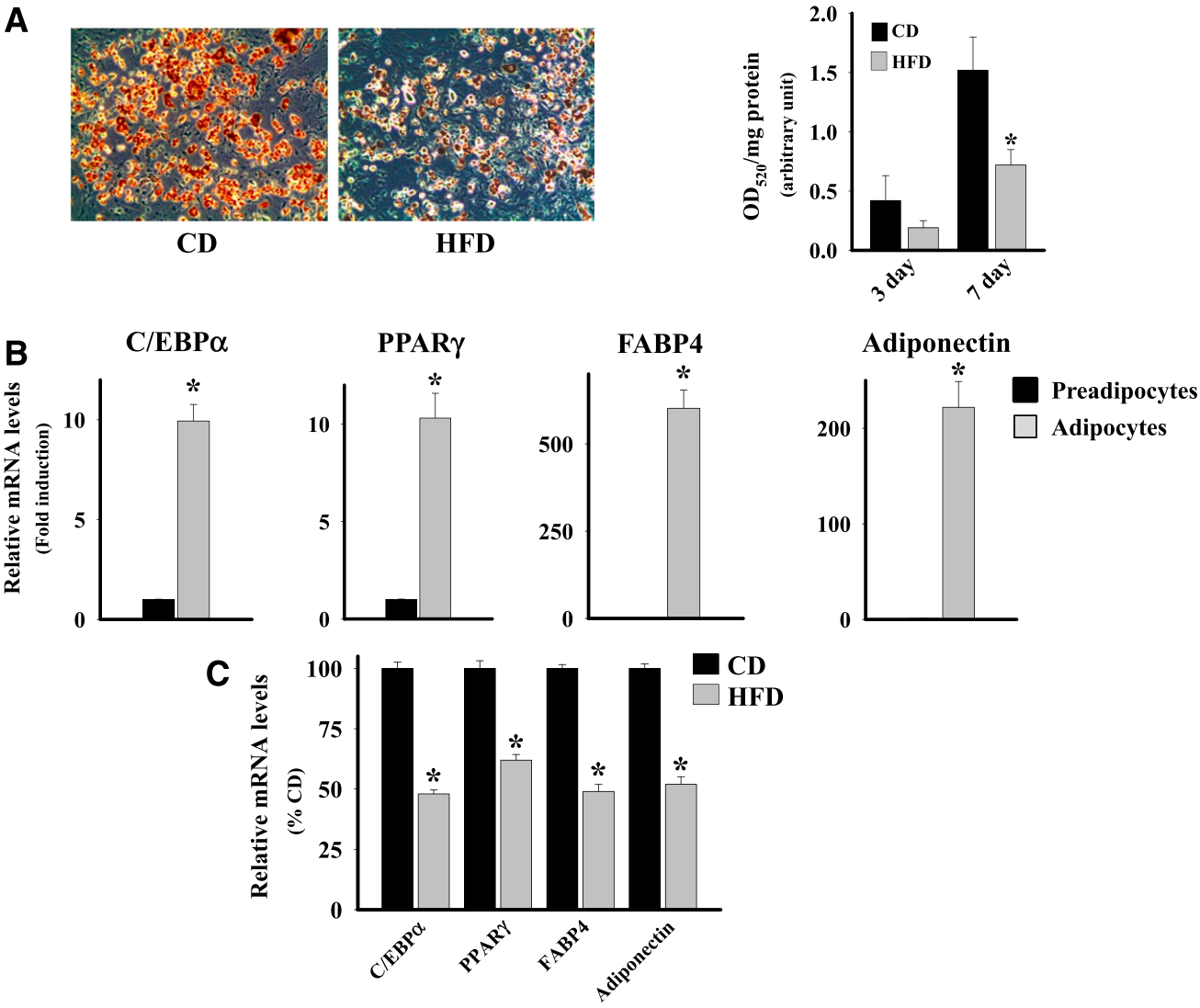

Figure 1—High-fat feeding impairs in vitro adipogenic differentiation of preadipocytes from mouse subcutaneous adipose tissue. *A*: Lipid droplet accumulation during in vitro adipogenic differentiation of preadipocytes from subcutaneous adipose depot of chow- and HFD-fed mice. Neutral lipids were labeled 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 (*$P < 0.05$). *B*: Induction of adipogenic differentiation-specific genes C/EBPα, PPARγ, FABP4, and adiponectin following 7-day in vitro differentiation of subcutaneous preadipocytes from chow-fed mice (*$P < 0.005$). *C*: Relative mRNA levels of adipogenic differentiation-specific genes in 7-day in vitro differentiated pre-adipocytes from chow- and HFD-fed mice. mRNA levels were quantified by qPCR and normalized to Arbp. Values represent mean ± SEM of fold increase from chow diet using preadipocytes from three different isolates (*$P < 0.005$). CD, chow diet.

differentiation (Fig. 2). Under identical conditions, preadipocytes from HFD mice exhibited blunted downregulation of HDAC9 mRNA and protein levels (Fig. 2). HDAC9 is constitutively a nuclear protein in preadipocytes from both chow- and HFD-fed mice, and the induction of adipogenic differentiation down-regulates nuclear HDAC9 protein levels only in pre-adipocytes 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 HFD-Fed Mice

The relationship between an 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 an 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

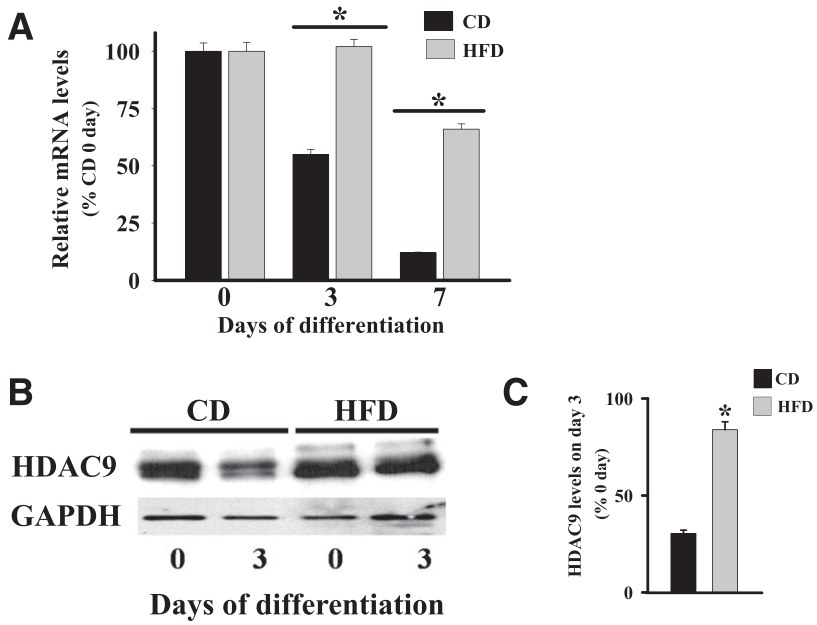

Figure 2—Impaired in vitro adipogenic differentiation parallels elevated HDAC9 expression. HDAC9 mRNA (*A*) and protein (*B*) expression were quantified by qPCR and Western blot analyses, respectively, on the indicated days of differentiation. Values represent percentage of change from preadipocytes (0 day) and are expressed as mean ± SEM of three different experiments. *C*: Western blot images were analyzed utilizing ImageJ software and expressed as percentage of 0 day levels in each condition after normalization to corresponding glyceraldehyde-3-phosphate dehydrogenase values. *$P < 0.05$. CD, chow diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

mice fed an 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 with chow-fed mice (Fig. 4*A*). 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. 4*B*). 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 g) compared with chow-fed wild-type mice (23.8 ± 1.5 g). Upon high-fat feeding, HDAC9 knockout mice gained significantly less weight compared with wild-type control mice (Fig. 5*A* and *B*). However, HDAC9 knockout and wild-type control mice exhibited similar locomotor activities (Fig. 5*C*) and consumed comparable amounts of food (Fig. 5*D*). Nuclear magnetic resonance evaluation of whole body adiposity demonstrated reduced fat mass both in chow- and HFD-fed HDAC9 knockout mice (Fig. 5*E*), and subcutaneous and epididymal adipose tissue weights were lower in HDAC9 knockout mice compared with wild-type mice (Fig. 5*F* and *G*). Histological analysis of the subcutaneous adipose tissues showed that adipocytes in HDAC9 knockout mice were smaller under HFD-fed conditions compared with their wild-type littermate controls (Fig. 5*H* and *I*) and that multilocular adipocytes were abundant in chow-fed HDAC9 knockout mice (Fig. 5*I*), 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 chow diet (Fig. 6*A*) and an HFD (Fig. 6*B*). The improved glucose tolerance in HDAC9 knockout mice was not due to enhanced insulin secretion, as similar plasma insulin levels were observed in both

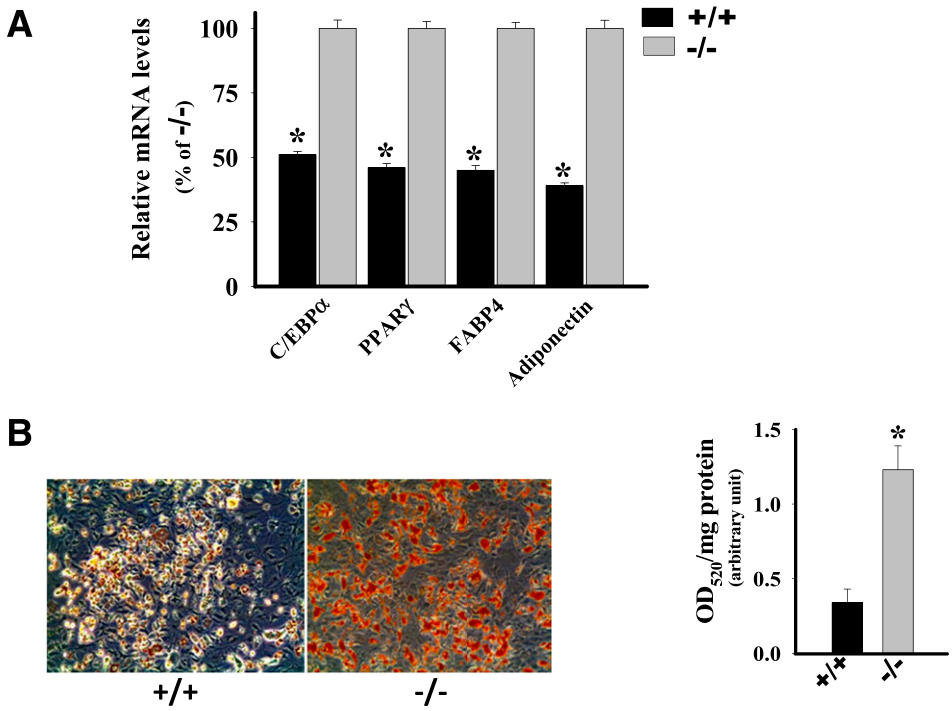

Figure 3—HDAC9 gene deletion improves adipogenic differentiation of preadipocytes from subcutaneous adipose tissues of HFD mice. Adipogenic gene expression (*A*) and lipid droplet accumulation (*B*) were determined as described in Fig. 1. Values represent mean ± SEM from four experiments. *$P < 0.05$.

groups of chow-fed mice (Fig. 6*C*), while plasma insulin levels were significantly lower in HFD-fed HDAC9 knockout mice as compared with wild-type mice (Fig. 6*D*). Insulin injection into chow-fed wild-type and HDAC9 knockout mice induced a rapid decrease in plasma glucose levels, consistent with preserved insulin sensitivity (Fig. 6*E*). Importantly, after an HFD, insulin sensitivity was improved in HDAC9 knockout mice as compared with wild-type mice (Fig. 6*F*). 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, an HFD did not significantly affect plasma adiponectin (data not shown) or lipid profile, except for total cholesterol levels (Supplementary Table 2). However, an HFD upregulated plasma proinflammatory adipokines leptin (Fig. 6*G*) and resistin (Fig. 6*H*) in wild-type mice, which was attenuated in HDAC9 knockout mice.

Because adipose tissue dysfunction promotes ectopic lipid deposition and accumulation in nonadipose tissues, we assessed lipid accumulation in the liver following an HFD. An HFD precipitated dramatic lipid accumulation in the livers of wild-type mice, which was completely prevented by HDAC9 gene deletion (Fig. 6*I*). Additionally, liver mass was significantly lower in HDAC9 knockout mice compared with wild-type mice (Fig. 6*J*). 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. 7*A*) and oxygen consumption (Fig. 7*B*) compared with their wild-type littermates under chow-fed conditions. No differences were detected in respiratory quotient between genotypes (data not shown). In addition, as compared with wild-type mice, HDAC9 knockout mice exhibited a higher basal body temperature and were protected against hypothermia following cold challenge (Fig. 7*C*), 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 with similarly maintained wild-type mice (Fig. 7*D*). Interestingly, histology demonstrated reduced lipid accumulation in brown adipose tissue of HDAC9 knockout mice fed an HFD as compared with wild-type mice (Fig. 7*E*). Thus we next investigated the impact of an HFD on the phenotype of brown adipose tissue. Unlike the dramatic changes observed in white adipose tissues (Fig. 1*C*), brown adipose tissue was

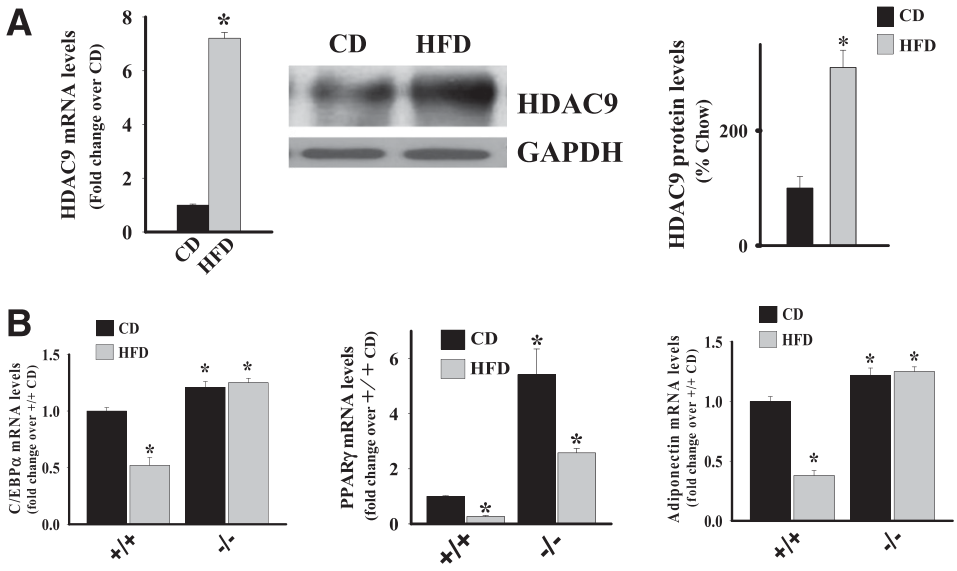

Figure 4—HDAC9 gene deletion prevents accumulation of inefficiently differentiated adipocytes in subcutaneous adipose tissue of HFD mice. *A*: Adipocytes (in vivo differentiated) were isolated from subcutaneous adipose depots of chow- and HFD-fed mice; HDAC9 mRNA levels in these cells were analyzed by qPCR. HDAC9 protein levels were determined in epididymal adipose tissue of chow- and HFD-fed mice by Western blot analysis. For mRNA expression, values represent mean ± SEM from four groups of mice (*$P < 0.001$), while ImageJ analysis of Western blot data were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase values and represent mean ± SEM from three groups of mice. *B*: Effects of an 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. Values are mean ± SEM of four experiments. *$P < 0.05$; CD, chow diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

largely unaffected by an HFD, as demonstrated by qualitatively minor changes in the expression of adipogenic differentiation-specific or thermogenic genes (Fig. 7*F*), 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 with wild-type mice in the setting of an HFD (Fig. 7*F*). 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 an HFD.

Effects of an 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 chow-fed wild-type and HDAC9 knockout mice. Expression of beige adipocyte marker genes PRDM16, UCP1, CIDEA, and PGC1α was significantly higher (Fig. 8*A*), and clusters of multiloculated beige adipocytes were more readily apparent (Fig. 5*I*) in HDAC9 knockout mice as compared with wild-type mice. Importantly, an HFD dramatically decreased expression of beige adipocyte marker genes in wild-type mice, which was strongly abrogated by HDAC9 gene deletion (Fig. 8*A*). By comparison, the effects of an HFD and HDAC9 gene deletion on beige adipocyte marker genes in adipocytes from epididymal adipose tissue were not significant (Fig. 8*B*). 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 chow- versus HFD-fed mice. An HFD profoundly reduced FGF21 levels in subcutaneous adipocytes of wild-type but not HDAC9 knockout mice (Fig. 8*C*), 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. An HFD, however, did not influence FGF21 levels in epididymal adipocytes.

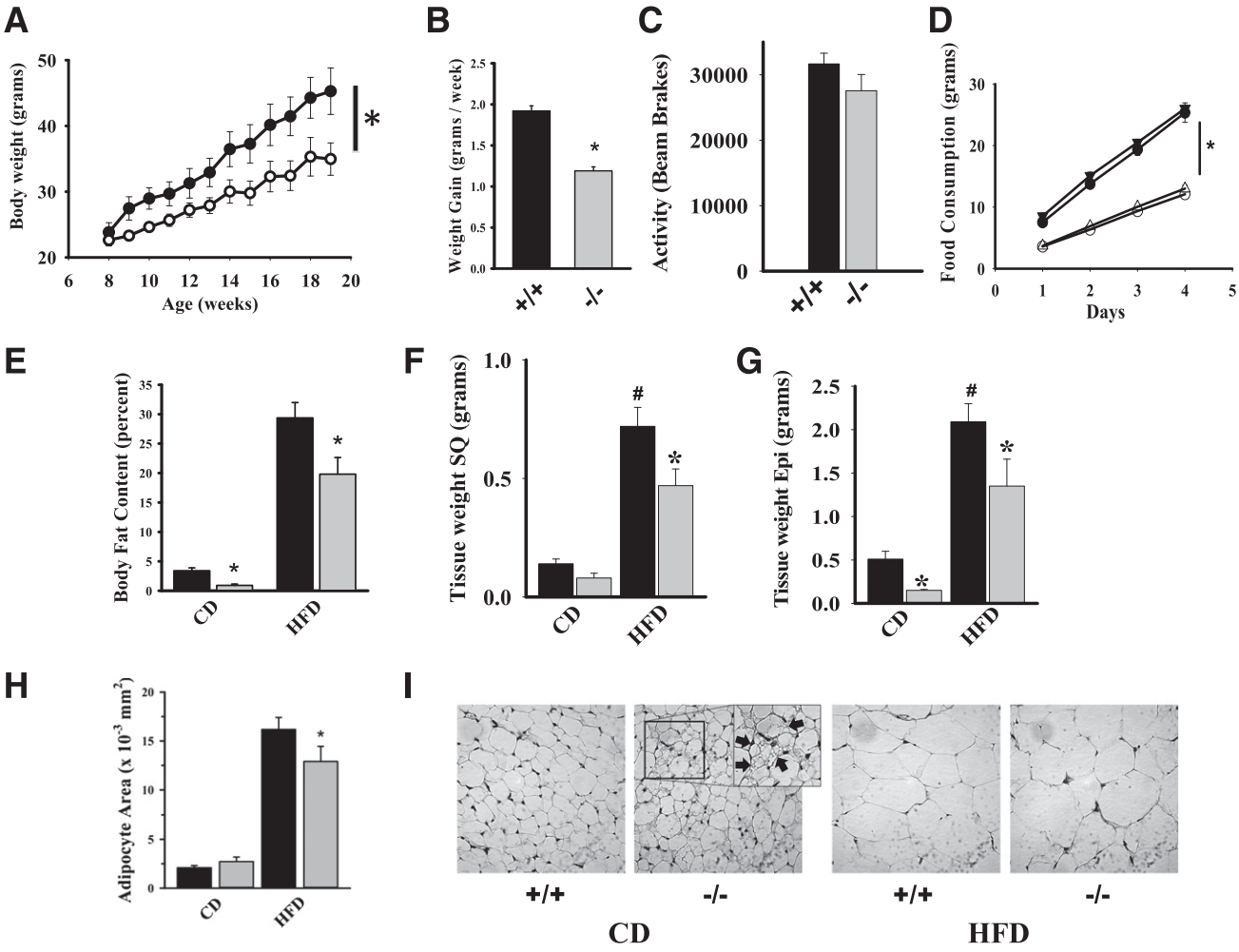

Figure 5—HDAC9 gene deletion attenuates the gain in body weight, adiposity, and adipocyte enlargement induced by an HFD. *A*: Growth curves of male wild-type (filled circles) and HDAC9 knockout mice (open circles) fed an HFD (*$P < 0.0001$). Slope of wild-type versus HDAC9 knockout growth curves. *B*: Mean weekly body weight gain of wild-type ($^{+/+}$) and HDAC9 knockout ($^{-/-}$) mice fed an HFD (*$P <$ 0.001). *C*: Cumulative locomotor activities over a 24-h period of wild-type ($^{+/+}$) and knockout mice ($^{-/-}$) on chow. *D*: Mean daily food consumption in wild-type mice fed chow (open circles) and an HFD (closed circles) versus comparably fed HDAC9 knockout mice (open and closed triangles, respectively). *E*: Whole body adiposity measured by nuclear magnetic resonance in chow and HFD mice. Black bars represent wild-type while gray bars represent HDAC9 knockout mice (*$P < 0.05$). *F*: Subcutaneous and (*G*) epididymal adipose tissue weights of wild-type (black bars) and HDAC9 knockout (gray bars) mice maintained on chow or an HFD for 12 weeks. Values are mean ± SEM from six mice (#$P < 0.05$ compared with wild-type CD mice; *$P < 0.05$ compared with wild-type HFD mice). *H*: Median adipocyte cross-sectional areas were determined from adipose tissues collected from mice fed chow or an HFD for 12 weeks. Data represent mean ± SEM of median adipocyte areas from $n = 6$ per group (*$P < 0.05$). *I*: Microscopic images of H&E stained subcutaneous adipose tissues from wild-type ($^{+/+}$) and HDAC9 knockout ($^{-/-}$) mice maintained on chow or an HFD for 12 weeks. Note the presence of multilocular adipocytes (arrows) within the inset. CD, chow diet; SQ, subcutaneous; Epi, epididymal.

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 an HFD, leading to blunted HDAC9 downregulation 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 downregulation in response to an HFD. HDAC9 gene deletion also prevents HFD-induced downregulation 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

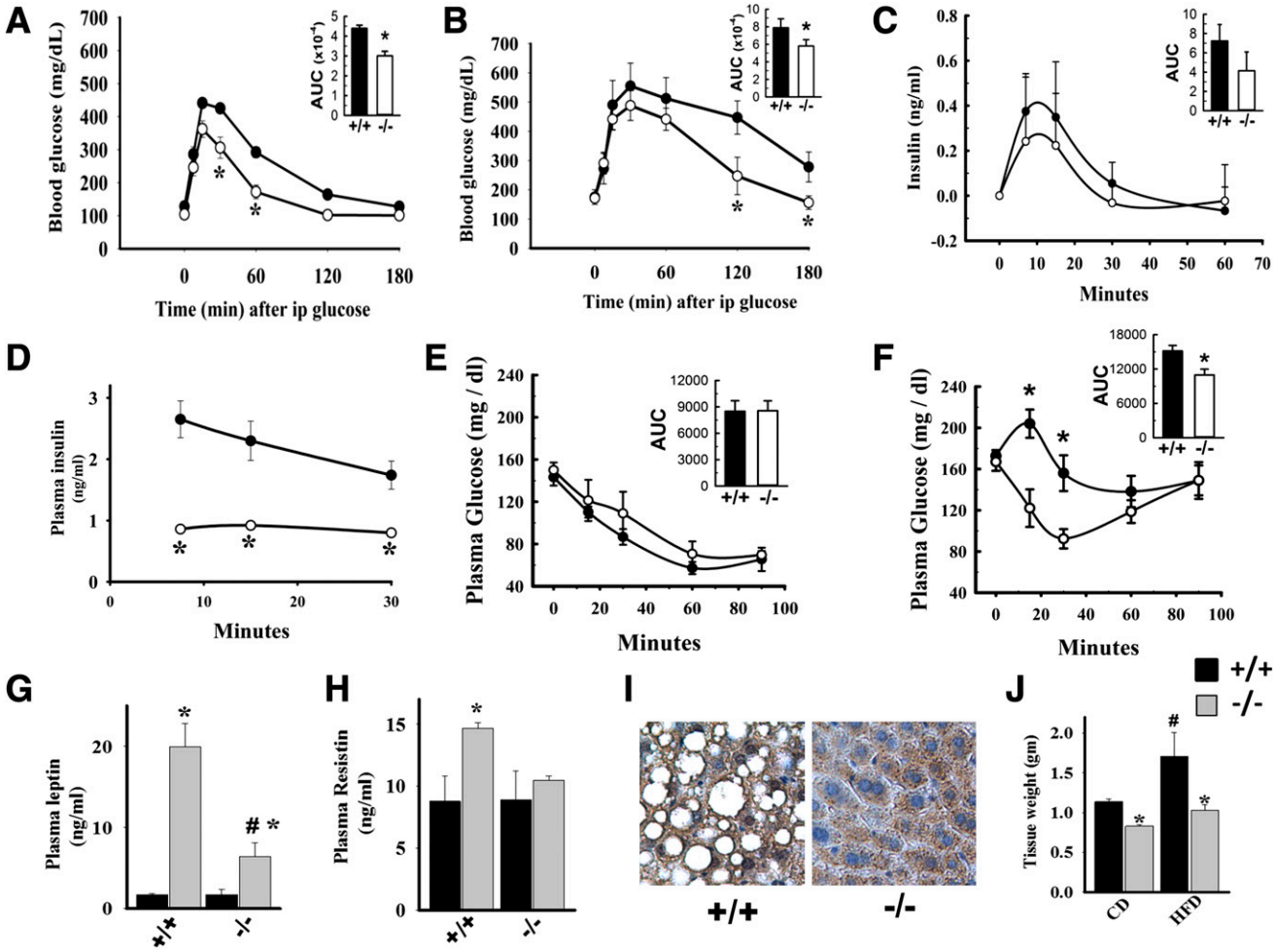

Figure 6—HDAC9 gene deletion improves glucose tolerance, insulin sensitivity, and lipid accumulation in liver. Glucose tolerance of mice fed chow (*A*) or an HFD (*B*) for 12 weeks and glucose-stimulated insulin secretion (*C*, *D*) were determined following an IP injection of a glucose solution (2 g/kg 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 with comparably fed wild-type mice). Systemic insulin sensitivity was assessed measuring plasma glucose concentrations after an IP injection of insulin (0.75 units/kg) in chow- (*E*) and HFD-fed (*F*) mice following a 6-h fast. Values represent mean ± SEM from six mice in each group (**P* < 0.05 compared with wild-type HFD). Plasma leptin (*G*) and resistin (*H*) levels were assayed in wild-type and HDAC9 knockout mice maintained on chow and an HFD. Values are mean ± SEM from six mice in each group (**P* < 0.05 compared with chow; #*P* < 0.05 compared with HFD fed wild-type mice). *I*: Representative histology of hemotoxylin and eosin–stained paraffin-embedded liver tissue sections from HFD-fed wild-type and HDAC9 knockout mice. *J*: Liver mass of wild-type and HDAC9 knockout mice fed chow and an HFD (**P* < 0.05). Values are mean ± SEM from 4–6 mice in each group. AUC, area under the curve; CD, chow diet.

regulating adipose tissue function during diet-induced obesity.

The mechanisms whereby an HFD disrupts HDAC9 downregulation and adipogenic differentiation of preadipocytes remain to be determined. An 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 downregulate 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 an HFD on the in vivo milieu of adipose tissues (e.g., hypoxia, neuronal input, and hormonal influences). Our results, therefore, suggest that an 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

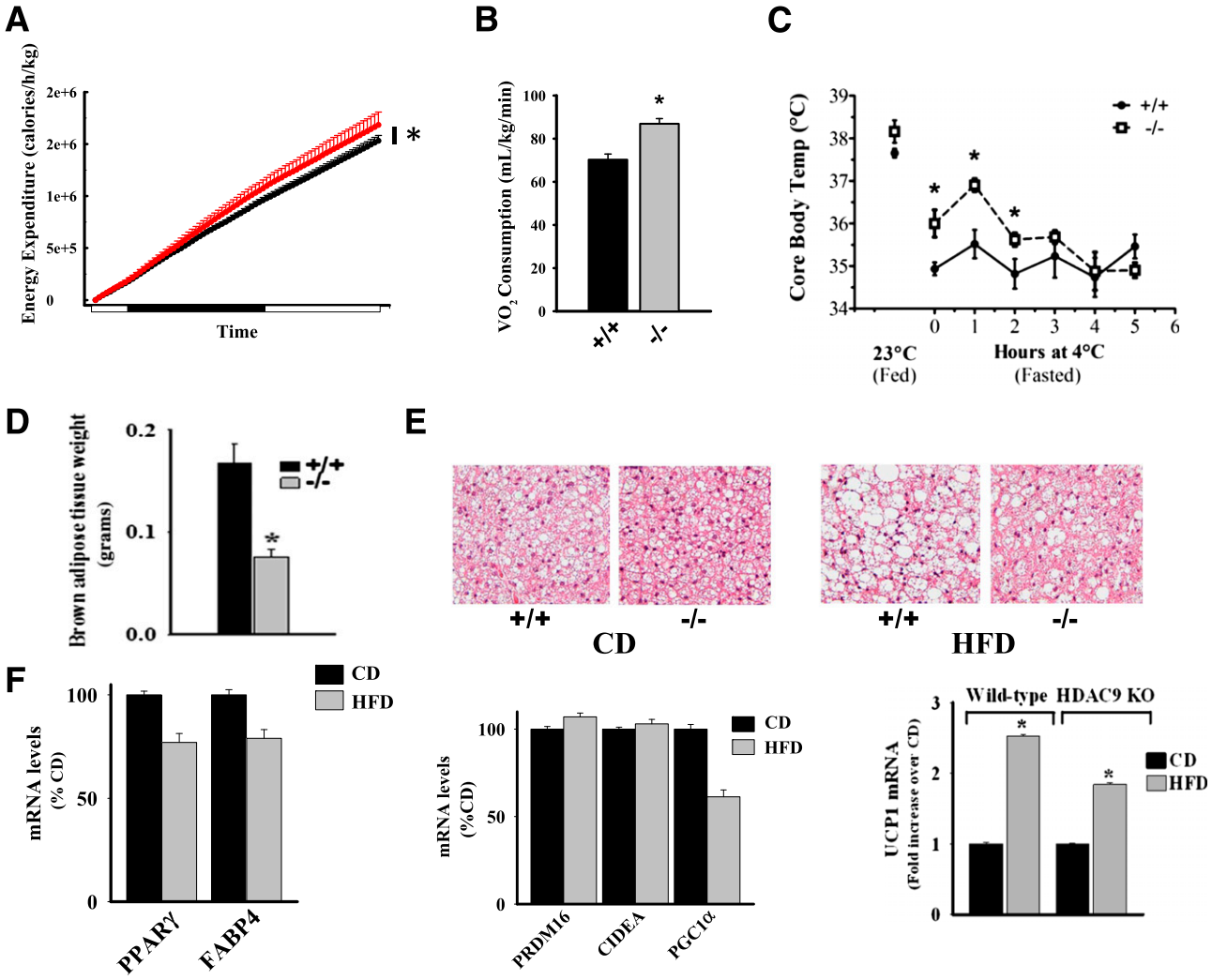

Figure 7—HDAC9 knockout mice exhibit increased energy expenditure, oxygen consumption, and resistance to cold challenge. *A*: Energy expenditure (24 h) was determined in wild-type (black line) and HDAC9 knockout (red line) mice maintained on chow. The thick black region in the *x*-axis represents dark period (12 h). *B*: O_2 consumption was quantified in wild-type ($^{+/+}$) and HDAC9 knockout ($^{-/-}$) mice maintained on chow. *C*: Body temperature was measured under basal conditions and following cold challenge. *D*: Brown adipose tissue (interscapular) depots were dissected and weighed in wild-type and HDAC9 knockout mice fed a chow diet. *E*: Histology (H&E staining) shows lipid accumulation in brown adipose tissue following an HFD in wild-type and HDAC9 knockout mice. *F*: Expression of adipogenic (PPARγ and FABP4) and brown adipose tissue (PRDM16, CIDEA, PGC1α, and UCP1) marker genes in brown (interscapular) adipose tissue following an HFD and chow was quantified in wild-type and HDAC9 knockout mice. *$P < 0.05$; CD, chow diet; KO, knockout.

with prolonged discontinuation of an HFD, remains to be determined.

Our data also suggest that endogenous HDAC9 directly contributes to the adverse systemic metabolic consequences of an 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 the 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 (Fig. 6). Rather, our data suggest that the metabolic benefits of HDAC9 gene deletion in a chronic HFD are potentially the result of increased thermogenic energy expenditure related to upregulated beige adipocyte gene expression. Thus beige-specific genes in subcutaneous adipose tissues are downregulated during an 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 an HFD, it had little

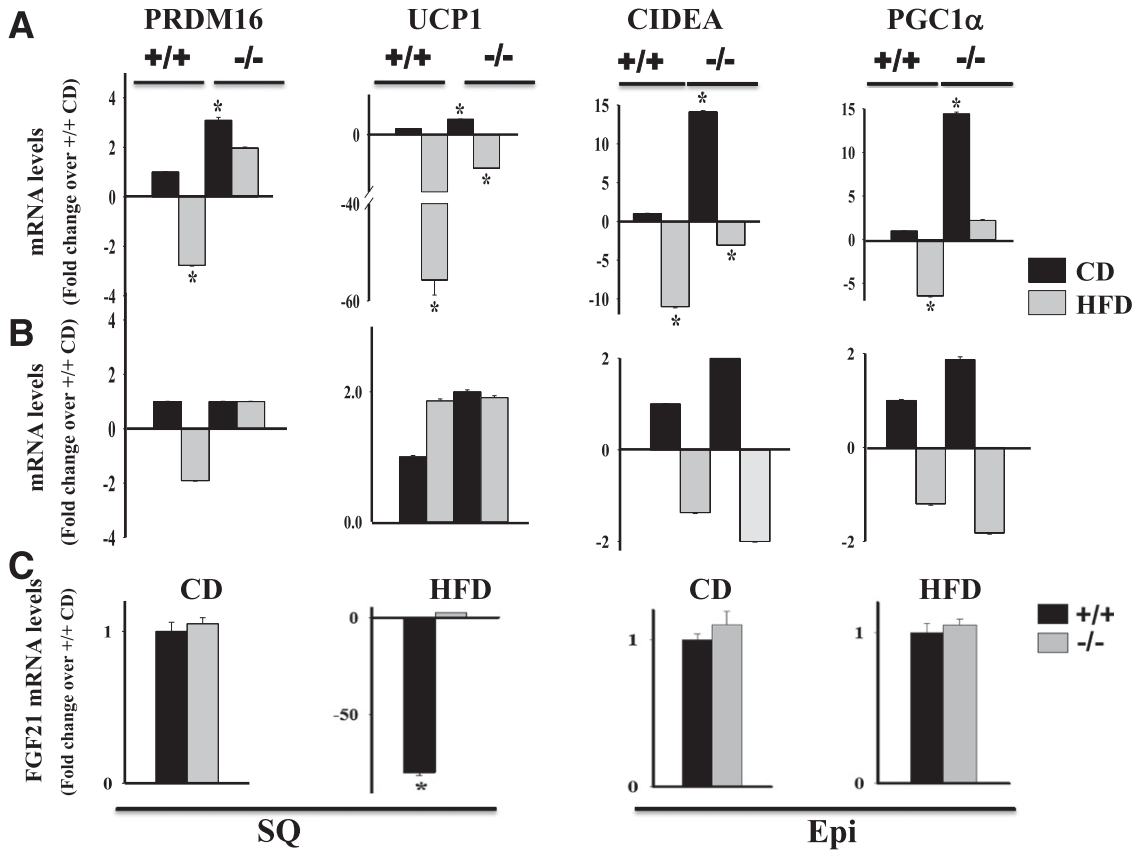

Figure 8—HDAC9 gene deletion prevents beige adipocyte marker genes' downregulation in subcutaneous adipocytes of HFD-fed mice. Expression of beige adipocyte marker genes in subcutaneous (*A*) and epididymal (*B*) adipocytes of wild-type and HDAC9 knockout mice fed chow and an HFD was determined by RT-qPCR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

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 downregulation of beige adipocytes contributes to the deleterious metabolic effects of an 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 downregulation 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 an 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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Author Contributions. T.K.C., D.Y.H., and N.L.W. researched data; contributed to the discussion; and wrote, reviewed, and edited the manuscript. J.E.B. researched data, contributed to the discussion, and wrote the manuscript. E.K. and W.S.T. researched data. V.B. and A.B.L. researched data and contributed to the discussion. A.L.B. and S.R. contributed to the discussion and reviewed and edited the manuscript. T.K.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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