HDAC6-mediated acetylation of lipid droplet-binding protein CIDEC regulates fat-induced lipid storage

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Obesity is characterized by aberrant fat accumulation. However, the intracellular signaling pathway that senses dietary fat and leads to fat storage remains elusive. Here, we have observed that the levels of histone deacetylase 6 (HDAC6) and the related family member HDAC10 are markedly reduced in adipose tissues of obese animals and humans. Mice with adipocyte-specific depletion of Hdac6 exhibited increased fat accumulation and reduced insulin sensitivity. In normal adipocytes, we found that reversal of P300/CREB-associated factor-induced (PCAF-induced) acetylation at K56 on cell death-inducing DFFA-like effector C (CIDEC, also known as FSP27) critically regulated lipid droplet fusion and lipid storage. Importantly, HDAC6 deacetylates CIDEC, leading to destabilization and reduced lipid droplet fusion. Accordingly, we observed elevated levels of CIDEC and its acetylated form in HDAC6-deficient adipocytes as well as the adipose tissue of obese animals and humans. Fatty acids (FAs) prevented CIDEC deacetylation by promoting the dissociation of CIDEC from HDAC6, which resulted in increased association of CIDEC with PCAF on the endoplasmic reticulum. Control of CIDEC acetylation required the conversion of FAs to triacylglycerols. Thus, we have revealed a signaling axis that is involved in the coordination of nutrient availability, protein acetylation, and cellular lipid metabolic responses.

Introduction

Levels of protein acetylation have been shown to affect the activity of many proteins, and its aberrant regulation is linked to the development of cancer and metabolic diseases (1–4). Protein acetylation is modulated by the antagonistic actions of histone acetyltransferases (HATs) and histone deacytases (HDACs) (5, 6). HDAC6, a member of the class IIb HDACs that contains two deacytase domains and a ubiquitin-binding domain at its C-terminus, is the major cytosolic deacytase. Its substrates include α-tubulin, peroxidases, and certain DNA repair proteins (7, 8). Recently, HDAC6 was shown to act as a specific deacytase and E3 ligase to regulate the autophagy-mediated clearance of aggregated proteins and defective mitochondria (9–11). HDAC6 was also suggested to be a tumor suppressor in hepatocellular carcinoma cells (12). HDAC10, another class IIb HDAC, has also been shown to regulate autophagy in neuroblastoma cells (13). P300/CREB-associated factor (PCAF), a HAT, has an important role in DNA damage and certain metabolic pathways (14). Despite our growing understanding of the scope of protein acetylation and its biological functions, the precise mechanism by which regulatory signals or nutrients control protein acetylation and its downstream metabolic consequences remains elusive. High extracellular glucose levels regulate the acetylation and activity of many metabolic enzymes in the glycolysis pathway (15–18). The role of fatty acids (FAs), a major nutritional component, in regulating acetylation and its downstream metabolic response remains unknown.

Obesity is characterized by excessive triacylglycerol (TAG) storage in adipose tissue and is a major risk factor for the development of other metabolic disorders, including diabetes, fatty liver disease, and cardiovascular disease (19, 20). The main cellular basis of obesity is the increased number of adipocytes (hyperplasia) and the increased size of adipocytes (hypertrophy). Adipocyte hypertrophy in adults was shown to be the dominant mechanism of adult fat mass expansion (21, 22). Adipocyte hypertrophy mainly results from the uncontrolled growth of lipid droplets (LDs) in the presence of excessive FAs (21). Local synthesis of TAG by LD-
Results

**HDAC6 is a negative regulator of obesity development.** We systematically measured the expression levels of HDAC1–HDAC10 in the gonadal white adipose tissue (GWAT) of WT and leptin-deficient (ob/ob) mice to assess whether the HDAC family of proteins has a functional role in the development of obesity. Levels of class IIB HDAC proteins (HDAC6 and HDAC10) (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI85963DS1) were markedly reduced in the GWAT of obese mice, whereas levels of other HDACs in the adipose tissue were similar in WT and obese mice (Figure 1A and Supplemental Figure 1B). Levels of Hdac6 and Hdac10 mRNAs were also reduced, although not as dramatically as protein levels (Supplemental Figure 1A). Markedly lower levels of HDAC6 and HDAC10 were also observed in the GWAT of mice fed a high-fat diet (HFD) (Supplemental Figure 1C). Importantly, HDAC6/10 levels were also substantially decreased in the GWAT of obese rhesus monkeys (Figure 1B, left) and in the intra-abdominal GWAT of obese patients who had undergone gastric bypass surgery (Figure 1B, right; Tables 1 and 2; and Supplemental Figure 1, D and E). These data indicate that class IIB HDACs negatively correlate with excess energy-induced lipid storage and the development of obesity.

We generated mice with a conditional Hdad6 allele (Hdad6<sup>fl/fl</sup>) and crossed them with adiponectin-Cre mice to generate an adipocyte-specific knockout of Hdad6 (i.e., Hdad6 AKO) to confirm that HDAC6 indeed has a negative role in the development of obesity. An analysis of HDAC6 protein levels indicated that the deletion was specific to white adipose tissue (GWAT) and brown adipose tissue (BAT) (Figure 1C); it was not observed in the liver (Supplemental Figure 1F). Levels of acetylated α-tubulin, a well-characterized downstream target of HDAC6, were markedly increased in the BAT and GWAT of Hdad6 AKO mice (Figure 1C), further confirming the deletion of Hdad6. Hdad6 AKO animals appeared to be obese upon gross morphological examination (Figure 1D). Body weights of Hdad6 AKO mice were increased compared with those of control mice under both normal diet (ND) and HFD conditions (Figure 1E). Weights of the fat pads from several anatomical locations (Tables 3 and 4) and adiposity indexes (Figure 1F) were higher in Hdad6 AKO mice. In addition, total fat mass measured by MRI was increased in animals with adipocyte-specific knockout of Hdad6, although total lean mass was similar in control and AKO mice under both ND and HFD conditions (Figure 1G). LD size and total amount of TAG in the GWAT and BAT were both increased in the Hdad6 AKO mice (Figure 1H), whereas total amounts of TAG in the liver and muscle were similar in control and Hdad6 AKO mice (Figure 1I and Supplemental Figure 1G). These data indicate that Hdad6 deficiency in adipose tissue results in the accumulation of more lipids and the development of obesity.

We performed metabolic cage experiments using 8 mice from each control and Hdad6 AKO group to further evaluate the physiological role of HDAC6 in controlling lipid storage. We observed significantly reduced oxygen consumption in Hdad6 AKO mice using a direct comparison method (Figure 2A), a ratio-based method (Supplemental Figure 2A), and analysis of covariance (ANCOVA) (Supplemental Figure 2B). Respiration exchange rate (Figure 2B) and energy expenditure (Figure 2C) were also lower in Hdad6 AKO mice. No differences in food intake (Supplemental Figure 2C) and physical activity were observed between control and AKO mice (Supplemental Figure 2D). Consistent with the reduced oxygen consumption rate, we observed lower levels of several mitochondrial proteins (CPT1, CPT2, COX4, and cytochrome c) in the GWAT of Hdad6 AKO mice (Figure 2D and Supplemental Figure 2E). Levels of COX4 and cytochrome c in the BAT of AKO mice were also reduced (Figure 2E). Next, we observed similar levels of BAT mitochondrial DNA in control and Hdad6 AKO mice, indicating that their BAT contained similar numbers of mitochondria (Supplemental Figure 2F). Furthermore, we directly evaluated the function of mitochondria in the BAT of control and Hdad6 AKO mice by isolating their mitochondria and measuring the oxygen consumption rate by use of a XFe96 Seahorse analyzer (Seahorse Bioscience). We observed an approximately 20% reduction in basal respiration, ADP-stimulated respiration, and maximal respiration after the addition of the uncoupler FCCP in the BAT mitochondria of Hdad6-deficient compared with control mice (Figure 2F). The

**Notes:**

- Figures 1A and 1B: Images showing HDAC6 and HDAC10 expression levels in GWAT and BAT of control and obese mice.
- Figures 1C and 1D: Experimental setup and results of metabolic cage experiments.
- Figures 1E and 1F: Analysis of body weight and total body fat mass.
- Figures 1G and 1H: Comparison of LD size and TAG amounts in GWAT and BAT.
- Figures 1I and 1J: Additional metabolic parameter comparisons between control and Hdad6 AKO mice.

**Articles Cited:**

- Cidec display markedly reduced lipid storage in their adipose tissue (30, 31) and develop a lipodystrophic phenotype (32, 33).
- Acetylation of CIDEC at K56 by PCAF was abolished by HDAC6.
- Our observations revealed a regulatory pathway involved in the coordination of nutrient availability, protein acetylation, and cellular lipid metabolic responses.

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**Conflicts of Interest:**

- The authors have no conflicts of interest to disclose.

**References:**

- 23, 25] and CIDE/CSP27-mediated LD fusion and growth (26–29) have been shown to be the two crucial mechanisms that control adipocyte hypertrophy. Animals deficient in Cidec display markedly reduced lipid storage in their adipose tissue (30, 31) and develop a lipodystrophic phenotype (32, 33).

**Highlights:**

- HDAC6 is a negative regulator of lipid storage.
- HDAC6 deficiency in adipocytes results in increased body weight and total fat mass.
- HDAC6 is a negative regulator of energy metabolism in adipocytes.
- HDAC6 deficiency results in increased oxygen consumption and mitochondrial function in adipocytes.
expression levels of white fat browning genes (Prdm16, Cidea, Ucp1, and Dio2), genes in the TAG synthesis pathway (Acsl3, Agpat4, Gpat4, Lpin1, Dgat1, and Dgat2), and genes in the FA biosynthesis pathways (Fas and Accl) in the GWAT of control and Hdac6 AKO mice were similar (Supplemental Figure 2, G and H). In addition, we observed a similar rate of FA uptake in control and Hdac6-deficient white adipocytes (Supplemental Figure 2I). However, the rate of hormone-stimulated lipolysis was slightly lower in Hdac6 AKO mice (Figure 2G). The reduced mitochondrial activity in the BAT and lower lipolysis activity in the animals with adipose-specific depletion of Hdac6 likely contributed to their reduced whole body metabolic activity and increased adiposity index.

To evaluate the insulin sensitivity of animals with adipose tissue-specific knockout of Hdac6, we first performed glucose (GTT) and insulin (ITT) tolerance tests. Both the GTT and ITT showed that Hdac6 AKO mice had lower insulin sensitivity compared with control mice (Supplemental Figure 2J). We then evaluated the insulin sensitivity of Hdac6 AKO mice using a hyperinsulinemiceuglycemic clamp. Consistent with the results of the GTT and ITT, mice with adipocyte-specific knockout of Hdac6 were insulin resistant compared with control mice. This was manifested by a decreased glucose infusion rate (GIR) (Figure 2H), lower glucose disposal rate (GDR) (Figure 2I), and lower insulin-stimulated GDR (IS-GDR) (Figure 2J). These animals did not show a change in the ability of insulin to suppress hepatic glucose production (HGP) (Supplemental Figure 2K) and reduce circulating free FA (FFA) levels (Supplemental Figure 2J). These data indicate that deletion of Hdac6 in adipose tissue leads to systemic and skeletal muscle insulin resistance. We found that serum levels of adiponectin in adipose tissue leads to systemic and skeletal muscle insulin resistance. We found that serum levels of adiponectin by native gel analysis indicated that levels of both the high-molecular-weight (HMW) and medium-molecular-weight (MMW) forms of adiponectin were decreased in Hdac6 AKO mice (Supplemental Figure 2M). Expression levels of several inflammatory cytokines, including Tnfa and Il18, were also increased in the GWAT of the Hdac6 AKO mice (Supplemental Figure 2N). The reduced secretion of adiponectin and increased expression of inflammatory cytokines may contribute to insulin resistance in Hdac6 AKO mice. Overall, our data strongly suggest that HDAC6 is an important negative regulator of obesity development.

CIDE C is a downstream target of HDAC6. We isolated the microsomal fraction from the GWAT of control and Hdac6 AKO mice to identify the potential downstream target of HDAC6 that mediates its function in controlling lipid storage (34). Proteins were digested with trypsin, and acetylated peptides were enriched with an antibody against acetylated lysine residues. These peptides were subjected to mass spectrometry (MS) analysis by tandem liquid chromatography-tandem MS (LC-MS/MS). CIDEC was identified as one of the potential downstream targets of HDAC6, as its acetylation was increased in the GWAT of Hdac6 AKO mice (see Supplemental material, Data sheet 1). As CIDEC was shown to have an important role in promoting lipid storage by controlling LD fusion, we further evaluated its acetylation by immunoprecipitating acetylated proteins from the GWAT of control and Hdac6 AKO mice using an antibody against pan-acetylated lysine. The immunoprecipitated products were blotted with antibodies against several LD-associated proteins (ADRP, TIP47, PLIN1, and CIDEC). Indeed, we observed dramatically increased acetylation of CIDEC in Hdac6-deficient adipocytes (Figure 3A). No acetylation of ADRP and TIP47 was observed in the GWAT. PLIN1 was detected in the immunoprecipitated product, but its

Table 2. Information on normal and obese rhesus monkeys

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<th>Insulin (μU/l)</th>
<th>TAG (mmol/l)</th>
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CE, cholesteryl ester.
levels were similar in control and Hdac6-deficient GWAT. Levels of acetylated α-tubulin, a well-characterized downstream target of HDAC6 used as a positive control, were substantially increased (Figure 3A). Therefore, CIDEC appears to be a downstream target of HDAC6. We ectopically expressed CIDEC in 293T cells to further confirm that CIDEC is a downstream target of HDAC6 and showed that levels of acetylated CIDEC were increased in cells treated with trichostatin A (TSA), an inhibitor of HDACs, and in cells where Hdac6 was depleted using siRNA (Figure 3B). These data suggest that CIDEC is a specific downstream target of HDAC6 in adipocytes.

PCAF acetylates CIDEC at K56. We coexpressed Flag-tagged CIDEC with different acetyltransferases (i.e., P300, CBP, PCAF, GCN5, and TIP60) in 293T cells to identify the acetyltransferase responsible for CIDEC acetylation. Levels of acetylated CIDEC were specifically increased when it was coexpressed with PCAF, but not when it was coexpressed with other acetyltransferases (Figure 3C). Similar to a previous observation, levels of P53 acetylation were increased when it was coexpressed with all the tested acetyltransferases (Figure 3C). The enzymatically inactive form of PCAF that harbored mutations at its active site (Phe568, Thr569, and Glu570) was not able to acetylate CIDEC (Supplemental Figure 3B). An single mutation at K34 (i.e., K34R) slightly decreased CIDEC acetylation levels, whereas a mutation at K56 (i.e., K56R) significantly reduced its acetylation (Figure 3D–F). A comparison of the amino acid sequences revealed that K56 in CIDEC was conserved across many species (Figure 3F). We then generated antibodies against the acetylated form of CIDEC (i.e., AcK56) and observed that this antibody was able to specifically recognize acetylated CIDEC in 3T3-L1 adipocytes. Using this antibody, we observed increased CIDEC acetylation at K56 in HDac6-knockdown cells (Supplemental Figure 3I). Mutations in K56 (i.e., K56Q, acetylation mimic; K56R, acetylation defective) markedly reduced levels of CIDEC protein (Figure 3E). When the in vitro acetylated CIDEC was subjected to mass spectrometric analysis, several lysine residues on CIDEC were identified as potential acetylation sites (Supplemental Figure 3E). We then generated serial mutations of these lysine residues and coexpressed them with PCAF. Mutations at two lysine residues (i.e., K34R and K56R) completely abolished CIDEC acetylation (Supplemental Figure 3, D–F). A comparison of the amino acid sequences revealed that K56 in CIDEC was conserved across many species (Figure 3F). We then generated antibodies against the acetylated form of CIDEC (i.e., AcK56) and observed that this antibody was able to specifically recognize acetylated CIDEC in 3T3-L1 adipocytes. Using this antibody, we observed increased CIDEC acetylation at K56 in HDac6-knockdown cells (Supplemental Figure 3I). Mutations in K56 (i.e., K56Q, acetylation mimic; K56R, acetylation defective) markedly reduced levels of CIDEC acetylation (Figure 3E). Furthermore, levels of CIDEC protein and its acetylated form were significantly increased in the GWAT and BAT of Hdac6 KO mice (Figure 3I). Overall, these data indicate that PCAF acetylates CIDEC at K56 and HDAC6 promotes CIDEC deacetylation.

| Table 3. Adipose tissue weight and blood chemistry of control (Ctrl) and Hdac6 KO mice under ND conditions |
|-------------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| ND                                              | Ctrl                              | AKO                              | P value                          |
| Subcutaneous fat (g)                            | 8 1.40 ± 0.08                      | 8 1.83 ± 0.12                    | 0.003466a                        |
| Gonadal fat (g)                                 | 8 1.32 ± 0.07                      | 8 1.65 ± 0.06                    | 0.002558a                        |
| Mesenteric fat (g)                              | 8 0.24 ± 0.03                      | 8 0.42 ± 0.04                    | 0.005141a                        |
| Retroperitoneal fat (g)                         | 8 0.28 ± 0.03                      | 8 0.37 ± 0.03                    | 0.034893a                        |
| Liver (g)                                       | 8 1.31 ± 0.06                      | 8 1.4 ± 0.06                     | 0.363280                         |
| BAT (g)                                         | 8 0.11 ± 0.01                      | 8 0.13 ± 0.01                    | 0.161311                         |
| Glucose (mmol/l)                                | 6 9.06 ± 0.21                      | 6 9.15 ± 0.33                    | 0.828478                         |
| Insulin (ng/ml)                                 | 6 1.55 ± 0.12                      | 6 1.26 ± 0.08                    | 0.121785                         |
| Serum NEFA (mEq/l)                              | 11 0.64 ± 0.04                     | 9 0.71 ± 0.03                    | 0.190201                         |
| Serum TAG (mg/ml)                               | 11 0.53 ± 0.04                     | 11 0.65 ± 0.05                   | 0.080436                         |

Data represent mean ± SEM. aP < 0.01, bP < 0.05, by 2-tailed Student’s t test.

| Table 4. Adipose tissue weight and blood chemistry of control (Ctrl) and Hdac6 KO mice under HFD conditions |
|-------------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| HFD                                            | Ctrl                              | AKO                              | P value                          |
| Subcutaneous fat (g)                            | 6 1.95 ± 0.16                      | 6 2.44 ± 0.19                    | 0.018930a                        |
| Gonadal fat (g)                                 | 6 1.51 ± 0.15                      | 6 1.95 ± 0.14                    | 0.035202a                        |
| Mesenteric fat (g)                              | 6 0.48 ± 0.07                      | 6 0.74 ± 0.07                    | 0.035904a                        |
| Retroperitoneal fat (g)                         | 6 0.56 ± 0.04                      | 6 0.82 ± 0.09                    | 0.043104a                        |
| Liver (g)                                       | 6 1.61 ± 0.12                      | 6 1.66 ± 0.11                    | 0.778081                         |
| BAT (g)                                         | 6 0.10 ± 0.01                      | 6 0.12 ± 0.01                    | 0.337525                         |
| Glucose (mmol/l)                                | 6 10.11 ± 0.23                     | 6 10.34 ± 0.30                   | 0.753491                         |
| Insulin (ng/ml)                                 | 6 2.13 ± 0.19                      | 6 2.29 ± 0.24                    | 0.536759                         |
| Serum NEFA (mEq/l)                              | 6 0.61 ± 0.05                      | 6 0.64 ± 0.03                    | 0.688199                         |
| Serum TAG (mg/ml)                               | 6 0.77 ± 0.08                      | 6 0.93 ± 0.11                    | 0.370233                         |

Data represent mean ± SEM. aP < 0.05, by 2-tailed Student’s t test.
Figure 2. Animals with adipose tissue–specific knockout of Hdac6 have decreased metabolic activity and lower insulin sensitivity. (A) Oxygen consumption (VO$_2$) of control and Hdac6 AKO mice monitored for 48 hours ($n$ = 8 mice per group). (B) Respiratory exchange rate analysis according to dark and light phases. From 8pm to 8am (next day) refers to dark phases. From 8am to 8pm refers to light phases. ($n$ = 8 mice per group). (C) Energy expenditure analysis of control and Hdac6 AKO mice monitored for 48 hours ($n$ = 8 mice per group). (D) Protein expression of CPT1, CPT2, COX4, and cytochrome c (CytoC), which are important in mitochondrial respiration in the GWAT of control and Hdac6 AKO mice ($n$ = 3 mice per group). (E) Protein expression of CPT1, CPT2, COX4, and cytochrome c, which are important in mitochondrial respiration in the BAT of control and Hdac6 AKO mice ($n$ = 3 mice per group). (F) Quantitative analysis of oxygen consumption rate (OCR) of mitochondria isolated from the BAT of control and Hdac6 AKO mice using Seahorse equipment ($n$ = 3 mice per group). (G) Lipolysis in control and Hdac6 AKO mice under fed, fasted, and isoproterenol–stimulated conditions ($n$ = 6 mice per group). (H) Levels of GIR of control and Hdac6 AKO mice ($n$ = 6 mice per group). (I) Levels of GDR of control and Hdac6 AKO mice ($n$ = 6 mice per group). (J) Rate of insulin–stimulated glucose disposal (IS–GDR) of control and Hdac6 AKO mice ($n$ = 6 mice per group). (K) Reduced serum levels of adiponectin in control and Hdac6 AKO mice ($n$ = 6 mice per group). Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, by 2–tailed Student’s t test.
Acetylation of CIDEC enhances its stability. As we observed increased levels of the CIDE protein and its acetylated form in Hdad6-deficient adipocytes, we hypothesized that CIDEC acetylation would increase its stability. Therefore, we knocked down Hdad6 in 3T3-L1 adipocytes and examined CIDEC stability. Although CIDEC levels decreased rapidly in WT 3T3-L1 adipocytes (with a half-life of approximately 45 minutes), in the presence of cycloheximide (CHX), which inhibits protein translation, CIDEC was very stable in Hdad6-depleted adipocytes (Figure 4A). Indeed, the acetylated form of CIDEC was much more stable (Figure 4B). Coexpression of CIDE with PCAF significantly increased its stability, whereas expression of HDAC6 abrogated PCAF-induced CIDEC stability (Supplemental Figure 4A). The increased CIDEC stability in the presence of PCAF was likely due to its role in inhibiting CIDEC ubiquitination, as we observed reduced levels of CIDEC ubiquitination when it was coexpressed with PCAF (Supplemental Figure 4B). HDAC6 markedly increased CIDEC ubiquitination (Supplemental Figure 4B). To further confirm that CIDEC acetylation enhances its stability, we conducted 3 sets of experiments. First, we tested the stability of the acetylation-mimicking mutant (K56Q) and acetylation-defective mutant (K56R) of CIDEC by ectopically expressing them in 293T cells. We observed that CIDEC K56Q was much more stable than WT CIDE protein, whereas K56R was less stable (Supplemental Figure 4C). Next, we knocked down HDAC6 in 293T cells using its specific siRNA and then expressed WT CIDEC and K56R in these cells. K56R was less stable than WT CIDEC protein in control cells treated with scramble siRNA. When HDAC6 was knocked down, the stability of CIDEC increased dramatically, as no obvious protein degradation was observed after 1 hour of CHX treatment. On the contrary, K56R was much less stable than WT protein (Supplemental Figure 4D). Third, we evaluated CIDEC stability by knocking in WT or CIDEC K56R using an adeno-associated virus (AAV) system in Hdad6-deficient adipocytes. WT CIDE protein in control adipocytes was rapidly degraded, whereas it was very stable in Hdad6-deficient adipocytes. However, CIDEC K56R was degraded much faster in Hdad6-deficient adipocytes compared with WT CIDE protein (Figure 4C). These data further confirm that HDAC6-mediated deacetylation of CIDEC significantly reduced its stability. Despite reduced protein stability for CIDEC K56R, its ubiquitination levels were similar to those of WT CIDEC (Supplemental Figure 4E), indicating that K56 is not the ubiquitination site. Consistent with the positive role of CIDEC acetylation in controlling its stability, levels of CIDE protein and its acetylated form were increased in the GWAT of obese mice, monkeys, and human patients (Figure 4, D–F, and Supplemental Figure 4F).

Acetylation of CIDEC enhances LD fusion activity. We then evaluated the function of CIDEC acetylation in controlling lipid storage in adipocytes by measuring LD size in 3T3-L1 adipocytes that were depleted of either Pcaf or Hdad6. 3T3-L1 adipocytes with Pcaf knockdown displayed smaller LDs and lower cellular TAG levels, whereas Hdad6 depletion resulted in the accumulation of more TAGs and large LDs in 3T3-L1 adipocytes (Figure 5, A and B). Lipid exchange activity, a hallmark of CIDE-mediated LD fusion, was reduced in Pcaf-knockdown 3T3-L1 adipocytes but increased in Hdad6-knockdown adipocytes (Figure 5C). Coexpression of CIDEC and PCAF in 3T3-L1 preadipocytes also resulted in the accumulation of larger LDs, whereas coexpression of CIDEC and HDAC6 led to the accumulation of smaller LDs (Figure 5D). When expressed in 3T3-L1 preadipocytes, the acetylation-defective mutant of CIDEC (K56R) had significantly lower LD fusion activity (Figure 5, E–G). By contrast, cells expressing an acetylation-mimicking mutant of CIDEC (i.e., K56Q) displayed increased rates of lipid exchange and LD fusion, resulting in the accumulation of larger LDs, primarily due to the increased levels of CIDEC K56Q (Figure 5, E–G). Overall, these data indicate that CIDEC acetylation increases its stability and ability to promote LD fusion and lipid storage in adipocytes.

FA s increase CIDEC stability and acetylation. We examined CIDEC levels in mature 3T3-L1 adipocytes treated with various FAs and glucose to evaluate the role of nutrients in regulating CIDEC expression. CIDEC protein (but not RNA) levels were markedly increased in the presence of palmitic acids (PAs) and oleic acids (OAs), but not glucose (Figure 6A and Supplemental Figure 5A). Time course analysis showed that the stability of CIDEC and levels of acetylated CIDEC in mature adipocytes were markedly increased in the presence of OA (Figure 6B and Supplemental Figure 5B). In addition, when ectopically expressed, the relative stoichiometry of acetylated K56 in CIDEC is 1.75%. In the presence of OA, the ratio was increased to approximately 2.6%, a more than 1-fold increase in K56 acetylation (Supplemental Figure 5C). Levels of endogenous CIDEC acetylation were also increased in OA-treated 3T3-L1 adipocytes (Figure 6C). OA-induced CIDEC acetylation was abolished by the K56R mutation (Supplemental Figure 5D). When PCAF was knocked down in 293T cells using a specific siRNA, OA-induced CIDEC acetylation was decreased compared with that in control cells that were treated with a scrambled siRNA (Figure 6D). Overexpression of HDAC6 completely abolished OA-induced CIDEC acetylation (Figure 6E). Consistent with their roles in enhancing CIDEC acetylation and stability, both OA and TSA reduced CIDEC ubiquitination (Figure 6F). The dosage analysis indicated that the levels of acetylated CIDEC increased as the amount of OA increased (Supplemental Figure 5E). CIDEA, a protein closely related to CIDEC, also showed increased acetylation and stability in the presence of OA and TSA (Supplemental Figure 5, F and G). Overall, these data suggest that CIDEC acetylation is induced by extracellular FAs, and PCAF and HDAC6 mediate FA-induced CIDEC acetylation.

FA induces a dynamic association among CIDEC, PCAF, and HDAC6. We investigated the potential interaction of CIDEC with PCAF and HDAC6 in 3T3-L1 adipocytes to delineate the underlying mechanism of FA-induced CIDEC acetylation. When CIDEC was pulled down by its antibody, PCAF, HDAC10, and HDAC6 were detected in the immunoprecipitated products, suggesting that PCAF and HDAC6 can interact with CIDEC in adipocytes (Figure 6G). Next, we assessed whether FAs could affect the interaction between CIDEC and PCAF and CIDEC and HDAC6 in mature 3T3-L1 adipocytes that were cultured under starvation conditions (to reduce extracellular lipid supply) and then with the addition of OA. Under starvation condition, an association between CIDEC and HDAC6 was detected, whereas the association between CIDEC and PCAF was reduced (Figure 6H). In the presence of OA, the amount of HDAC6 that associated with CIDEC gradually decreased, whereas the amount of PCAF that associated
Figure 3. CIDEC is deacetylated by HDAC6 and acetylated by PCAF at K56. (A) Levels of CIDEC acetylation were increased in the GWAT of Hdac6 AKO mice. Levels of α-tubulin were used as a positive control. IgG was used as a loading control. To detect CIDEC acetylation in vivo, the GWAT tissue sample was incubated with A/G beads conjugated to antibodies against acetylated lysine, and the immunoprecipitates were blotted with antibodies against CIDEC and other LD-associated proteins (PLIN1, ADRP, TIP47). Data represent results from at least 3 independent experiments. (B) Ectopically expressed CIDEC was acetylated in the presence of TSA or when Hdac6 was knocked down. Data represent results from at least 3 independent experiments. (C) PCAF acetylates CIDEC. Flag-CIDEC was coexpressed in 293T cells with different acetyltransferases. Upper panel shows levels of CIDEC acetylation; lower panel shows levels of P53 acetylation when Flag-CIDEC and Flag-P53 were coexpressed with various acetyltransferases. Data represent results from at least 3 independent experiments. (D) HDAC6 deacetylates CIDEC. Data represent results from at least 3 independent experiments. (E) PCAF acetylates CIDEC in vitro. Bacterially isolated CIDEC-MBP was incubated with GST-PCAF containing the enzymatic domain (active) in vitro. Data represent results from at least 3 independent experiments. (F) Identification of the conserved K56 residue in difference species. (G) Ratio of acetylated CIDEC at K56 in the adipose tissue of control and Hdac6 AKO mice. (H) Characterization of anti–acetyl-K56 (AcK56) antibody. Data represent results from at least 3 independent experiments. (I) Increased levels of CIDEC protein and its acetylated form in the GWAT and the BAT of Hdac6 AKO mice. The immunoprecipitated CIDEC was normalized (n = 3 mice per group).
similar to its interaction with the WT protein, whereas the interaction between PCAF and acetylation-mimicking mutant of CIDEC (K56Q) was reduced (Figure 6L). By contrast, the interaction between HDAC6 and the acetylation-defective mutant (K56R) was significantly reduced (Figure 6M). Therefore, FAs enhanced CIDEC acetylation by promoting the dissociation between CIDEC and HDAC6 and the association between CIDEC and PCAF.

**TAG synthesis is vital for FA-induced CIDEC acetylation.** FAs exert their cellular effects in two ways: (a) by binding to FA receptors to control the downstream signaling pathway; and (b) by being transported into cells for esterification to generate TAGs that are incorporated into nascent LDs. We first knocked down several FA receptors, including GPR40, GPR120, and GPR84, in 3T3-L1 adipocytes using their corresponding siRNAs. The depletion of these FA receptors did not affect CIDEC acetylation (data not shown). Then, we tested the effect of FA transport on CIDEC acetylation by knocking down Fabp4 and Cd36, proteins that are responsible for FA transport. When Fabp4 or Cd36 was knocked...
down, levels of acetylated CIDEC were markedly reduced in adipocytes in the presence of OA (Figure 7A, Fbp4 knockdown; and Supplemental Figure 6A; Cid36 knockdown, \( P < 0.01 \)). Consistent with this finding, the CIDEC-HDAC6 interaction was increased, whereas the CIDE-CIDEC interaction was decreased (Figure 7A), suggesting that intracellular FA transport was required for CIDEC acetylation. Next, we checked whether TAG synthesis was required for FA-induced CIDEC acetylation by adding 2-BrO, which blocked the last step of TAG synthesis by inhibiting DGAT activity. Intriguingly, when TAG synthesis was blocked by 2-BrO, the levels of acetylated CIDEC and the association between PCAF and CIDEC were substantially reduced (Supplemental Figure 6B). However, the interaction between CIDEC and HDAC6 was increased (Supplemental Figure 6B). Consistent with this observation, the depletion of Dgat1 and Dgat2 in adipocytes using their corresponding siRNAs also resulted in a decreased association between CIDEC and PCAF but an enhanced association between CIDEC and HDAC6 (Figure 7B). The depletion of several other enzymes (ACS3, GPT4, AGPAT4, and lipin 1) in the TAG synthesis pathway also reduced CIDEC acetylation (data not shown). By contrast, coexpression of CIDEC with DAG1, DAG2, or AGPAT4 markedly increased CIDEC acetylation (Supplemental Figure 6C). We obtained mice deficient in lipin 1 (an enzyme in the TAG synthesis pathway) and tested the effect of defective TAG synthesis on controlling CIDEC acetylation in a lipodystrophic model to further confirm that TAG synthesis is required for FA-induced CIDEC acetylation. Consistent with a previous observation, lipin 1-deficient animals displayed a typical lipodystrophic phenotype, with very low body fat accumulation (Supplemental Figure 6D). Importantly, we observed lower levels of the CIDEC protein and its acetylated form in the adipose tissue of lipin 1-deficient compared with WT mice. Lipin 1 deficiency did not affect PCAF and HDAC6 protein levels in the GWAT (Figure 7C). Therefore, intracellular FA trafficking and the conversion of FAs to TAG are required for disruption of the interaction between CIDEC and HDAC6 and the accumulation of acetylated CIDEC.

**Discussion**

CIDEC, along with CIDEA and CIDEB, constitute the CIDE family of proteins, which play a vital role in the development of obesity and hepatic steatosis by controlling LD fusion and lipid storage. Here, we revealed that HDAC6 is a major regulatory factor for obesity development. Moreover, the interplay among PCAF, HDAC6, and CIDEC constitutes an evolutionarily conserved regulatory circuit that senses FA levels and controls lipid storage, and its dysregulation leads to the development of obesity and insulin resistance.

First, our analyses indicate that protein levels of class IIb HDACs (HDAC6 and HDAC10) were markedly reduced in the

**ER is the regulatory site for FA-induced CIDEC acetylation.** We biochemically isolated ER-enriched microsomal, LD, and nuclear fractions from 293T cells expressing CIDEC in the absence or presence of OA to determine the site that regulates FA-induced CIDEC acetylation. Endogenous HDAC6 was detected in the cytosolic fraction in the absence of CIDEC. Endogenous PCAF was predominantly detected in the nuclear fraction, with lower amounts in the cytosolic and ER fractions (data not shown). Interestingly, when coexpressed with CIDEC, HDAC6 was detected in the ER fraction in the absence of OA but displayed a reduced ER association in the presence of increasing amounts of OA (Figure 7D and Supplemental Figure 6E), whereas the amount of PCAF that associated with CIDEC was increased in the ER fraction in the presence of OA (Figure 7E).

In mature 3T3-L1 adipocytes, PCAF was detected in the ER fraction, and its association with CIDEC was increased in the presence of OA. HDAC6 was also present in the ER fraction, but it was not readily detectable when the adipocytes were incubated with OA. Levels of the CIDEC protein and its acetylated form were markedly increased in the ER fraction in the presence of OA (Figure 7F). Intriguingly, neither PCAF nor HDAC6 was detected in the LD fractions, despite the presence of higher levels of CIDEC and its acetylated form (Figure 7F). Consistent with this finding, when treated with MG132, increased levels of ubiquitinated CIDEC were detected in the ER fraction, but not the LD fraction (Supplemental Figure 6F), whereas the levels of ubiquitinated CIDEC in the ER were decreased when adipocytes were treated with OA (Supplemental Figure 6G).

We assessed the subcellular localization of CIDEC in the presence or absence of OA by immunofluorescence staining to further confirm the localization of CIDEC, PCAF, and HDAC6. The majority of CIDEC (approximately 80%) exhibited a diffuse cytosolic pattern in cells grown under starvation conditions (Supplemental Figure 6H). In the early course of FA treatment, CIDEC was localized to the ER; then, the association of CIDEC with LD rapidly increased 1 hour after FA treatment (Supplemental Figure 6H). Although PCAF was predominantly localized to the nucleus, PCAF localization to the ER was evident and not dependent on the presence of OA (data not shown) or CIDEC expression (Supplemental Figure 6I). However, HDAC6 alone exhibited a diffuse cytosolic distribution in the absence or presence of FAs (data not shown). When coexpressed with CIDEC, HDAC6 showed a transient but specific colocalization to the ER 30 minutes after FA treatment (Figure 7G). Overall, these data strongly indicate that the ER is the regulatory site of FA-induced CIDEC acetylation.
Figure 6. FA induces CIDE acetylation and dynamic association among CIDE, Pcaf, and HDAC6. (A) FAs increase CIDE protein levels. Data represent results from at least 3 independent experiments. (B) OA enhances the stability of endogenous CIDE in 3T3-L1 adipocytes. Data represent results from at least 3 independent experiments. NC, negative control that without OA treatment. (C) OA-induced CIDE acetylation was at the K56 residue (AcK56) in 3T3-L1 adipocytes. Data represent results from at least 3 independent experiments. (D) Knockdown of Pcaf abolished OA-induced CIDE acetylation. Data represent results from at least 3 independent experiments. (E) OA-induced CIDE acetylation was inhibited by the expression of HDAC6. Data represent results from at least 3 independent experiments. (F) Levels of CIDE ubiquitination (Ub) were decreased in the presence of OA or TSA. Data represent results from at least 3 independent experiments. (G) Interaction among CIDE, Pcaf, HDAC6, and HDAC10 in differentiated 3T3-L1 adipocytes. Data represent results from at least 3 independent experiments. (H) OA inhibited the interaction between CIDE and HDAC6 but stimulated the interaction between CIDE and Pcaf in the 3T3-L1 adipocytes. Data represent results from at least 3 independent experiments. (I) OA induced Pcaf and CIDE interaction in a dose-dependent manner. Data represent results from at least 3 independent experiments. (J) OA inhibited CIDE and HDAC6 interaction in a dose-dependent manner. Data represent results from at least 3 independent experiments. (K) HDAC6 had high affinity to the acetylation-defective form of CIDE. Data represent results from at least 3 independent experiments. (L) Pcaf showed high binding affinity to the acetylation-mimicking form CIDE. Data represent results from at least 3 independent experiments.
GWAT of HFD-fed mice, obese mice, monkeys, and humans. HDAC6 and HDAC10 protein levels were also markedly reduced in the BAT of obese and HFD-fed mice. Interestingly, *Hdac6* and *Hdac10* mRNA levels were only moderately decreased in the GWAT of the obese mice. As the stability of HDAC6 was not controlled by the ubiquitin-dependent protein degradation pathway (data not shown), the significant reduction in HDAC6 protein levels in the adipose tissue of the *ob*/*ob* or HFD-fed mice may be due to more complex regulatory mechanisms, such as translational regulation or specific protein degradation pathways activated in response to long-term FA exposure and/or increased lipid storage in the adipocytes.

Consistent with a negative role of HDAC6 in lipid storage, animals with adipose tissue–specific knockout of *Hdac6* showed enlarged LDs and increased fat accumulation in the GWAT and BAT. *Hdac6* deficiency resulted in slightly lower lipolysis but did not affect other lipid metabolic pathways, including FA uptake, FA biosynthesis, and TAG synthesis. The increased fat accumulation may be due to the lower metabolic activity in the adipose-specific *Hdac6*-deficient animals, as these animals displayed reduced oxygen consumption, lower respiration exchange, and lower energy expenditure. The lower metabolic activity may be partially contributed by reduced mitochondrial activity due to the lower expression of genes in the mitochondrial oxidative pathway in the BAT and GWAT of animals with adipose-specific *Hdac6* deficiency. As we did not observe differences in food intake and physical activity between control and *Hdac6* AKO mice, it was not likely that the adipose-specific knockout of *Hdac6* influenced the CNS activity.

The GTT and ITT experiments showed that animals with an adipose-specific *Hdac6* deficiency exhibited decreased insulin sensitivity. The hyperinsulinemic-euglycemic clamp experiment further confirmed the insulin-resistant phenotype of these animals, as they had decreased glucose infusion, glucose disposal, and insulin-stimulated GDRs. Interestingly, these animals did not show changes in HGP or circulating FFA levels. Therefore, *Hdac6* deletion in the adipose tissue led to systemic and skeletal muscle insulin resistance. The reduced secretion of adiponectin and increased expression of inflammatory cytokines in the *Hdac6* AKO animals may contribute to their reduced systemic insulin sensitivity.

We found that CIDE2, a protein responsible for lipid storage and LD fusion in adipocytes, is a downstream target of HDAC6 based on the following evidence. First, levels of acetylated CIDE2 were increased in the adipose tissue of *Hdac6*-knockout mice. Knockdown of *HDAC6* in other cell types also reduced levels of ectopically expressed CIDE2 acetylation. We also identified PCAF as a specific acetyltransferase for CIDE2 and K56 as the specific acetylation site. HDAC6 can antagonize the effect of PCAF-induced CIDE2 acetylation. Importantly, CIDE2 acetylation enhances its stability by inhibiting its ubiquitination and degradation. Functionally, LD fusion and growth and lipid storage activities were increased with expression of the acetylation-mimicking CIDE2 mutant and in *Hdac6*-depleted adipocytes.

Interestingly, we found that FA can effectively enhance CIDE2 stability by increasing its acetylation. FA-induced CIDE2 acetylation was mediated by PCAF and inhibited by HDAC6. Notably, CIDEA, a close homolog of CIDE2, was also acetylated in response to FA treatment. FA-induced acetylation may represent a general mechanism for the regulation of lipid metabolism in response to dietary FAs. Mechanistically, we have obtained clear evidence showing that FA-induced CIDE2 acetylation was regulated by its ability to modulate the dynamic “ping-pong” association between CIDE2 and PCAF and CIDE2 and HDAC6. Under FA-free conditions, HDAC6 exhibited a stronger association with CIDE2, and this association decreased with the addition of FAs. By contrast, the association between PCAF and CIDE2 increased in the presence of FAs in a dose-dependent manner. Imaging and biochemical fractionation analyses showed that PCAF and CIDE2 were colocalized in the ER. By contrast, HDAC6 was only detected in the ER fractions when it was coexpressed with CIDE2, and HDAC6 was dissociated from the ER in the presence of FAs.

How do FAs regulate the interactions between CIDE2 and HDAC6 and CIDE2 and PCAF? In adipocytes, FAs are delivered to ER and then converted into phospholipids, TAG, or other intermediates through various enzymes in the lipid metabolic pathway. We showed that the ER is the primary site for FA-induced CIDE2 acetylation. Importantly, we observed that the depletion of enzymes involved in TAG synthesis, such as a lipin 1 deficiency, results in reduced CIDE2 acetylation. Therefore, TAG synthesis is required for this regulatory cascade. TAG may be able to directly disrupt the interaction between CIDE2 and HDAC6 or promote the CIDE2-PCAF association. Alternatively, newly synthesized TAG may form a subdomain in the ER and promote nascent LD formation, resulting in changes in CIDE2 configuration and disassociation of the CIDE2-HDAC6 interaction. In addition, ER- but not LD-associated CIDE2 was ubiquitinated in vivo, suggesting that the configuration of CIDE2 was different in these two subcellular organelles. Because PCAF binds to acetylated CIDE2 at lower levels, acetylated CIDE2 will be released from the ER and mobilized to the LD surface, where it promotes LD fusion and lipid storage.

Although the relative stoichiometry of K56 acetylation in CIDE2 was not very high in control mouse adipocytes, it was dramatically increased in *Hdac6*-deleted or FA-treated adipocytes, consistent with the role of HDAC6 in mediating FA-induced CIDE2 acetylation. The low stoichiometry of acetylated CIDE2 is probably due to the limited availability of acetyl-CoA, as the primary site of CIDE2 acetylation is on the ER. This is also consistent with the previously observed relatively low stoichiometry of acetylation of many proteins, in particular cytosolic proteins (36–38). Intriguingly, we observed that acetylated CIDE2 is highly enriched at the LD fraction (Figure 7F). This local enrichment of acetylated CIDE2 may dramatically increase the stoichiometry of CIDE2 acetylation. In addition, acetylation of CIDE2 at K56 may lead to its conformational change and render it inaccessible for ubiquitination and degradation. Therefore, we postulate that this local enrichment of acetylated CIDE2 on LDs could dramatically enhance its stoichiometry and induce a conformational change to prevent it from undergoing ubiquitin-mediated protein degradation. In addition, another acetylation site (K34) on CIDE2 may cooperate with K56 acetylation to control its stability and enhance its function in promoting LD fusion and lipid storage.

Previous research has shown that a *Cidec* deficiency results in reduced LD fusion activity, reduced lipid storage, and increased lipolysis in GWAT. When crossed to leptin-deficient *ob*/*ob* or BAT-less obese mice, *Cidec*-deficient mice displayed hepatic steatosis.
and insulin resistance (33). Interestingly, CIDEA, CIDEK, and PLIN1 expression is positively correlated with insulin sensitivity in humans. Therefore, the lipid storage capacity of adipose tissues must be carefully monitored. The moderate lipid storage observed when CIDEK is expressed at normal or slightly higher levels is beneficial for the animal. Excess lipid storage in adipose tissue (under CIDEC-deficient conditions) leads to insulin resistance. Although we cannot rule out the involvement of other downstream targets of HDAC6, CIDEC contributes to its role in controlling lipid storage and obesity development.

Based on the above evidence, we propose a model to explain the molecular mechanism of FA-induced CIDEK acetylation and obesity development (Figure 7H). In the absence of extracellular FAs, CIDEK is associated with HDAC6, resulting in its lower acetylation and rapid degradation. In the presence of high levels of FAs, HDAC6 is dissociated from CIDEK, resulting in the association between CIDEK and PCAF and increased CIDEK acetylation. Acetylated CIDEK is mobilized to LD to promote fusion and lipid storage.

Methods

Details on plasmid construction, antibodies, reagents, recombinant protein, cell culture, RNAi, transfection, immunofluorescence staining, live cell imaging, immunoprecipitation, and MS; and lists of primers and siRNAs are described in Supplemental Methods. MS. Samples for proteomics analysis were prepared as previously described (34). For LC-MS/MS analysis, digestion products were separated by a 60-minute gradient elution at a 0.250-μl/min flow rate with a Thermo-Dionex Ultimate 3000 HPLC system, which was directly interfaced with a Thermo LTQ Orbitrap Velos mass spectrometer. The analytical column was a homemade fused silica capillary column (75 μm ID, 150 mm length; Upchurch) packed with C-18 resin (300 Å, 5 μm; Varian). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. An LTQ Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.07 software. A single full-scan mass spectrum (400–1,800 m/z, 30,000 resolution) was acquired, followed by 20 data-dependent MS/MS scans in an ion trap at 35% normalized collision energy.

In vitro acetyltransferase assays. CIDEK-MBP recombinant protein was subjected to immunoprecipitation with MBP beads. The immunoprecipitates were mixed with a final volume 50 μl of acetylation buffer (40 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM TSA, 0.1 mM acetyl-CoA, 2 mM sodium butyrate, and 2 mM DTT) containing 1 μg of recombinant PCAF, HDAC6, or GST and were incubated for 60 minutes at 30°C. The reaction was terminated by the addition of 40 μl of 2x SDS sample buffer, the samples were boiled, and portions (10 μl) of each mixture were subjected to immunoblot analysis.

Isolation of ER and LD fractions. ER was isolated from 293T cells treated with OA or 3T3-L1 adipocytes differentiated for 8 days according to the procedures for the Endoplasmic Reticulum Isolation Kit (Sigma-Aldrich). In brief, cells were detached and washed with PBS; and hypotonic extraction buffer (10 mM HEPES, pH 7.8, 1 mM EGTA, 25 mM KCl) was used to allow the cells to suspend and swell before they were centrifuged at 600 g for 5 minutes. The pellet was suspended in isotonic extraction buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 1 mM EGTA, 25 mM KCl) and homogenized in a 7-mL Dounce homogenizer with 10 strokes. The homogenate was centrifuged at 1,000 g for 10 minutes, and the supernatant was then centrifuged at 12,000 g for 15 minutes. After the lipid pad was discarded, the supernatant was the post-mitochondrial fraction. The crude ER was the pellet collected after centrifugation of the post-mitochondrial fraction at 100,000 g for 60 minutes. All procedures were performed at 4°C, and protease inhibitors cocktails (Roche) were added.

The LD fraction was isolated according to a previously described method (39), with slight modifications. In brief, 8-day-differentiated 3T3-L1 adipocytes or 293T cells treated with OA were harvested, washed with PBS, and resuspended in TES buffer (20 mM Tris, pH 7.4, 1 mM EDTA, and 250 mM sucrose). Cells were homogenized with a Dounce homogenizer with a loose-fitting pestle 25 times. The postnuclear supernatant after centrifugation at 1,000 g was overlaid with floating buffer (100 mM NaCl, 20 mM Tris, pH 7.4, and 1.5 mM MgCl2) and centrifuged at 200,000 g for 1 hour. The pellet and middle gradient were collected as total membrane and cytosol, respectively. The LDs on the top were collected and re-floated using the 2-layer gradient mentioned above for another 30 minutes at 200,000 g. All procedures were performed at 4°C, and protease inhibitor cocktails (Roche) were added. Protein concentration was determined using the Bradfold method (Bio-Rad).

TAG measurement. The measurement method for TAG in adipose tissue and liver was the same as described previously (30). For quantitative analysis, adipose and hepatic TAG spots of samples and...
standard on TLC plates (Sigma-Aldrich) were scanned and quantified using Quantity One software (Bio-Rad). TAG levels were normalized with the protein concentration of each sample.

Generation of adipose tissue-specific knockout mice and animal maintenance. The Hdac6 adipose tissue-specific knockout mice were generated on a C57BL/6j background. We generated a conditional Hdac6 allele (Hdac6^Cre) by engineering loxp sites flanking exons 9 and 14. We next crossed Hdac6^Cre (control) and adiponectin-Cre mice from the Jackson Laboratory to generate mice with adipocyte-specific knockout of Hdac6 (AKO). Genotyping primer sequences used to confirm Hdac6^Cre were 5′-CATGGTTTGGCAGGAGCAG-3′ and 5′-TGTTGGATCTCCT-GAACTGG-3′. The primer sequences used to test adiponectin-Cre were 5′-CATGGTTTGGCAGGAGCAG-3′ and GAACGCTAGACCT-GTTTGGACGGTTC. Routine maintenance of mouse strains was performed in the laboratory animal facility at Tsinghua University. All of the mice used in the studies were male. The animals were fed ND (5053, Picolab Rodent Diet 20, Research Diets) and HFD (58% of kilocalories from fat; D12331, Research Diets) for 8 weeks.

Statistics. Data were subjected to statistical analysis with GraphPad Prism 5 (GraphPad Software) and were plotted by AI Illustrator (Adobe). Results represent the mean ± SEM of at least 3 independent experiments as indicated in the figure legends. Two-tailed Student’s t test based on ANOVA was used for 2-group comparisons. For multiple comparisons made in Figure 1, F and G, and Figure 5, B, C, F, and G, we conducted 1-way ANOVA with Dunnett’s correction to analyze differences among the control group and 1 or more independent treatment groups. Differences were considered statistically significant at P < 0.05, with significance indicated in figures as * P < 0.05, ** P < 0.01, *** P < 0.001. NS represents no significant difference. We used 3 different statistical methods for comparison of oxygen consumption, including a direct comparison method, a ratio-based method, and ANCOVA using a multiple linear regression model. Statistical procedures in the 3 methods are discussed in detail in Supplemental materials. We used 2-way repeated-measures ANOVA to evaluate the data in Figure 1E (left), Figure 4A, and Supplemental Figure 4A with Holm–Šidák post hoc test (P < 0.05, * P < 0.01, and *** P < 0.001 in these figures indicate that the 2 groups have significant differences).

Study approval. The Center of Biomedical Analysis at Tsinghua University was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the IACUC. All animal protocols were approved by the animal facility at Tsinghua University. The human study was approved by Air Force General Hospital, PLA, and Shanghai Sixth People’s Hospital. Human patients who provided fat tissues were informed and consented with this study. The rhesus monkey study was approved by the Institute of Molecular Medicine, Peking University.

Author contributions
HQ and YC designed and performed most of the experiments, analyzed the data, and wrote the manuscript. FJC and LY helped with the image analysis. HD and YG helped with MS analysis. WX helped with protein purification. PL and SH helped with insulin clamp experiments. J. Li helped to generate Lpin1^−/− mice. J. Liu performed the experiments. HY, XZ, Hongchao Zhang, Haihong Zhang, YB, RPX, and WJ helped to collect human and monkey fat samples. LZ, LS, ZN, JY, and LY participated in the experimental design, discussion of results, and manuscript preparation. SZ helped with plasmid and reagent preparation. PL was responsible for forming the hypothesis, project development, data coordination, and writing, finalizing, and submitting the manuscript. All authors discussed and approved the manuscript.

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