Supplementary Information for

Loss of adipose TET proteins enhances β-adrenergic responses and protects against obesity by epigenetic regulation of β3-AR expression

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Adipose TET deficiency protects against the detrimental metabolic effects of obesity.

Obesity is often accompanied by deleterious metabolic complications, including glucose intolerance and insulin resistance, which is reflected by hyperglycemia and hyperinsulinemia (1). As anticipated, an HFD substantially increased plasma glucose and insulin concentrations in WT mice, which were potently inhibited in Tet TKO mice (SI Appendix, Fig. 13 A and B). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) score also indicated that adipose TET deficiency antagonized obesity-induced insulin resistance (SI Appendix, Fig. S14A). Consistently, glucose and insulin tolerance tests revealed that Tet TKO mice fed an HFD showed improved glucose tolerance and insulin sensitivity (SI Appendix, Fig. 13 C and D). These parameters were comparable between CD-fed mice groups (SI Appendix, Fig. S14 B and C). To directly analyze insulin signaling, we stimulated mice in vivo with insulin and then examined Akt phosphorylation in insulin-responsive tissues (i.e., adipose depots, liver, and muscle). HFD-fed Tet TKO mice displayed an enhanced Akt phosphorylation in all tissues tested compared to WT littermates, thus confirming more efficient insulin signaling (SI Appendix, Fig. 13 E and F and SI Appendix, Fig. S14 D-G); however, there was no difference in CD-fed mice (SI Appendix, Fig. S14H). In response to insulin resistance, a compensatory homeostatic mechanism in pancreatic β-cells causes islet hypertrophy and hyperinsulinemia (2). Consistent with metabolic improvements, the amplification of pancreatic islets and insulin-secreting β-cells was not observed in HFD-fed Tet TKO mice (SI Appendix, Fig. 13 G and SI Appendix, Fig. S14I).

Chronic HFD feeding causes hepatic steatosis and increased liver mass that are both associated with hepatic dysfunction (3). As expected, HFD-fed WT mice possessed enlarged livers exhibiting large LDs and high TG contents (SI Appendix, Fig. 13 H and I and SI Appendix, Fig. S15A), and elevated levels of circulating alanine aminotransferase, aspartate aminotransferase, cholesterol, low- and high-density lipoproteins, and TG (SI Appendix, Fig. 13J and SI Appendix, Fig. S15 B and C). All these alterations were dramatically mitigated in Tet TKO mice. Circulating free fatty acid concentrations were substantially increased in HFD-fed WT mice but not in Tet TKO mice (SI Appendix, Fig. 13J). Obesity also profoundly alters adipokine profiles, and this aggravates glucose homeostasis and insulin sensitivity (4). HFD-fed Tet TKO mice possessed significantly higher serum concentrations of antidiabetic adiponectin and elevated adiponectin mRNA levels in eWAT (SI Appendix, Fig. 13K). Feeding a HFD significantly increased the serum leptin concentrations in WT mice, as
reflected by the upregulation of leptin mRNA levels in eWAT. However, circulating leptin concentrations and adipose leptin mRNA levels remained low in the HFD-fed TKO mice, reflecting reduced fat mass (SI Appendix, Fig. 13L).

Chronic low grade inflammation is a hallmark of obesity that is initiated early during AT expansion, particularly in eWAT, and it progresses during the development of obesity to permanently skew the inflammatory balance toward a pro-inflammatory status that impairs insulin signaling (5). As expected, macrophage infiltration was substantially blocked in eWAT of HFD-fed Tet TKO mice, as assessed by a marked decrease in the frequency of crown-like structures and CD11b\(^+\) F4/80\(^+\) macrophages (SI Appendix, Fig. 13M and SI Appendix, Fig. S15 D and E). Consistent with these observations, the mRNA levels of inflammatory chemokines (e.g., Ccl2, Ccl5, etc.), proinflammatory molecules (e.g., Tnf-\(\alpha\), Il-1b, Il6, and Nos), and macrophage markers (e.g., F4/80, Ccr2, and Ccr5) were also dramatically reduced (SI Appendix, Fig. 13N and SI Appendix, Fig. S15 F and G). Taken together, these data indicate that ablation of adipose TET proteins substantially protects mice from obesity-associated metabolic complications.

**TET deficiency prevents HFD-induced transcriptional alterations in visceral adipose tissues.**

To further analyze the mechanism of TET-mediated metabolic regulation, we performed RNA sequencing of eWAT from CD- or HFD-fed WT and TKO mice. There was no significant variation among triplicate samples (SI Appendix, Fig. S16A). Comparisons of gene expression levels in four different conditions revealed that 3,898 genes were differentially expressed in at least one condition, and these genes could be clustered into 10 distinct groups based on their expression patterns (SI Appendix, Fig. S16B). In WT mice, 2,162 genes (clusters 1, 2, 3, and 5) were significantly upregulated after HFD feeding (SI Appendix, Fig. S16 B–D). Strikingly, 2,030 (~93.89%) of these genes (clusters 1, 2, and 3) were not upregulated or remained at lower levels in Tet TKO mice. Gene ontology (GO) enrichment analysis revealed that these genes are involved in hypoxia, inflammation, TNF signaling via NF-\(\kappa\)B, interferon response, and KRAS and E2F signaling (SI Appendix, Fig. S16B). In contrast, 1,250 genes (clusters 7, 8, and 9) were significantly downregulated in WT mice after HFD feeding. Of these, 753 (60.24%) genes (clusters 7 and 8) were not downregulated or expressed at higher levels in Tet TKO mice (SI Appendix, Fig. S16 B–D). These genes were enriched for GO terms related to
adipogenesis, fatty acid metabolism, oxidative phosphorylation, and xenobiotic metabolism. Thus, these data suggest that TET proteins are required for HFD-induced transcriptional reprogramming in eWAT during the development of obesity.

To further analyze the cellular pathways, we categorized genes differentially expressed in HFD-fed WT and Tet TKO mice into two main classes. Ingenuity pathway analysis (IPA) indicated that genes expressed at lower levels in TKO cells (class 1; clusters C1, 2, 3, and 4) were primarily implicated in the innate or adaptive immune cell infiltration, signaling, differentiation, or activation (SI Appendix, Fig. S17A). In contrast, IPA of genes that were expressed at higher levels in TKO cells (class 2; clusters 6, 7, and 8) indicated that TET deficiency blocked HFD-induced suppression of genes affiliated with WAT browning, fat lipolysis, fatty acid oxidation, reactive oxygen species detoxification, AMPK signaling, and epigenetic gene regulation (SI Appendix, Fig. S17B). RNA-seq also confirmed that TET deficiency resisted HFD-induced reduction of β3-AR expression (SI Appendix, Fig. S17C). Thus, these results suggest that TET proteins are critical mediators of HFD-induced alterations in gene expression programs in adipose tissues during promotion of diet-induced obesity.
SI Materials and Methods

Animals

Mice carrying LoxP-targeted (floxed) Tet1, Tet2, and Tet3 genes (Tet1^{fl/fl}, Tet2^{fl/fl}, and Tet3^{fl/fl}) have been described previously (6-8), and kindly provided by Dr. Anjana Rao (La Jolla Institute, CA, USA). Adiponectin-Cre (#010803) and Cre-ERT2 (#8085) transgenic mice were obtained from the Jackson Laboratory. To delete all Tet-floxed alleles in adipose tissues, Tet1^{fl/fl} Tet2^{fl/fl} Tet3^{fl/fl} mice were crossed with Adiponectin-Cre mice. For inducible excision of Tet-floxed alleles, Tet1^{fl/fl} Tet2^{fl/fl} Tet3^{fl/fl} mice were crossed with Cre-ERT2 transgenic mice. Male mice on a C57BL/6J background were used for all studies unless stated otherwise. Mice were maintained in a temperature-controlled (22 ± 2 °C) facility under a 12-hr light/dark cycle. Mice were bred and housed in the In Vivo Research Center (IVRC) at Ulsan National Institute of Science and Technology (UNIST, Ulsan, Korea) under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of the UNIST (UNISTACUC-17-35, 20-23), and performed in accordance with the institutional guidelines.

Cell Lines

HEK293T cells from American Type Culture Collection were maintained in Dulbecco's Modified Eagle's medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Utah, USA), 2 mM L-glutamine, and 1X penicillin/streptomycin (Invitrogen). Immortalized preadipocytes from iWAT of 4–6-week-old Tet1^{fl/fl} Tet2^{fl/fl} Tet3^{fl/fl} ERT2-Cre mice were maintained and differentiated in DMEM/F12 (Thermo Fischer Scientific) supplemented with 10% FBS and 1X penicillin/streptomycin (Invitrogen) at 37 °C in an incubator with 5% CO2.

Establishment of Immortalized Preadipocytes

The stromal vascular fraction (SVF) was isolated from iWAT of 4–6-week-old Tet1^{fl/fl} Tet2^{fl/fl} Tet3^{fl/fl} ERT2-Cre mice. Briefly, iWAT was washed with phosphate-buffered saline (PBS), minced in HBSS (Gibco, #14175-095), and digested with 1.5 mg/mL collagenase type II (Sigma, C6885) for 40 min at 37 °C with gentle shaking. After incubation, digestion was stopped by adding the same volume of DMEM/F12 medium supplemented with 10% FBS and 1X penicillin/streptomycin (Invitrogen), and the digested tissue was filtered through a 100 μm nylon cell strainer (BD Falcon). After centrifugation at 700 g for 5 min, the pellet was resuspended in ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2-EDTA) for 1 min at room temperature to remove red blood cells. After centrifugation, cells were plated onto
dishes, and the medium was aspirated after 1–2 h to remove immune cells and other contaminants. Then, cells were further washed with PBS twice and cultured in fresh medium at 37 °C in an incubator with 5% CO₂. An immortalized preadipocyte cell line was established by transducing cells with retroviruses expressing SV40 large T antigen, followed by selection with geneticin (G418, 500 μg/mL).

**Adipogenic Differentiation *in vitro***

Immortalized preadipocytes were maintained and differentiated in DMEM/F12 supplemented with 10% FBS and 1X penicillin/streptomycin (Invitrogen) at 37 °C in an incubator with 5% CO₂. Two days after cell confluency, adipogenic differentiation was induced with an adipogenic cocktail consisting of 5 mg/mL insulin (Sigma, #I6634), 1 nM T3 (Sigma, #T2877), 125 μM indomethacin (Sigma, #I7378), 1 μM dexamethasone (Sigma, #D1756), 0.5 mM IBMX (Sigma, #I5879), and 0.5 μM rosiglitazone (Sigma, #R2408). After 2 days, cells were maintained in culture medium in the presence of insulin and T3, and the media was replaced every two days.

To induce Tet gene deletion in cells derived from Tet1<sup>fl/fl</sup> Tet2<sup>fl/fl</sup> Tet3<sup>fl/fl</sup> ERT2-Cre mice, the cells were treated with 4-hydroxytamoxifen (1 μM, Sigma, #H7904) dissolved in ethanol at the indicated points until day 6. For Oil Red O staining, the cells were washed twice with 1X PBS, fixed with 10% neutral buffered formalin (MEDILAB, #0151) for 30 min, washed twice with distilled water, and then stained with 0.2% Oil Red O dye (Sigma, #O0623) in isopropanol and water (6:4) for 1 h at room temperature. The cells were then washed with distilled water ten times. The stained cells were visualized under a microscope and de-stained with 100% isopropanol for 10 min under gentle shaking. Optical density was measured by spectrometry at a wavelength of 492 nm. For the majority of the experiments involving *in vitro* differentiated adipocytes, WT and Tet TKO adipocytes were generated by treating differentiating cells with either ethanol or 4-OHT (1 μM) at day 2, followed by further differentiation until day 6. Differentiated adipocytes were treated with HDAC inhibitors including SAHA (Selleckchem, #S1047) and MS-275 (Cayman, #13284).

**Lentivirus transduction**

Lentiviral particles were generated by co-transfection of HEK293T cells with the lentiviral vector together with packaging vectors psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) using PEI transfection reagent (Polyscience, #23966). The Myc-tagged WT (Addgene #79554) or mutant (Addgene #79611) Tet2 catalytic domain (Tet2-CD) in pScalps_Puro lentiviral plasmid was provided by Dr. Silvia Moticelli (Institute for Research in Biomedicine,
Switzerland). The virus-containing medium was collected, filtered through 0.45 μm filter, and added to the cell culture together with 8 μg/ml polybrene (Millipore, PR-1003) for 2 days, followed by selection with 2 μg/ml puromycin (Gibco, A1113803) for 2–3 days. Stable expression of WT or Mut Tet2-CD was validated by immunoblot analysis.

**Diet-Induced Obesity, Cold Exposure, and CL-316,243-Induced Browning**

To induce diet-induced obesity, all mice were fed a regular diet (13.5% fat; #R03-10, SAFE, Rosenberg, Germany) until 8 weeks of age. Subsequently, mice were randomly divided into two groups and fed either a chow diet (CD, 10% fat, Research Diets, NJ, USA) or a high-fat diet (HFD, 60% fat, Research Diets, NJ, USA) for 12 weeks, and water was provided *ad libitum*. Individual animals were weighed weekly. At the end of the study, blood samples were collected from the orbital venous plexus. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C for further analyses. For cold exposure, mice were housed at 4 °C for 7 days with access to food and water. Rectal temperatures were measured at different time intervals during the cold challenge using a digital thermometer (TD-300, Shibaura Electronics, Tokyo, Japan). Thermal images of the dorsal view were acquired using a thermal camera (Fortric, #226) and analyzed using AnalyzIR software (Fortric). In addition, the β3-AR agonist CL-316,243 (Tocris, Catalog #1499, 1 mg/kg body weight/d) was administered intraperitoneally daily to 8-week-old WT and *Tet* TKO mice for 3 consecutive days.

**Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)**

For the oral glucose tolerance test (OGTT), mice were administered an oral injection of D-glucose (1 g/kg body weight; ThermoFischer, #15023–021) after overnight fasting, and serum glucose concentrations were measured at the indicated time points using a glucometer (Accu-check Performa, Roche). For insulin tolerance tests (ITT), mice were fasted for 6 h before intraperitoneal injection of insulin (0.75 unit/kg body weight; Sigma, #91077C), and blood glucose concentrations were measured at the indicated time points. HOMA-IR (homeostatic model assessment of insulin resistance) was calculated as fasting blood glucose (mg dL⁻¹) × fasting plasma insulin (mU mL⁻¹)/405 (9).

**Metabolic Analyses**

For indirect calorimetry, mice were individually housed for 3 days prior to measurements to allow them to acclimate to the housing conditions. Animals were weighed before being placed in designated cages. Mice in each chamber had free access to food and water. Oxygen
consumption (VO₂), carbon dioxide production (VCO₂), and heat production were measured using the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) and calculated using built-in software (Oxymax). The raw data were normalized to body weight. Movement was measured by counting horizontal beam breaks. Ambient temperatures were set to automatically drop from 30 °C to 22 °C to 4 °C at 2-day intervals. Recording was performed at regular intervals throughout the duration of the experiment.

ELISA and Lipid Analyses
Blood glucose levels were measured using a glucometer (Accu-check Performa, Roche). Plasma levels of insulin (Cayman, #589501), adiponectin (Cayman, #10007620), leptin (Cayman, #10007609), and hepatic TG (Cayman, #10010303) were determined by ELISA using commercial kits. Serum ALT, AST, cholesterol, low and high-density lipoprotein (LDL, HDL), triglycerides, and free fatty acids were analyzed using a BS-390 Automated Chemistry Analyzer (POHANG TECHNOPARK, Bioengineering Center, Pohang, Korea). Plasma samples were stored at −80 °C until analysis.

Histology
Tissue biopsies were fixed at 4 °C overnight in 10% neutral buffered formalin (MEDILAB, #0151), dehydrated, and then embedded in paraffin according to the standard laboratory protocol. Paraffin blocks were sectioned at 5 μm thickness, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) following the standard protocol. Adipocyte cell area was determined from H&E-stained sections using the virtual microscopy (Olympus). Adipocyte size was analyzed using built-in software (imaging software). For immunohistochemistry, specimens were deparaffinized and endogenous peroxidase was blocked in 0.3% H₂O₂. Heat-induced antigen retrieval with sodium citrate buffer (10 mM, pH 6.0) was performed before blocking with PBS containing 1% bovine serum albumin for 30 min at room temperature in a humidifying chamber. Then, tissue sections were stained with anti-Ucp1 (Abcam, #ab10983, 1:500), anti-F4/80 (Abcam, #ab6640, 1:500), or anti-insulin (Abcam, #ab181547, 1:200) at 4 °C overnight. After washing with PBS, tissue sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG for 1 h, followed by incubation in 3,3-di-amino-benzidine (DAB) solution (Dako, #L346811) and subsequent imaging by microscopy. The relative Ucp1 intensity was quantified using ImageJ (iij150, USA). For the immunofluorescence assay, Alexa Fluor 594-conjugated goat anti-rat secondary antibodies
were used, and imaging was performed using a LSM880 confocal microscope (ZEISS).

**Lipolysis Assays**

For the *in vivo* lipolysis assay, mice were injected intraperitoneally with CL316,243 (Tocris Bioscience, #1499) at 1 mg/kg body weight. Plasma samples were collected before injection and at 7.5, 15, and 30 min after injection. For *ex vivo* and *in vitro* assays, small pieces of adipose tissue explants (0.5 ~ 1 cm²) or *in vitro* differentiated adipocytes were stimulated with isoproterenol (Sigma, #I2760, 1–10 μM), forskolin (Selleckchem, #S2449, 20 μM), or isobutylmethylxanthine (IBMX) (Sigma, #I5879, 0.2 mM) for 90 min. Concentrations of glycerol or free fatty acids were measured using an EZ-free Glycerol Assay kit (Dogenbio, #DG-FGC100, Seoul, Korea) or a Free Fat Assay kit (Dogenbio, #DG-FFA100, Seoul, Korea), respectively, according to the manufacturer's instructions. For *ex vivo* samples, the quantification results were normalized to tissue weights as an index of lipolysis.

**Fatty Acid Oxidation**

Fatty acid oxidation (FAO) was measured using the Fatty Acid Oxidation kit (Biomedical Research Service, E-141, New York, USA) following the manufacturer's instructions. Briefly, tissues or cells were homogenized or resuspended in ice-cold lysis solution and centrifuged at 14,000 rpm for 5 min at 4 °C, and supernatants were then transferred to new tubes. Twenty microliters of each supernatant were added into each well of a 96-well plate and mixed with 50 μL of FAO Assay solution or control solution. After incubation at 37 °C for 60 min, the plate was read at an optical density of 492 nm (OD492) using a spectrophotometer (SpectraMax 190, Molecular Devices).

**Assessment of oxygen consumption rate (OCR)**

OCR was measured using a Seahorse XF-24 analyzer (Seahorse Bioscience, Billerica, MA, USA), according to the manufacturer's instructions. For OCR measurements, SVF cells (20,000 cells) were seeded in a 24-well Seahorse assay plate (Agilent, San Jose, CA, USA) and differentiated into adipocytes as described above. One day before measurement, the sensor cartridge was placed into calibration buffer (XF calibrant, pH 7.4) and incubated in a non-CO₂ incubator at 37°C. After 6 days of differentiation, adipocytes were washed twice and pre-incubated in pre-warmed XF assay media (pH 7.4), supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, and 2% bovine serum albumin in a non-CO₂ incubator for 30 min. During measurements, adipocytes were sequentially treated with CL316,243 (10 μM),
oligomycin (1.25 μM), and rotenone/antimycin A (1 μM). The basal OCR was measured three times, and three readings were taken after the addition of each mitochondrial OXPHOS inhibitor. The OCR was automatically recorded and calculated by the sensor cartridge and Seahorse XF-24 software.

Mitochondrial DNA quantification
Tissues were digested with 200 μg/mL proteinase K (Roche, Basel, Switzerland) overnight at 55°C with gentle agitation. Then, genomic DNA was isolated sequentially with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1), followed by precipitation with two volumes of ethanol. Total DNA was quantified using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Mitochondrial DNA was quantified by measuring the abundance of DNA regions corresponding to mitochondrial cytochrome c oxidase subunit 2 (Cox2), NADH dehydrogenase subunit 1, and cytochrome c, relative to that of the intronic regions of nuclear-encoded β-actin, β-globin, and glucagon using SYBR Green real-time PCR. Primer sequences are listed in Table S1.

mRNA Sequencing (RNA-Seq)
Total RNA was extracted from three biological replicates of eWAT from CD- or HFD-fed WT or Tet TKO mice using a Purelink RNA Micro kit (Invitrogen). Total RNA integrity was assessed using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value. mRNA sequencing libraries were prepared using the Truseq stranded mRNA library prep kit (Illumina) according to the manufacturer’s instructions. The quality of the libraries was verified by capillary electrophoresis (Bioanalyzer, Agilent). High-throughput sequencing (100 bp, paired end) was performed using a NovaSeq 6000 sequencing system (Illumina).

Analysis of RNA Sequencing Data
After quality filtering according to the Illumina pipeline, paired-end reads were mapped to the reference mouse genome (mm10 assembly) using STAR aligner version 2.7.3a with default parameters. Differentially expressed genes (DEGs) were identified using DESeq2 1.30.0. Read counts for DESeq2 analysis were obtained using featureCounts v2.0.0. After eliminating absent and low features (zero or < 1 CPM), the raw counts were normalized using DESeq2, followed by differential expression analysis. Differentially expressed genes were identified according to a P-value of ≤ 0.01 and fold change thresholds of ≥ 2 or ≤ 0.5. To generate the heatmap of K-mean clusters using RNA-seq, we used the Morpheus
K-means algorithm. K was chosen at 10, as lower values failed to identify all meaningful clusters and higher values subdivided meaningful clusters. Clusters were further grouped into two classes based on gene expression levels in HFD-fed WT and TKO mice. The canonical pathway was determined according to the IPA canonical pathway analysis of genes within classes 1 and 2. One representative category based on $P$-value was presented if more than one similar category was identified. Principal component analysis (PCA) was conducted using R tools, and RNA-seq data were visualized by preparing custom tracks for the UCSC Genome Browser.

**Flow Cytometry**

Stromal vascular fraction (SVF) cells were harvested as described and washed with 1X PBS. The cells were then suspended in FACS buffer (1X PBS containing 0.2% heat-inactivated FBS and 0.1% sodium azide), blocked with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™), and stained with anti-mouse monoclonal antibodies (BioLegend), including APC-Cy7-conjugated CD45.2 (104), APC-conjugated CD11b (M1/70), and PE-Cy7-conjugated F4/80 (BM8), at 4 °C for 30 min in the dark. The cells were then washed and resuspended in FACS buffer for flow cytometry. Flow cytometric analyses were performed using a FACS LSR Fortessa (BD Biosciences), and the data were analyzed using FlowJo software (BD Biosciences).

**Cloning of TET Expression Vectors and Co-Immunoprecipitation**

The open reading frame (ORF) of full-length (FL), N-terminal region lacking catalytic domain (ΔCD), or C-terminal catalytic domain (CD) of human TET1, TET2, and TET3 was amplified by polymerase chain reaction (PCR) and cloned into pOZ-FH-N vector that adds Flag-HA (FH) tandem tag to the N-terminal region of the inserted protein. Then, FH-tagged FL, CD, ΔCD of each TET was amplified by PCR using FH-TET1,2,3 in pOZ-FH-N vector as templates, followed by insertion into pEF1/V5 vector (Invitrogen). The amino acid numbers of the cloned regions are shown in Fig. S16 D-F. HEK293T cells were transfected with FH-tagged TET1, TET2, and TET3 plasmids described above. After 48 h, cells were harvested and washed twice with 1X PBS. Cells were then lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with protease/phosphatase inhibitor cocktail (10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μM leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)), incubated on ice for 20 min, and centrifuged at 12,000 rpm for 15 min at 4 °C. Co-
immunoprecipitation was performed using an anti-FLAG® M2 affinity gel (Sigma). Whole-cell lysate (500 μg) in a final 500 μL volume of lysis buffer was incubated with protein A/G agarose for 1 h at 4 °C with gentle rotation, followed by centrifugation for 2 min at 1,400 rpm. The supernatant was transferred to a tube containing 40 μL of anti-FLAG® M2 affinity gel and incubated overnight at 4 °C with gentle rotation. The protein-bead complexes were washed with 1X wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100) six times at 4 °C. Each wash consisted of a 5 min incubation at 4 °C with gentle rotation. The bound proteins were eluted by boiling in a sample buffer and then visualized by electrophoresis on SDS-PAGE and subsequent immunoblotting.

**Chromatin Immunoprecipitation Coupled with qPCR**

ChIP assays were performed using the Chromatin Immunoprecipitation (ChIP) Assay kit (Merck, #17–295) according to the manufacturer’s instructions. Briefly, cells were cross-linked using 1% (w/v) formaldehyde for 10 min at 37 °C. After washing twice with cold PBS, cells were collected and resuspended in SDS lysis buffer (Merck, #20–163). Lysates were sonicated to shear DNA to lengths that were between 200 and 500 bp using a Q800R3 sonicator (Qsonica). The sonicated cell supernatants were diluted 10-fold in ChIP Dilution Buffer and immunoprecipitated with 2 μg of anti-H3K27Ac (Abcam, #ab4729) or 10 μg of anti-HDAC1 (GeneTex, #GTX100513), anti-HDAC3 (Abcam, #ab7030), anti-Tet1 (GeneTex, #GTX125888), anti-Tet2 (Abcam, #ab94580), anti-Tet3 (GeneTex, #GTX121453), or IgG (Cell Signaling Technology, #2729). After washing, the immunoprecipitated DNA was recovered using phenol/chloroform extraction and analyzed via qPCR with TOPreal™ qPCR 2X PreMIX (Enzynomics) and QuantStudio 5 and 6 real-time PCR systems. Percentages of input recovery were calculated. The primer sequences are listed in Table S1.

**Dot Blot Analysis**

Cells and tissues were digested by incubation with 200 μg/mL proteinase K (Roche) overnight at 55 °C, followed by isolation of genomic DNA by phenol-chloroform extraction as described above. DNA samples were denatured in 0.4 M NaOH, 10 mM EDTA at 95 °C for 10 min, and then neutralized with cold 2 M ammonium acetate (pH 7.0). Two-fold serial dilutions of the denatured DNAs were spotted on a nitrocellulose membrane in an assembled Bio-Dot apparatus (Bio-Rad) according to the manufacturer’s instructions. The membrane was washed with 2X SSC buffer, air-dried, and vacuum-baked at 80 °C for 2 h, then blocked with 5% non-fat milk for 1 h and incubated with anti-5hmC (1:6,000, gifted from Dr. Anjana Rao) overnight at 4 °C.
After incubating with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody, the membrane was visualized by chemiluminescence (Biorad). To ensure equal loading of DNAs on the membrane, the same blot was stained with 0.04% toluidine blue in 0.3 M sodium acetate (pH 5.2).

**RNA Purification, Reverse Transcription, and Real-Time RT-PCR**

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen) and reverse transcription was performed using SuperScript IV (Invitrogen), according to the manufacturer’s instructions. Diluted cDNAs were analyzed by real-time PCR using QuantStudio 5, 6, or 7 Flex (Applied Biosystems) and TOPreal™ qPCR 2X PreMIX (Enzymomics). Data were analyzed by QuantStudio 5, 6, or 7 real-time PCR software (Applied Biosystems). Levels of gene expression were normalized to *Gapdh*. Primer sequences are listed in Table S1.

**Immunoblot Analysis**

Cells and tissues were lysed using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease/phosphatase inhibitor (20 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium o-vanadate, 10 μM leupeptin, 10 μg/mL aprotinin, 1 mM freshly prepared phenylmethylsulphonyl fluoride (PMSF) and incubated on ice for 15 min. Adipose tissues were lysed with TissueLyser LT in lysis buffer (Qiagen). After centrifuging at 13,000 rpm for 15 min at 4 °C, the protein concentration was determined by Bradford protein assay. Cell lysates were mixed with SDS sample buffer before boiling for 5 min. Then, cell lysates were separated by 7.5% or 10% SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were detected by immunoblotting in TBST (150 mM NaCl, 10 mM Tris-Cl, pH 8.0, 0.5% Tween-20) containing 5% low-fat milk and antibodies against proteins of interest, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen) and enhanced chemiluminescence (Bio-rad). The following antibodies were used in this study. Anti-Ucp1 (Abcam, #ab10983), anti-Pgc-1α (Novus, #NBp1-04676), anti-β3-AR (Santa Cruz, #sc-515763), anti-p-PKA substrate (CST, #9624), anti-p-Hsl (Ser563) (CST, #4139), anti-Hsl (CST, #4107), anti-p-Akt (Ser473) (CST, #9271), anti-p-Akt (Thr308) (CST, #9275), anti-Akt (CST, #9272), DDK (Flag) epitope (Origene, TA50011-100), anti-HDAC1 (GeneTex, #GTX100513), anti-HDAC3 (Abcam, #ab7030), anti-α-Tubulin (Sigma, #T5168), and anti-β-actin (Sigma, #A5441).
Luciferase Reporter Assay

Adipocytes or HEK293T cells were transfected with pREP4-Luc reporter constructs (Addgene, #124892) driven by the \( \beta_3 \)-AR promoter, or pGL3-promoter vector (Promega) driven by \( Ppargc1a \) 2 kb promoter (Addgene, #8887) or \( Ppargc1a \) promoter ΔCRE (Addgene, #8888) using Lipofectamine 2000 reagent (Invitrogen). The \( Renilla \) luciferase reporter construct (pRL-TK, Promega) was used to monitor transfection efficiency. To assess the effect of the catalytic activity of TET proteins, mammalian expression vectors for WT or catalytically inactive mutants of TET1, TET2, or TET3 were co-transfected with reporter constructs and a \( Renilla \) luciferase construct. Mutant TET constructs were generated by site-directed mutagenesis of the HxD catalytic motif by PCR using Pfu polymerase, Dpn I treatment, and transformation (10, 11). Lysates were collected 48 h after transfection, and firefly and \( Renilla \) luciferase activities were measured using a Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized to the activity of \( Renilla \) luciferase. The \( \beta_3 \)-AR promoter 6 kb-driven luciferase plasmid was described elsewhere (12). The serially truncated versions of upstream fragments of the mouse \( \beta_3 \)-AR gene shown in were cloned by PCR. The ENCODE cCRE in the \( \beta_3 \)-AR locus was amplified by PCR. Primer sequences used for cloning are listed in Table S1. The truncated versions of the \( \beta_3 \)-AR upstream fragments and \( \beta_3 \)-AR promoter (1 kb) in both orientations were originally cloned into pGL3-promoter vector using primers shown in Table S1, followed by subcloning into the pREP4-Luc vector using Not I and Xho I enzymes.

Statistical Analysis

All statistics were performed with GraphPad Prism 8.0 software (GraphPad, CA, USA). All data are presented as the mean ± s.d., except where noted. Statistical significance was analyzed by a two-tailed, unpaired Student’s \( t \)-test unless stated otherwise. \(* \ P < 0.05\), \(** \ P < 0.005\), and \(*** \ P < 0.0005\) were considered significant. For RNA-seq data, significance was estimated by Kolmogorov-Smirnov test. The relationship between metabolic rate and body weight was statistically tested using ANCOVA (Pinguin v0.3.12 Python package) with the significance threshold of \( p \leq 0.05\).

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Establishment of immortalized pre-adipocytes for inducible Tet deletion

**A**

Adipose tissue dissection
Collagenase digestion
Stromal vascular fraction
SV40 large T infection
Geneticin selection

**B**

| Gene | Time after 4-OHT treatment (hr) |
|------|----------------------------------|
| Tet1 | 0 12 24 36 48 60                |
| Tet2 | 0 12 24 36 48 60                |
| Tet3 | 0 12 24 36 48 60                |

**C**

**Establishment of immortalized pre-adipocytes (iWAT)**

| Event | Day |
|-------|-----|
| Cell plating | -3 |
| Confluence | -2 |
| Media change (adipogenic induction) | 0 |
| Media change (differentiation) | 2 |
| Media change (differentiation) | 4 |
| Media change (differentiation) | 6 |

**Assay**

- Oil Red O staining
- qRT-PCR/ immunoblotting
- Lipolysis assay
- Fatty acid oxidation assay

**D**

**Vehicle (WT)**

| Condition | Day |
|-----------|-----|
| No diff. | -3 |
| Differentiation | 0 |
| Day 2     |     |

**4-OHT (TKO)**

| Condition | Day |
|-----------|-----|
| No diff. | -3 |
| Differentiation | 0 |
| Day 2     |     |

**Oil Red O stain (OD 492 nm)**

**E**

| Gene   | Condition | Day |
|--------|-----------|-----|
| Pparg  | No diff.  | -3 |
|        | Differentiation | 0 |
|        | Day 2     |     |

**F**

| Gene   | Condition | Day |
|--------|-----------|-----|
| Tet1   | No diff.  | -3 |
|        | Differentiation | 0 |
|        | Day 2     |     |

**G**

| Gene   | Condition | Day |
|--------|-----------|-----|
| β3-AR  | No diff.  | -3 |
|        | Differentiation | 0 |
|        | Day 2     |     |

**Note:**

- **Differentiation** refers to the process of adipocyte formation.
- **Early deletion** and **Late deletion** indicate the timing of geneticin selection.
- **4-OHT addition** is the treatment of inducible Tet deletion.
- **β3-AR** refers to the β3-adrenergic receptor.
Fig. S1. Inducible Tet deletion in immortalized preadipocytes in vitro.

(A) Schematic representation of the establishment of immortalized preadipocytes for inducible Tet deletion.

(B) mRNA levels of Tet1, Tet2, and Tet3 relative to Gapdh in immortalized preadipocytes after treatment with 4-OHT (1 μM) for the indicated time periods. n = 3.

(C) Schematic representation of induction of Tet deletion during preadipocytes differentiation in vitro.

(D) Representative Oil Red O staining images of differentiated adipocytes after 4-OHT treatment at the indicated time points (left) and quantification of accumulated lipids by spectrometry (right). n = 3. Scale bar, 50 μm.

(E and F) mRNA levels of the indicated genes relative to Gapdh in differentiated adipocytes after 4-OHT (1 μM) treatment at the indicated time points. n = 3.

(G) mRNA levels of the indicated genes relative to Gapdh in WT and Tet TKO adipocytes. Preadipocytes were treated with or without 4-OHT on day 2 of differentiation. n = 3.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. 0 h (B), no diff (D-F), and WT (G); bP < 0.005.
Fig. S2. TET proteins suppress thermogenic gene expression. 
(A and B) mRNA levels of the indicated genes relative to Gapdh in WT and Tet TKO adipocytes stimulated with or without forskolin (20 μM) for 6 h. Adipogenic marker gene expression (B) served as a negative control. n = 3. 
All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. **P < 0.005, ***P < 0.0005 vs. WT; bP < 0.005, cP < 0.0005.
Fig. S3. TET proteins cooperate with HDAC1 to suppress Ppargc1a and Ucp1 transcription. 

(A) Luciferase reporter activities driven by Ppargc1a (2 kb) promoters (WT or ΔCRE) in WT and Tet TKO adipocytes. Results were normalized to Renilla luciferase activity. RLU, relative light units. n = 3.

(B and C) Luciferase reporter activities driven by Ppargc1a (2 kb) promoters (WT or ΔCRE) in HEK293T cells transiently transfected with WT or mutant TET1, TET2, and TET3 plasmids. WT, wildtype; Mut, catalytically inactive mutant. n = 3.

(D and E) ChIP-qPCR for IgG, HDAC1, and HDAC3 (D) or H3K27ac (E) at the Ppargc1a promoter and Ucp1 promoter/enhancer regions in WT and Tet TKO adipocytes. n = 3.

(F) mRNA expression of the indicated genes relative to Gapdh in WT and Tet TKO adipocytes after stimulation with or without CL316,243 (1 μM, 24 h). Cells were either untreated or pre-treated for 1 h with SR59230A (10 μM), a β3-AR antagonist, prior to CL216,243 treatments. n = 3. All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT (A, D-F) and reporter only (B and C); ⁶P < 0.0005.
Fig. S4. TET proteins suppress thermogenic gene expression in an enzymatic activity-independent manner.

(A-C) mRNA expression of Ppara (A), Elovl3 (B), and Cidea (C) relative to Gapdh in WT adipocytes or Tet TKO adipocytes expressing empty vector, WT, or mutant Tet2-CD with or without CL-316,243 stimulation. n = 3.

(D) mRNA expression of Ppargc1a and Ucp1 relative to Gapdh in adipocytes shown in Fig. 2C. n = 3.

(E and F) Quantification of Pgc-1α (E) and Ucp1 (F) protein levels shown in Fig. 2C. n = 4.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; aP < 0.05, bP < 0.005, cP < 0.0005.
**Fig. S5. Generation of adipocyte-specific Tet triple knockout mice.**

(A) mRNA expression of Tet1, Tet2, and Tet3 relative to Gapdh in the indicated organs from WT and Tet TKO mice at 8 weeks of age. n = 6 mice per genotype.

(B and C) Dot blot analyses to quantify 5hmC levels in adipose tissues (B) and the indicated organs (C) isolated from WT and Tet TKO mice. Toluidine blue staining was performed to confirm blotting of equal amounts of DNAs.

(D and E) mRNA expression of Tet1, Tet2, and Tet3 relative to Gapdh in mature adipocytes or stromal vascular fraction (SVF) cells separated from iWAT (D) and eWAT (E) of WT and Tet TKO mice. n = 3–4 (for adipocytes) and n = 6 (for SVF) mice per genotype.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT.
Fig. S6. Expression of genes related to lipolysis and fatty acid oxidation in CL-316,243-stimulated wildtype and Tet TKO mice.

WT and Tet TKO mice were administered intraperitoneally with CL-316,243 (1 mg/kg body weight/d) daily for 3 consecutive days.

(A and B) mRNA expression of genes related to lipolysis (A) or fatty acid oxidation (B) relative to Gapdh in iWAT of CL-316,243-stimulated mice. n = 3.

(C and D) mRNA expression of genes related to lipolysis (C) or fatty acid oxidation (D) relative to Gapdh in BAT of CL-316,243-stimulated mice. n = 3.

(E and F) Relative mitochondria DNA quantification of iWAT (E) and BAT (F) from CL-316,243-stimulated mice. n = 3

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT.
Fig. S7. Tet TKO mice resist high-fat-diet-induced obesity.

(A) TET1, TET2, and TET3 mRNA levels in lean, obese, and type 2 diabetic human samples. RNA-sequencing data were from GSE133099.
(B) Representative photographs of WT and Tet TKO mice fed either CD or HFD for 12 weeks. n = 6. Scale bar, 1 cm.
(C) Daily food intake of WT and Tet TKO mice fed CD or HFD. n = 30.
(D) Representative photographs of dissected WT and Tet TKO mice fed either CD or HFD for 12 weeks. n = 10. Scale bar, 0.5 cm.
(E) Weights of internal organs of WT and Tet TKO mice fed either CD or HFD. n = 6 for CD and n = 10 for HFD.
(F) Average adipocyte volume of iWAT, eWAT, and BAT from WT and Tet TKO mice fed either CD or HFD.

(G-J) mRNA expression of representative adipogenic markers relative to Gapdh in eWAT and iWAT of WT and Tet TKO mice fed either CD (G and H) or HFD (I and J) for 12 weeks. n = 3. All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; †P < 0.0005 vs. CD.
**Fig. S8.** TET-deficient female mice are resistant to diet-induced obesity.

(A) Changes in body weight of WT and Tet TKO female mice fed either CD or HFD. n = 5–6. 

(B) Summary of body weight gain in WT and Tet TKO female mice fed either CD (n = 6) or HFD (n = 14) for 12 weeks. 

(C) Daily food intake of WT and Tet TKO female mice fed either CD or HFD. n = 30. 

(D) Representative photographs of iWAT, eWAT, and BAT from WT and Tet TKO female mice fed either CD or HFD (top). A summary of the weights of each fat pad is also shown (bottom). n = 5. Scale bar, 1 cm. 

(E) Absolute amount of fat mass (left) and lean mass (right) from WT and Tet TKO female mice fed either CD or HFD. n = 5. 

(F) Weights of non-adipose organs of WT and Tet TKO female mice fed either CD or HFD. n = 5. All data are presented as the mean ± s.d. The *P*-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; cP < 0.0005 vs. CD. n.s. not significant.
Fig. S9. Tet TKO leads to the maintenance of higher levels of β3-AR and lipolytic capacity in adipose tissues on a high-fat diet.

(A) β3-AR mRNA levels relative to Gapdh in BAT from WT and Tet TKO mice fed either CD or HFD. n = 3.

(B) Ex vivo lipolysis in eWAT (left) or iWAT (right) isolated from HFD-fed WT and Tet TKO mice stimulated with or without ISO at the indicated concentrations. n = 3–4.

(C) Glycerol concentrations in eWAT (left) and iWAT (right) isolated from HFD-fed WT and Tet TKO mice stimulated with or without ISO, forskolin, or IBMX as indicated. n = 3–4.

(D) Ex vivo lipolysis in BAT isolated from HFD-fed WT and Tet TKO mice stimulated with or without ISO at the indicated concentrations. n = 3–4.

(E) Glycerol concentrations in BAT explants isolated from HFD-fed WT and Tet TKO mice stimulated with or without ISO, forskolin, or IBMX as indicated. n = 3–4.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005 vs. WT.
Fig. S10. High-fat-diet-fed Tet TKO mice maintain a higher metabolic rate with no alterations in locomotor activity.

(A and B) Indirect calorimetry of HFD-fed WT and Tet TKO mice maintained at 30 °C, 22 °C, and 4 °C to obtain VCO₂. n = 5.

(C) Locomotor activity of HFD-fed WT and Tet TKO mice during the dark and light phases at the indicated temperatures. n = 5.

(D) Average respiratory exchange ratio (RER) of HFD-fed WT and Tet TKO mice at the indicated temperatures. n = 5.

(E) Regression-based analysis of absolute MR against body weight. n = 5. Data were analyzed using ANCOVA with body weight as covariate.

(F) Predicted MR at a given body mass of 40 g. n = 5.

(G) Rectal temperatures measured in HFD-fed WT and Tet TKO mice at RT for 12 weeks. n = 7 for WT and n = 9 for Tet TKO.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. **P < 0.005, ***P < 0.0005 vs. WT.
Fig. S11. High-fat-diet-fed Tet TKO mice maintain higher levels of Ppargc1a expression, mitochondrial content, and fatty acid oxidation capacity.

(A) Ppargc1a mRNA levels relative to Gapdh (A) in the indicated adipose depots (iWAT, eWAT, and BAT) from WT and Tet TKO mice fed either CD or HFD. n = 3.

(B and C) Relative mitochondria DNA content (B) and ex vivo fatty acid oxidation (C) in the indicated adipose depots from WT and Tet TKO mice fed a HFD. n = 3.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; aP < 0.05, bP < 0.005 vs. CD.
A. Relative Ppara mRNA expression

B. Relative Usp1 mRNA expression

C. mRNA expression of various genes:
   - Cox5b: X 1.6
   - Cox7a: X 3.1
   - Cox8b: X 2.4
   - Ppara: X 2.2
   - Elov13: X 3.1
   - Cidea: X 1.2
   - Pparg: X 2.5
   - Fabp4: X 0.3

D. TET1 Core catalytic domain
   - IP: anti-Flag
   - Input
   - kDa: Flag (TET1), HDAC1, HDAC3, ACTIN

E. TET2 Core catalytic domain
   - IP: anti-Flag
   - Input
   - kDa: Flag (TET2), HDAC1, HDAC3, ACTIN

F. TET3 Core catalytic domain
   - IP: anti-Flag
   - Input
   - kDa: Flag (TET3), HDAC1, HDAC3, ACTIN

G. Putative β3-AR silencer-like element (5' -> 3')
   - TCTTGGGCTATTTTCTGGGCTCTGTCCATGGGGTGAAGGGTGTGGGTGGTGGG
   - AGAGCGCAAGGGACGAGGTACATTCCTTTGGATGAGATGCTGGTGGAGAACTTATGA
   - CACCCTGTGACAGACGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGA
Fig. S12. TET proteins cooperate with HDACs to repress thermogenic gene expression.

(A and B) mRNA expression of Ppargc1a (A) and Ucp1 (B) relative to Gapdh in WT and Tet TKO adipocytes stimulated with or without the indicated HDAC inhibitors (MS-275: 10 μM, 24 h or SAHA: 20 μM, 6 h). n = 3.

(C) mRNA expression of the indicated genes relative to Gapdh in WT and Tet TKO adipocytes stimulated with or without MS-275 (5 μM) for 24 h. n = 3.

(D-F) Domain structures of TET1 (D), TET2 (E), and TET3 (F) proteins and co-immunoprecipitation with HDAC1 and HDAC3 in HEK293T cell lysates.

(G) Sequences of the putative silencer-like element at the β3-AR locus.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; bP < 0.005, cP < 0.0005 vs. DMSO.
Fig. S13. TET deficiency protects against the detrimental metabolic effects of obesity.

(A) Fasting serum glucose concentrations in WT and Tet TKO mice fed either CD (n = 6) or HFD (n = 12) for 12 wk, starting at 8 wk of age.

(B) Fasting serum insulin concentrations in WT and Tet TKO mice fed either CD or HFD for 12 wk. n = 5.

(C and D) Oral glucose tolerance test (OGT T; n = 12) and intraperitoneal insulin tolerance test (IP IT T; n = 10) in WT and Tet TKO mice fed a HFD for 12 wk.

(E and F) Immunoblot analysis of p-Akt (Ser-473), p-Akt (Thr-308), and total Akt proteins in eWAT (E) and the livers (F) from HFD-fed WT and Tet TKO mice stimulated with or without insulin (1.5 U/kg). α-Tubulin served as a loading control. Quantification results are shown in SI Appendix, Fig. S14 D and E.

(G) Representative immunohistochemical staining for insulin in the pancreas of WT and Tet TKO mice fed either CD or HFD (Top) (n = 6). A summary of the average β-cell area is shown (Bottom). n = 8. (Scale bar, 50 μm.)

(H) Representative hematoxylin and eosin staining of the livers from WT and Tet TKO mice fed either CD or HFD. n = 6. (Scale bar, 100 μm.)

(I) Triglyceride levels in the livers from WT and Tet TKO mice fed either CD or HFD. n = 5.

(J) Plasma concentrations of cholesterol, low-density lipoprotein (LDL), and free fatty acid (FFA) in WT and Tet TKO mice fed either CD or HFD. n = 4–8.

(K) Plasma adiponectin concentrations in WT and Tet TKO mice fed either CD or HFD (Left; n = 4–8) and adiponectin mRNA levels relative to Gapdh in eWAT. n = 3.

(L) Plasma leptin concentrations in WT and Tet TKO mice fed either CD or HFD (Left; n = 4–8) and leptin mRNA levels relative to Gapdh in eWAT. n = 3.

(M) Representative immunohistochemical staining for F4/80 in eWAT of HFD-fed WT and Tet TKO mice. n = 4. (Scale bar, 100 μm.)

(N) mRNA expression of proinflammatory molecules relative to Gapdh in eWAT of WT and Tet TKO mice fed an HFD. n = 4. All data are presented as the mean ± SD. The P values were determined by unpaired Student’s t test. *P < 0.05, **P < 0.005, ***P < 0.0005 versus WT; bP < 0.005, cP < 0.0005 versus CD. IHC, immunohistochemistry.
Fig. S14. Effect of TET loss on glucose tolerance and insulin sensitivity in vivo.

(A) HOMA-IR score in WT and Tet TKO mice fed a HFD. n = 5.

(B and C) Oral glucose tolerance (OGTT) (B) and insulin tolerance (IPITT) (C) tests in WT and Tet TKO mice fed a CD. n = 3–5.

(D and E) Quantification results of SI Appendix, Fig 13 E and F. Levels of p-Akt (Ser473) and p-Akt (Thr308) proteins relative to total Akt proteins in eWAT (D) and liver (E) of HFD-fed WT and Tet TKO mice stimulated with or without insulin (1.5 U/kg) are shown.

(F and G) Immunoblot analysis (left) and quantification (right) of p-Akt (Ser473), p-Akt (Thr308), and total Akt proteins in muscle (F) and iWAT (G) of HFD-fed WT and Tet TKO mice stimulated with or without insulin (1.5 U/kg). α-Tubulin served as a loading control. n = 2.

(H) Immunoblot analysis of p-Akt (Ser 473), p-Akt (Thr 308), and total Akt proteins in eWAT, iWAT, and liver from CD-fed WT and Tet TKO mice stimulated with or without insulin (1.5 U/kg) for the indicated time periods. α-Tubulin served as a loading control.

(I) Representative hematoxylin and eosin (H&E) staining (left) and quantification of the islet area (right) in the pancreas in WT and Tet TKO mice fed either CD or HFD. n = 8. Scale bar, 50 μm.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05 **P < 0.005, and ***P < 0.0005 vs. WT.
Fig. S15. TET deficiency protects against the detrimental metabolic effects of obesity.

(A) Representative photographs (left) and absolute weights (right) of the livers from WT and Tet TKO mice fed either CD or HFD. n = 6 for CD and n = 10 for HFD. Scale bar, 1 cm.

(B) Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations in WT and Tet TKO mice fed either CD or HFD. n = 5–6.

(C) Plasma concentrations of high-density lipoprotein (HDL) and triglycerides (TG) in WT and Tet TKO mice fed either CD or HFD. n = 4–8.

(D) Representative immunohistochemical staining for F4/80 in eWAT from HFD-fed WT and Tet TKO mice (left). Quantification results are also shown (right) (n = 10). ICC, immunocytochemistry. Scale bar, 50 μm.

(E) Flow cytometric analysis of macrophage infiltration in eWAT of HFD-fed WT and Tet TKO mice. CD45⁺ CD11b⁺ F4/80⁺ cells were defined as macrophages. Representative contour plots (left) and quantification results (right) are shown. n = 6.

(F and G) mRNA expression of inflammatory chemokines (F) and macrophage markers (G) relative to Gapdh in eWAT from WT and Tet TKO mice fed a HFD. n = 4.

All data are presented as the mean ± s.d. The P-values were determined by unpaired Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005 vs. WT; *P < 0.05, bP < 0.005, cP < 0.0005 vs. CD.
Inflammatory response
KRAS signaling up E2F targets
E2F targets
Interferon response
Tumor necrosis factor (TNF) signaling via NF-κB
E2F targets
Hypoxia
Inflammatory response
Cholesterol homestasis
Adipogenesis
Myogenesis
Xenobiotic metabolism
Adipogenesis
Fatty acid metabolism
Oxidative phosphorylation
Coagulation
Estrogen response
Bile acid metabolism

Class 1

Up by HFD in WT
(N = 2,162)
WT, HFD > TKO, HFD
(N = 2,168)

Class 2

Down by HFD in WT
(N = 1,250)
WT, HFD < TKO, HFD
(N = 966)
Fig. S16. TET proteins are critical for high fat diet-induced transcriptomic alterations in visceral adipose tissue.

(A) Principal component analysis (PCA) plot of RNA-seq data of eWATs from WT and Tet TKO mice fed either CD or HFD.

(B) Clustering of differentially expressed genes (P-value ≤ 0.01, fold change ≥ 2 or ≤ 0.5) in eWAT from WT or Tet TKO mice fed either CD or HFD for 12 weeks. GO categories of the MSigDB hallmark gene set of the genes within each cluster are shown on the right. N, number of genes.

(C) Violin plots presenting normalized read counts of genes within each cluster as shown in (B). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (Kolmogorov-Smirnov test).

(D) Venn diagrams indicating the number of differentially expressed genes.
Fig. S17. Ingenuity pathway analysis and β3-AR expression in RNA-seq analysis.

(A-B) Ingenuity pathway analysis (canonical pathways) of differentially expressed genes in eWAT of HFD-fed WT or Tet TKO mice (class 1, downregulated (A); class 2, upregulated genes (B) in HFD-fed Tet TKO vs. WT mice)

(C) UCSC genome browser tracks of β3-AR mRNA expression in eWAT from WT and Tet TKO mice fed either CD or HFD.
Table S1-List of oligonucleotides used in this study

| Target      | Forward primer (5' -> 3') | Reverse primer (5' -> 3') | Assay             |
|-------------|---------------------------|---------------------------|-------------------|
| Tet1        | GAGCCTGTTCCTCGATGTGG      | CAAACCCACCTGAGGCTGTT     | Mitochondrial DNA quantification |
| Tet2        | AACCTGGCTACTGTCATTGCTCCA | ATGCTCTGCTGGTGCTCTGCTGA | ChIP-qPCR         |
| Tet3        | TCCGGATTGAGAAGTCACTCG     | CACAGGGGCGAGAACTGTT      | Reporter assay    |
| β1-AR       | CTCATCGTGGTGGGTAACGTG     | ACACACACACACACACACACAC  |                      |
| β2-AR       | AACCTGGCTACTGTCATTGCTCCA | ATGCTCTGCTGGTGCTCTGCTGA |                      |
| β3-AR       | TCCGGATTGAGAAGTCACTCG     | CACAGGGGCGAGAACTGTT      |                      |
| Ucp1        | TCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Prdm16      | CCAGCGAGGGCCAGGGG        | AGTCTGCTTGGTGGTGAATT    |                      |
| Ppargc1a    | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Pparα       | AAGACTACCTGCTACCGAAATG   | AACATTGGGCCGGTTAAGG     |                      |
| Dio2        | CATTGATGAGGCTGCTCTCTCT  | GGCTTGGATCTGACTGTTCT    |                      |
| Cidea        | TGCTCTGCTGCTGCTGCTGCT   | CTCAGCTGCTGCTGCTGCTGCT |                      |
| Cox5b       | GCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1-promoter | GGCTTACTTCACTGCCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1-promoter | GCTCTCTTAGCTCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1-promoter | GCCAGGCTGCTGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1-enhancer | CCCAGTTACGACACCTAAGG     | AGTCTGCTGCTGCTGCTGCT    |                      |
| β3-AR-A      | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-B      | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-C      | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-D      | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-E      | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-upstream | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-common reverse | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| β3-AR-cCRE   | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Cox2         | GCGCTAAGAGAGGCAAGA      | CAAAGGCATAAGAGCTG        |                      |
| mifD2        | ACCATGCTGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Cca3        | CTCTCTGCTGCTGCTGCTGCT   | CTCAGCTGCTGCTGCTGCTGCT |                      |
| Ppargc1a    | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Pparα       | AAGACTACCTGCTACCGAAATG   | AACATTGGGCCGGTTAAGG     |                      |
| Dio2        | CATTGATGAGGCTGCTCTCTCT  | GGCTTGGATCTGACTGTTCT    |                      |
| Cidea        | TGCTCTGCTGCTGCTGCTGCT   | CTCAGCTGCTGCTGCTGCTGCT |                      |
| Cox5b       | GCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1        | TCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Prdm16      | CCAGCGAGGGCCAGGGG        | AGTCTGCTTGGTGGTGAATT    |                      |
| Ppargc1a    | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Pparα       | AAGACTACCTGCTACCGAAATG   | AACATTGGGCCGGTTAAGG     |                      |
| Dio2        | CATTGATGAGGCTGCTCTCTCT  | GGCTTGGATCTGACTGTTCT    |                      |
| Cidea        | TGCTCTGCTGCTGCTGCTGCT   | CTCAGCTGCTGCTGCTGCTGCT |                      |
| Cox5b       | GCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Ucp1        | TCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Prdm16      | CCAGCGAGGGCCAGGGG        | AGTCTGCTTGGTGGTGAATT    |                      |
| Ppargc1a    | ACGCTGTCGAGGCTGCTGCT    | GGCTTGGATCTGACTGTTCT    |                      |
| Pparα       | AAGACTACCTGCTACCGAAATG   | AACATTGGGCCGGTTAAGG     |                      |
| Dio2        | CATTGATGAGGCTGCTCTCTCT  | GGCTTGGATCTGACTGTTCT    |                      |
| Cidea        | TGCTCTGCTGCTGCTGCTGCT   | CTCAGCTGCTGCTGCTGCTGCT |                      |
| Cox5b       | GCTCTCGCCCGCTTAACTGCT    | GGCTTGGATCTGACTGTTCT    |                      |