Supplemental Figure 1

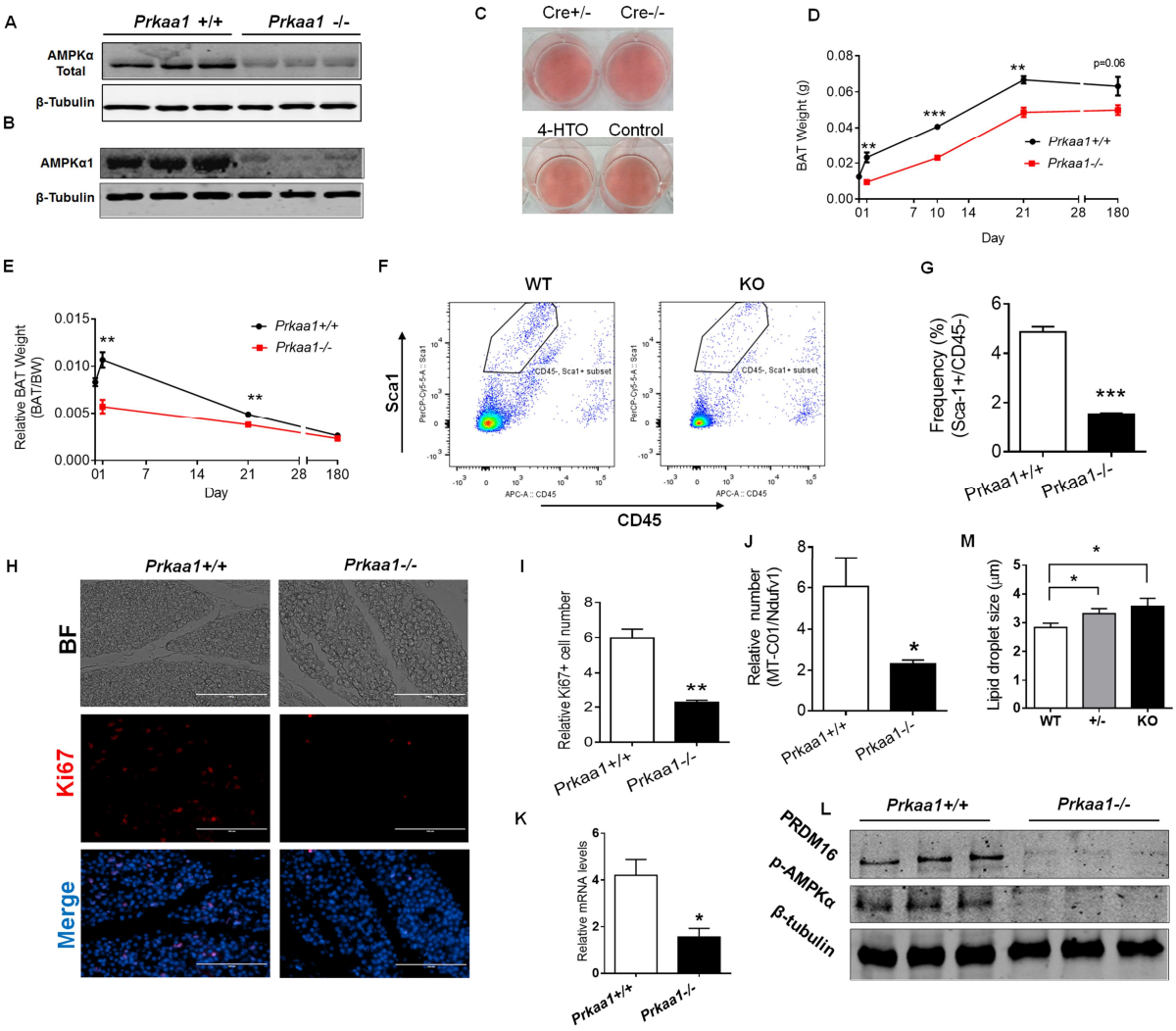

Figure S1 *Prkaa1* KO dramatically impairs BAT development, related to Figure1

(A-B) PRKAA1 is prominently expressed in BAT. Total PRKAA subunits, using an antibody recognizing total PRKAA was dramatically reduced in *Prkaa1* knockout mice (*A*); PRKAA1 specific antibody only detected trace amount of signal in *Prkaa1* -/- cells (*B*). (C) Activation of Cre recombinase and pretreatment of 4-hydroxytamoxifen (0.25 μM) did not affect brown adipogenesis. (D,E) BAT weights (*D*) and relative weights (*E*) of WT and *Prkaa1*-/- mice on a standard diet. (F,G) Flow cytometry plots of brown progenitor cells from interscapular BAT of WT and *Prkaa1*-/- mice at P21, with black cycles denoting the population of Lin-/Sca1+/CD45- brown adipogenic progenitors (*F*) and quantification of progenitor cells (*G*). (H,I) Proliferating cell density was reduced in *Prkaa1*-/- BAT of E18.5 fetuses. Immunofluorescence staining (*H,* Scale bar, 100μm) and quantification of Ki67+ cells (*I*). (J) Mitochondrial DNA copy number was reduced in P21 BAT due to *Prkaa1* KO ($n = 4$). (K,L) *Prdm16* mRNA expression (*K*) and protein content (*L*) were reduced due to *Prkaa1* ablation in E15.5 embryos. (M) Qualification of lipid droplet size (WT, $n = 73$; +/-, $n = 57$; KO, $n = 28$). Gene mRNA expression was normalization to the 18S rRNA. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; means ± s.e.m.; $n = 3$; Student's *t* test with a two-tailed distribution)

Supplemental Figure 2

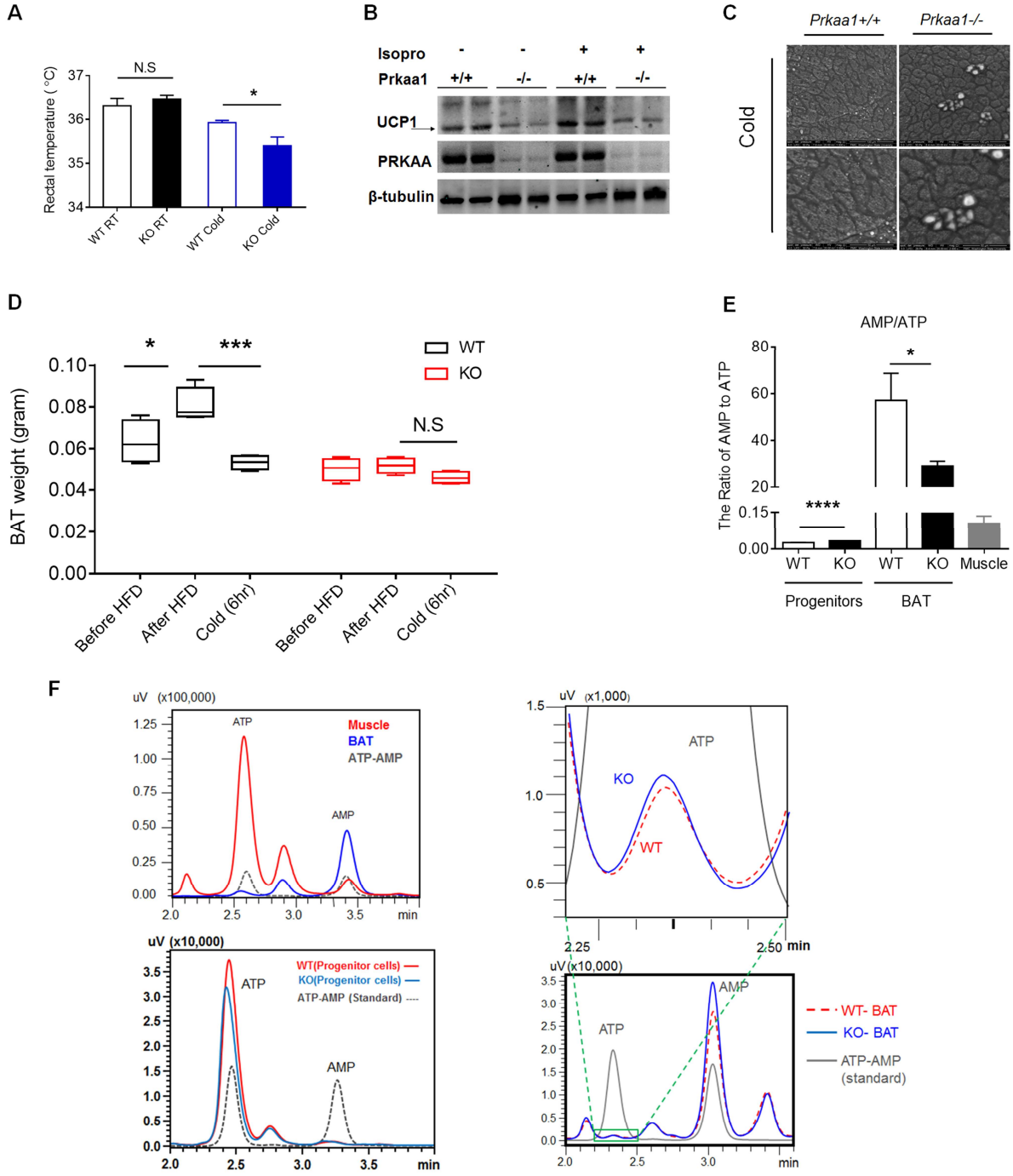

Figure S2 *Prkaa1* KO dramatically reduces thermogenesis, related to Figure 1.
(A) *Prkaa1* deletion impaired BAT thermogenesis of adult mice under cold exposure (6 h, 5 °C), rectal temperature. (B) BAT was separated from 6 month old WT and *Prkaa1* KO mice, sliced and, then, cultured in a medium containing 10 μM isoproterenol for 6 h. The UCP-1 expression stimulated by adrenergic agonist (isoproterenol) was attenuated in *Prkaa*1-/- BAT. (C) Scanning electron microscopic images of BAT of 2 month old after cold stimulation mice (Scale bar, 100μm and 50 μm). (D) Following 1 month HFD exposure, *Prkka1* deleted BAT was less responsive to HFD and cold stimulus in adult mice (6 h at 5 °C). (E,F) Quantification of AMP and ATP contents in progenitor cells and BATs (P10). (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; means ± s.e.m.; $n = 4$; one-way ANOVA with Tukey's *post hoc* test were used in D)

Supplemental Figure 3

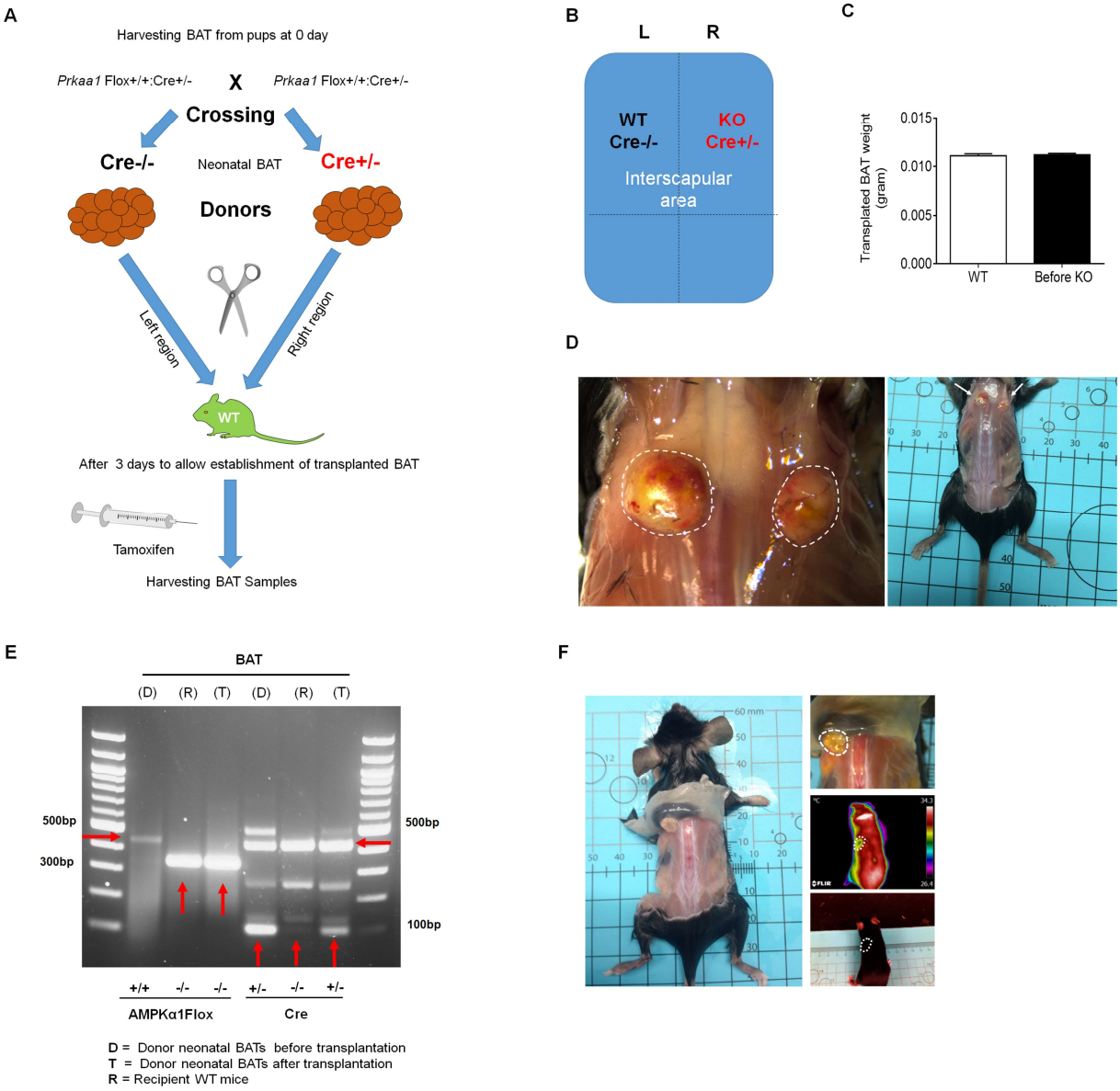

Figure S3 BAT transplantation scheme and the impact of *Prkaa1* deficiency on the function of transplanted BAT, related to Figure 1

(A,B) Scheme of BAT transplantation (A) and the location of transplants in recipient mice (B). (C) Exogenous BAT weight before transplantation. (D) After 21 days transplantation, the transplanted BAT was established in recipient mice (***R***). (E) PCR analysis confirmed the successfulness of *Prkaa*1 deletion in transplanted BAT. Donor neonatal BATs (***D***) were *Prkaa1* flox+/+:Cre+/- or Cre-/- before transplantation. BAT of the recipient mouse was *Prkaa1* flox-/-:Cre-/-. Three weeks following transplantation (***T***), *Prkaa1* was completely deleted in donor BAT (***T***) and Cre expression remained detectable. (F) At 21 day, transplanted visceral fat from WT mice showed no sign of thermogenesis. (***$P < 0.001$; Mean ± s.e.m.; $n = 3$; Student's *t* test with a two-tailed distribution)

Supplemental Figure 4

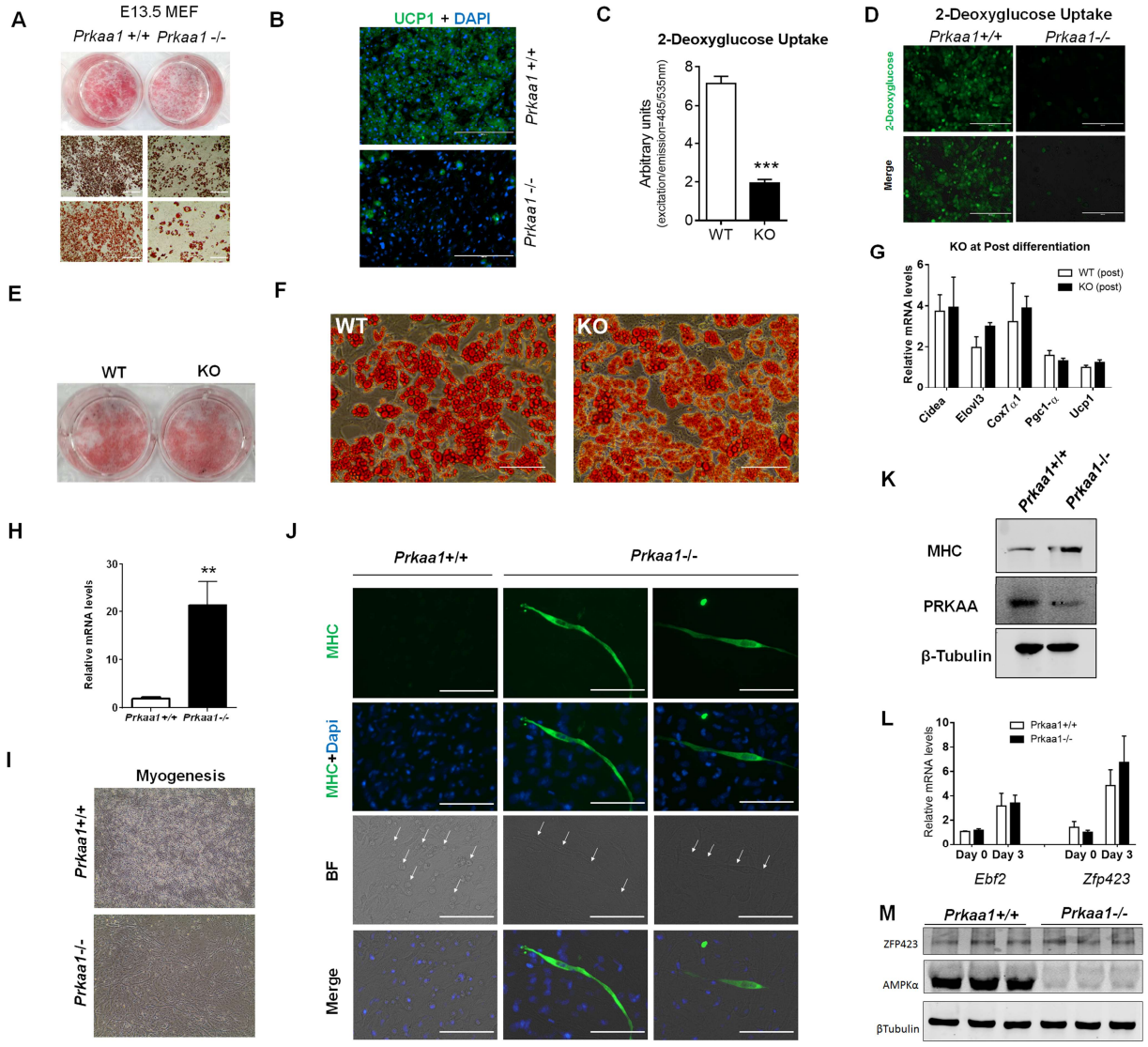

Figure S4 *Prkaa1* acute deletion suppresses brown adipogenesis in E13.5 MEFs, related to Figure 2

(A-D) Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 fetuses of *Prkaa1* conditional KO mice. Oil-red-O staining of WT and KO MEFs after 7 days of brown adipogenesis (*A*, Scale bar, 400 and 200μm); immunofluorescence images of UCP1 staining of brown adipocytes after 7 days of differentiation (*B,* Scale bar, 200μm). (C,D) 2-Deoxyglucose uptake in differentiated WT and inducible KO MEFs (*C*), and 2-Deoxyglucose concentration was correlated with green fluorescence density (*D*, Scale bar, 200μm).

Ablation of *Prkaa1* post-differentiation has no effect on the number of brown adipocytes but results in slightly different morphology of mature adipocytes.

(E,F) 4-hydrotamoxifen was added after 3 days of differentiation. Oil-Red-O staining of brown adipocytes at day 7 of differentiation (*E*) and lipid droplets were shown under microscopy (*F*, Scale bar, 100μm). (G) mRNA expression of related markers of brown adipocytes at day 7 of differentiation. ($n = 3$)

(H-K) *Prkaa1* deficiency enhances myogenesis of BAT stromal vascular cells. Stromal vascular cells separated from BAT were induced myogenesis. Expression of myogenin, a marker of myogenic differentiation, was enhanced in KO cells at day 3 of induction (*H,* $n = 4$); after 7 days of myogenic differentiation, long, tube-like cells were visible in AMPK ablated cells which was absent in WT cells (*I*); myotubes identified by immunohistochemical staining of myosin heavy chain (MHC) were present in *Prkaa1*-/- cells, but absent in WT cells (*J,* Scale bar, 100μm); MHC by immunoblotting was tested in BAT separated from E18.5 *Prkaa1*-/- fetuses compared to WT fetuses (*K*).

Prkaa1 loss has no effect on the expression of white adipogenic markers during the early stage of brown adipogenesis.

(L,M) *Ebf2* and *Zfp423* expression were examined between WT and *Prkaa1* KO during brown adipogenesis. Brown stromal vascular cells (BSVs) were prepared from *Prkaa1*$^{flox/flox}$ /ER-Cre neonatal mice, and *Prkaa1* KO was induced by 4-hydroxytamoxifen (4-HTO) after reaching confluence. (L) mRNA expression of *Ebf2* and *Zfp423* in WT and *Prkaa1*-/- cells at day 3 of brown adipogenic differentiation. (M) Immunoblotting for the expression of *Zfp423* at day 3 of brown adipogenic differentiation. Gene mRNA expression was normalization to the 18S rRNA. (***$P < 0.001$; Mean ± s.e.m.; $n = 3$; Student's *t* test with a two-tailed distribution)

Supplemental Figure 5

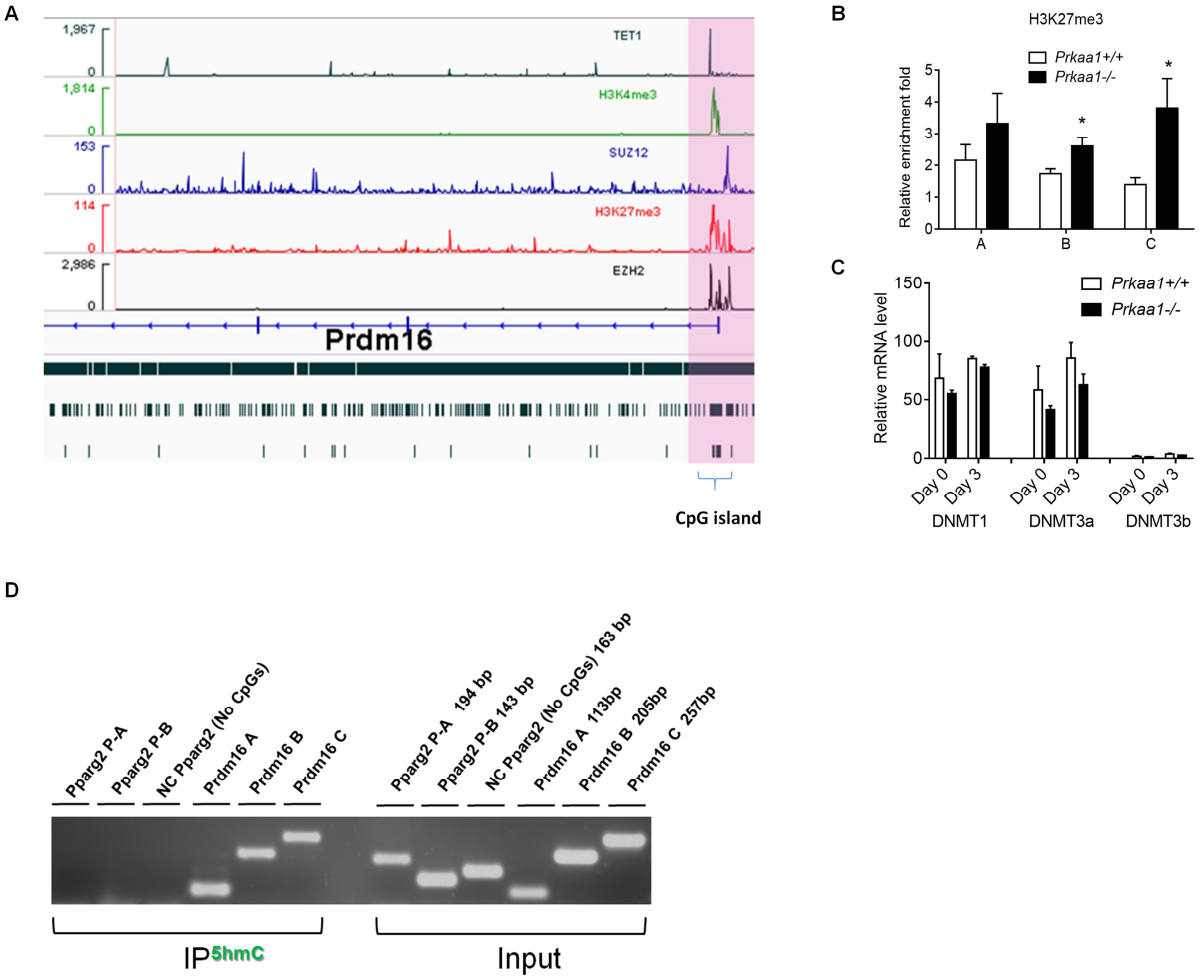

Figure S5 α-Ketoglutarate and epigenetic modifications in the *Prdm16* promoter, related to Figure 3 and 4.

(A) Genomic view of the *Prdm16* promoter based on ChIP-seq analysis by IGV (H3K27me3, H3K4me3, EZH2, and Tet1) in E13.5 MEFs. (B,C) Brown stromal vascular cells (BSVs) were separated from neonatal BAT. Enrichment of H3K27me3 histone modification in the *Prdm16* promoter of differentiated BSVs with *Prkaa1* KO at Day 3 (n = 5) (*B*); the expression of DNMT1, DNMT2 and DNMT3 before and after 3 days of brown adipogenic differentiation (*C*). (D) 5-hydroxymethylation enrichment occurred in the *Prdm16* promoter but not in the *Pparg2* promoter. 5hMedIP-PCR was performed. *Pparg2* P-A and P-B cover the *Pparg2* promoter area. NC *Pparg2* refers to a region in the *Pparg2* with no CpGs. Regions *Prdm16* A, B and C cover CpG-rich regions in the *Prdm16* promoter, where methylation occurs. Gene mRNA expression was normalization to the 18S rRNA. **P*<0.05; *P*** < 0.01; ****P* < 0.001; n = 3; means ± s.e.m.; Student's *t* test with a two-tailed distribution)

Supplemental Figure 6

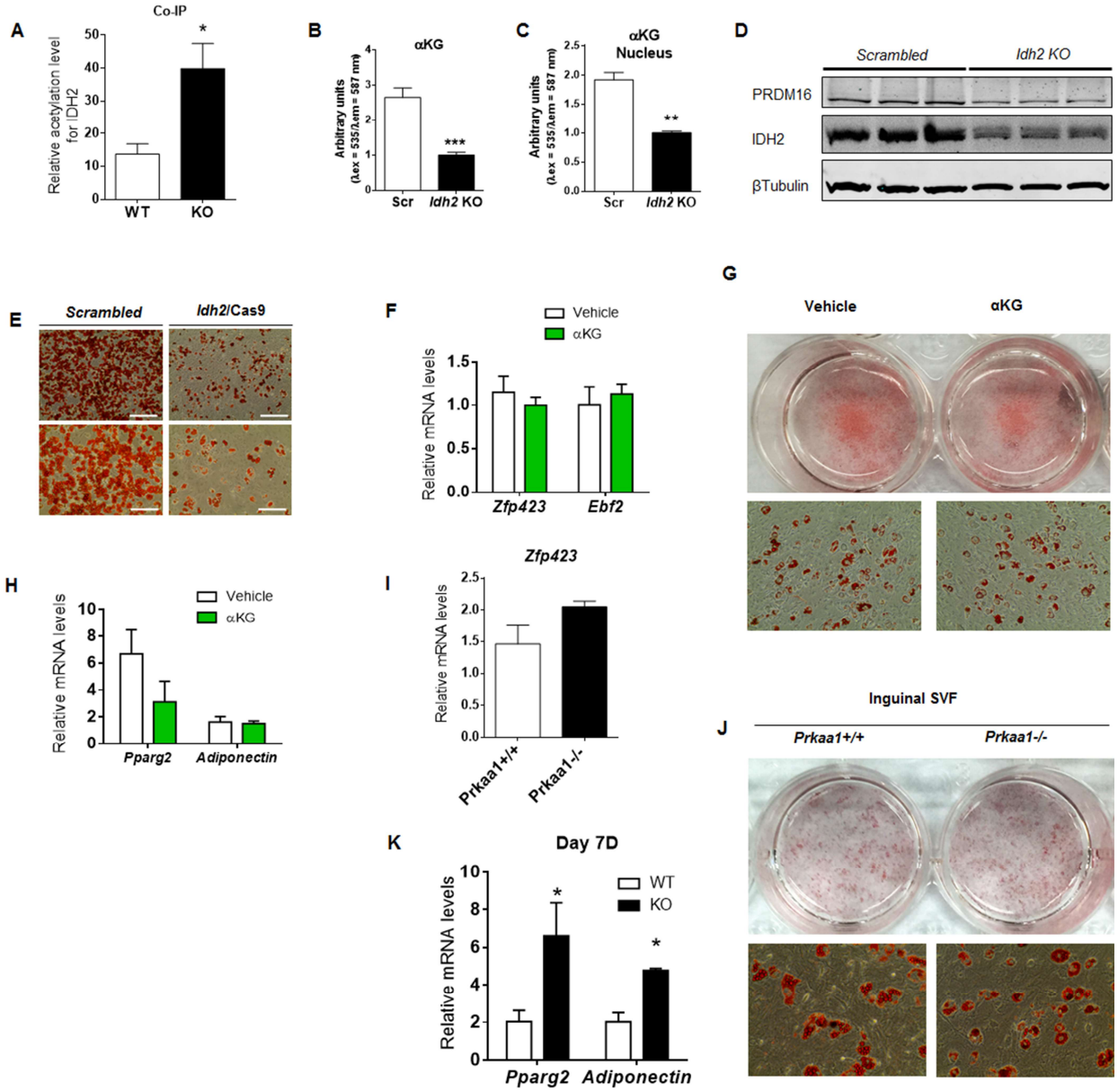

Figure S6 AMPK/aKG axis does not affect white adipogenic commitment, related Figure 4.

(A) Quantification of acetylation content of IDH2. (B-E) C3H10T1/2 cells were transfected with scrambled (Scr/Cas9) and *Idh2* CRISPR/Cas9 respectively, and then induced brown adipogenesis. Ablation of *Idh2* in C3H10T1/2 during brown adipogenesis decreases intracellular α-ketoglutarate content. Higher aKG concentration in *Scrambled* cells compared to *Idh2* ablated cells (*B*); *Idh2* KO by CRISPR/Cas9 system led to decreased α-ketoglutarate content in the nuclei (*C*); PRDM16 protein content was examined at day 3 of brown adipogenic differentiation (*D*); Oil-red-O staining of differentiated C3H10T1/2 transfected with *Scrambled* and *Idh2* KO plasmids after 7 days of brown adipogenesis (*E,* Scale bar, 400 and 200μm).

(F-H) aKG (4mM) was added during the initial 3 days of differentiation. *Ebf2* and *Zfp423* mRNA expression were examined (*F*). Oil-Red-O staining of brown adipocytes at day 7 of differentiation and lipid droplets were shown under microscopy (*G*). (H) mRNA expression of related markers of white adipocytes at day 7 of differentiation.

(I-K) lnguinal stromal vascular cells were prepared from the white adipose tissue *Prkaa1*$^{flox/flox}$ /ER-Cre mice, and *Prkaa1* KO was induced by 4-hydroxytamoxifen after reaching confluence. Then, cells were induced white adipogenesis. *Zfp423* mRNA expression at day 3 of differentiation (*I*). Oil-Red-O staining of white adipocytes at day 7 of differentiation and lipid droplets were shown under microscopy (*J*). mRNA expression of related markers of white adipocytes at day 7 of differentiation (*K*). Gene mRNA expression was normalization to the 18S rRNA. (**P*<0.05; *P*** < 0.01; ****P* < 0.001; *n* = 3; means ± s.e.m.; Student's *t* test with a two-tailed distribution)

Supplemental Figure 7

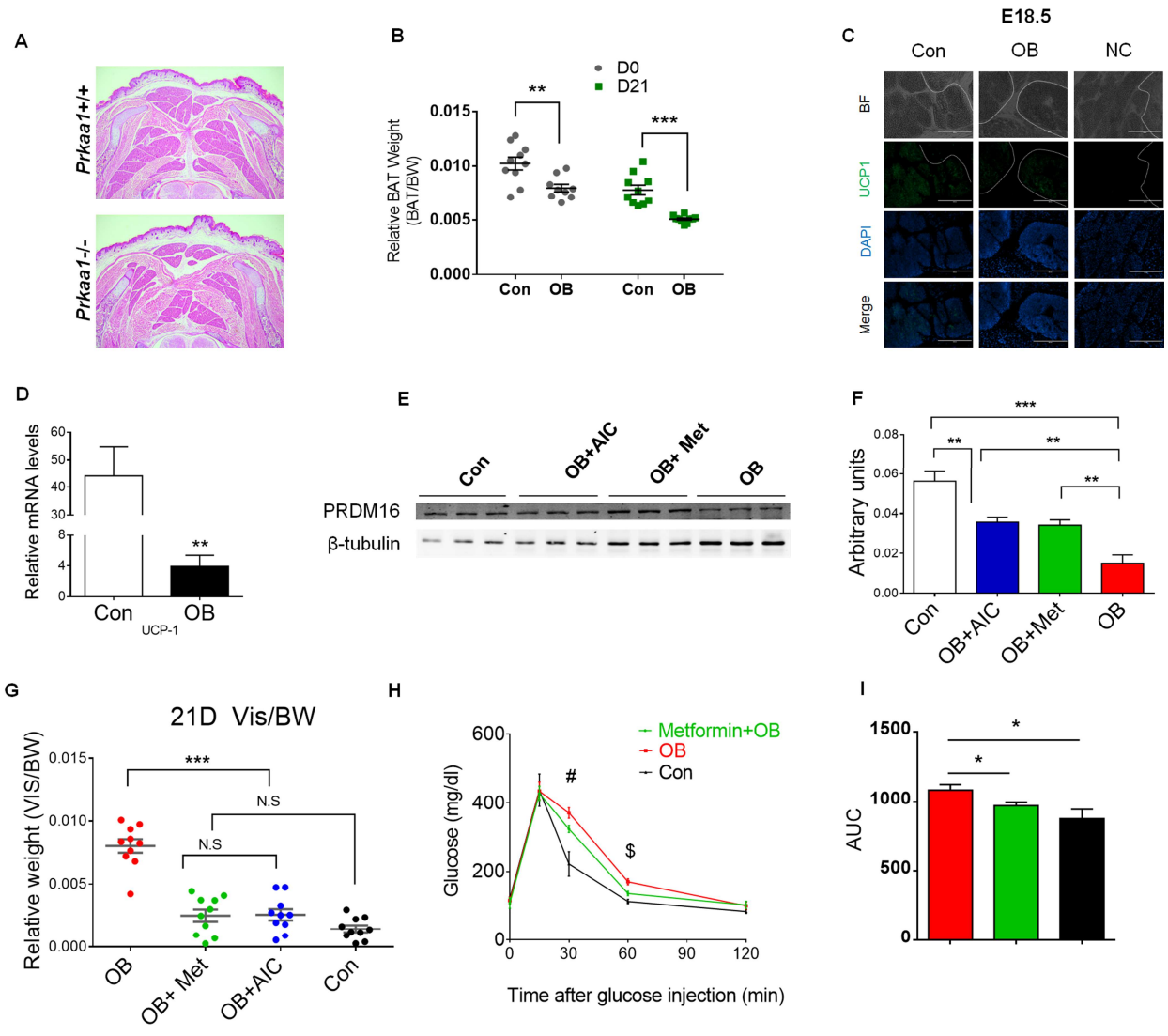

Figure S7 Brown fat weight and thermogenesis are attenuated due to maternal obesity, which is recovered by AMPK agonists, related to Figure 6 and 7.

(A-D) Brown fat development was impaired due to maternal obesity. Hematoxylin and eosin (H&E) staining of sections from the interscapular regions of E18.5 WT and *Prkaa1*-/- embryos (*A*); maternal obesity (OB) reduced the ratio of BAT weight to the body weight of offspring mice at 0 and 21 days of age (*B*, $n = 10$); immunofluorescence staining of UCP1 of Con and OB BAT at E18.5 (*C,* Scale bar, 200μm), and expression of UCP1 of BAT at E18.5 (*D*, $n = 5$ for Con, $n = 6$ for OB). (E-I) AMPKα agonists partially recover metabolic dysfunction in the offspring of obese mice. (E,F) PRDM16 expression was reduced due to maternal obesity but enhanced after AICRA and metformin treatments. BAT was separated from weaning mice treated with Con+Saline, obese (OB), OB+AICAR, and OB+metformin, and PRDM16 content was analyzed by immunoblotting (*E*) and statistic data (*F*). Visceral fat (epididymal fat) was reduced in OB mice by metformin and AICAR administration from birth to 21 days of age (*G*, $n = 10$); intraperitoneal glucose tolerance test (GTT) in female mice at postnatal 21 days (P21) of Con offspring, OB offspring, and OB offspring administered with metformin respectively (*H*, $^{\#}P < 0.01$, OB vs. OB+Metformin; $^{\$}P < 0.001$, OB vs. OB+Metformin) and under the curve areas (*I*, $n = 5$). Gene mRNA expression was normalization to the 18S rRNA. ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; mean ± s.e.m.; one-way ANOVA with Tukey's *post hoc* test was used in F and G).

Table S1, related to Experimental Procedures. Real time PCR and ChIP primers

Name		sequence
Prdm16 ChIP A	F	5'-AGAGAGAAGTGAGGTGAAGACCGAGAA-3'
	R	5'-ACACACTATCTTCATCTCCCTAGCATTGT-3'
Prdm16 ChIP B	F	5'-GCATGTGCGAAGGTGTCCAAA-3'
	R	5'-TGGATCGCATGGTGTCGGCT-3'
Prdm16 ChIP C	F	5'-CCAAAGCTTGAAGGAGAGAGACGTAAA-3'
	R	5'-TCGGTTCTTGGCCTCCAGAGAAG-3'
Pprag2 ChIP P-A	F	TCAGATGTGTGATTAGGAGTTTCAACC
	R	GTACAGTAGTTGGAATTACCAGAGCAGA
Pprag2 ChIP P-B	F	AATTCAGGCCTGATTCTTTCTGTGTTT
	R	GTCTATGTCTTGCAAAAGATTGGTTGG
Pparg2 ChIP NC	F	GAAGGAAGGAAGGAAAGAAGGATCATT
	R	AGGGGACCATCTAGGTTTGTTAGGA
Pparg2	F	AAACTCTGGGAGATTCTCCTGTT
	R	GCATCTCTGTGTCAACCATGGT
Adiponectin	F	GCCTTGTCTACAGAGTGAGTTCCAGG
	R	TGAAACTCACTCTGTAGACCAGGCTG
Ebf2	F	CACACCACACAATAATCAGGACATCA
	R	AAGAGTTGATACCCATCATACCGCTG
Zfp423	F	GTCACCAGTGCCCAGGAAGAAGAC
	R	AACATCTGGTTGCACAGTTTACACTCAT
Prdm16	F	5'-CAGCACGGTGAAGCCATTC-3'
	R	5'-GCGTGCATCCGCTTGTG-3'
IDH1	F	5'-ACATGCATATGGGGACCAATACAGA-3'
	R	5'-TTCAAAGTCATGTACCATGTATGTCACC-3'
IDH2	F	5'-GCAGTTCATCAAGGAGAAGCTCATC-3'
	R	5'-CACACTTGACAGCCACACTGTACTTCT-3'
IDH3	F	5'-TCAAGGAAGTGTTCAAGGCTGCTG-3'
	R	5'-GATGGCAACTTTGTTCTCCTTCATG -3'
Tet1	F	5'-CACCCTGTGACTGTGATGGAGGTA-3'
	R	5'-ACTATCTTCTCAATCCGGATTGCCTT-3'
Tet2	F	5'-AAGGATGCAATCCAGACAAAGATGAA-3'
	R	5'-TTTAGCAATAGGACATCCCTGAGAGCT-3'
Tet3	F	5'-GAACTCATGGAGGATCGGTATGGA-3'
	R	5'-CAGCTTCTCCTCCAGTGTGTGTCTT-3'
UCP1	F	5'-ACTGCCACACCTCCAGTCATT -3'
	R	5'-CTTTGCCTCACTCAGGATTGG-3'
Elovl3	F	5'-TCCGCGTTCTCATGTAGGTCT-3'
	R	5'-GGACCTGATCCAACCCTATGA-3'
18s	F	5'-GTAACCCGTTGAACCCCATT-3'
	R	5'-CCATCCAATCGGTAGTAGCG-3'

Fabp4	F	5'-CGACAGGAAGGTGAAGAGCATCATA-3'
	R	5'-CATAAACTCTTGTGGAAGTCACGCCT-3'
Ppargc1a	F	5'-CCATACACAACCGCAGTCGC-3'
	R	5'-GTGGGAGGAGTTAGGCCTGC-3'
Cox7a	F	5'-CAGCGTCATGGTCAGTCTGT-3'
	R	5'-AGAAAACCGTGTGGCAGAGA-3'
Cidea	F	5'-ATCACAACTGGCCTGGTTACG-3'
	R	5'-TACTACCCGGTGTCCATTTCT-3'
Glut4	F	5'-CTAGGCATCAATGCTGTTTTCTA-3'
	R	5'-CGAGACCAACGTGAAGACCGTATT-3'
	R	5'-: TGGTACTGTGCGTATTTAGCGATGGT -3'
Dnmt1	F	5'-CCGAGGCCTTTACTTTCAACATCA-3'
	R	5'-TCTCTTTTATCCGACCGATGCGATA-3'
Dnmt3a	F	5'-ATGGCAAGTTCTCAGTGGTGTGTGT-3'
	R	5'-CTGGAGGACTTCGTAGATGGCTTTG-3'
Dnmt3b	F	5'-GGAGACAGCACAGAGTATCAGGATG-3'
	R	5'-AGAAAACTTGCCATCACCAAACC-3'
AMPKα2 flox F	F	5'-GCTTAGCACGTTACCCTGGATGG-3'
AMPKα2 flox R	R	5'-GTTATCAGCCCAACTAATTACAC-3'
AMPKα1 KO		
Primer IRES Fa2	F	5'-GGGCTGCAGGAATTCGATATCAAGC-3'
Primer 3.0 Ra1	R	5'-CCTTCCTGAAATGACTTCTGGTGC-3'
Primer alpha WT	F	5'-AGCCGACTTTGGTAAGGATG-3'
Primer alpha WT	R	5'-CCCACTTTCCATTTTCTCCA-3'
Cre Primer		
oIMR1084		5'-GCGGTCTGGCAGTAAAAACTATC-3'
oIMR1085		5'-GTGAAACAGCATTGCTGTCACTT-3'
oIMR3621		5'-CGT GAT CTG CAA CTC CAG TC-3'
oIMR8546		5'-GGA GCG GGA GAA ATG GAT ATG-3'
AMPKα1flox		
primer-11528		5'-CCCACCATCACTCCATCTCT-3'
primer-11529		5'-AGCCTGCTTGGCACACTT AT-3'
MHC(Myosin heavy chain)	F	5'-TCCAAACCGTCTCTGCACTGTT-3'
	R	5'- AGCGTACAAAGTGTGGGTGTGT -3'

Supplemental Experimental Procedures

Animal Studies

To obtain obese mice, female C57BL/6 mice at 4 weeks of age were randomly separated into two groups and fed *ad libitum* either a control diet (Con, 10% energy from fat, D12450H, Research Diets, New Brunswick, NJ) or an obesogenic diet (OB, 45% energy from fat, D12451, Research Diets) for 8 weeks. Mice were housed in environmentally controlled rooms on 12 h-light/12 h-dark cycles. Females were mated with males maintained on a regular chow. During pregnancy and lactation, maternal mice were maintained on their respective diets before mating. Pups were weaned at postnatal 21 days (P21) and female mice were used for further studies. The constitutive *Prkaa1* and *Prkaa1* knockout (KO), and *Prkaa1*$^{flox/flox}$ and *Prkaa2*$^{flox/flox}$ mice were obtained as previously reported (Jorgensen et al., 2004). To achieve *Prkaa1* conditional KO, Rosa-Cre mice (Stock No: 004847, Jackson Lab, Bar Harbor, Maine) were crossed with *Prkaa1*$^{flox/flox}$ (Stock no: 014141) to obtain Rosa^{Cre}/*Prkaa1*. In these mice, AMPK KO was induced by tamoxifen injection (75 μg/g body weight). For cold stimulation, mice were housed at either 5 °C or 22 °C with 12-h light/12-h dark cycles. For administration of AMPK agonists or antagonists, newborn C57BL/6J mice were injected intraperitoneally with metformin (250 mg/kg body weight), AICAR (500 mg/kg), or saline for 15 consecutive days (up to P15).

Brown fat transplantation

BAT used for transplantation were removed from the interscapular region of neonatal mice, and put in pre-warmed PBS before transplantation. Then, 3 week-old C57BL/6 recipient mice were anesthetized by isoflurane inhalation. About 10 mg donor BAT from *Prkaa1* $^{flox/flox}$:*Cre*-/- mice and *Prkaa1* $^{flox/flox}$:*Cre*$^{+/-}$ mice were carefully transplanted into the left and right subcutaneous region, adjacent to the endogenous interscapular fat pads of recipient mice, respectively. Three days after transplantation, recipient mice were injected with tamoxifen daily for four days to induce *Prkaa1* KO in transplanted *Prkaa1* $^{flox/flox}$:*Cre*$^{+/-}$ BAT.

Cell line and primary cell culture

Primary mouse embryonic fibroblasts (MEFs) were isolated from embryos of wild-type mice at E13.5. Briefly, BAT was dissected from neonatal mice, washed with PBS and minced, which was digested in a medium containing 0.75 units/mL collagenase D (Roche, Pleasanton, CA) for 45 min in a shaking incubator at 37°C. The lysate was filtered sequentially through 100 and 40 μm strainers. Cells were collected by centrifugation at 400 x g for 5 min and seeded on plates.

For induction of *Prkaa1* KO, post-confluenced brown precursors were treated with 250 nM 4-hydroxytamoxifen (Sigma, St Louis, MO) for 3 days in F12 medium. The brown adipogenic differentiation was induced using a medium containing 15% FBS, 1 mg/mL insulin, 1 μM dexamethasone, 0.5 mM isobutyl-1-methylxanthine, 125 nM indomethacin and 1 nM T_3. Dimethyl 2-oxoglutarate and diethyl 2-oxoglutarate were purchased from Sigma-aldrich (Sigma, St Louis, MO), and diethyl 2-hydroxyglutarate was purchased from ARK Pharm Inc (Libertyville, IL), which were used for treating cells.

Plasmid Transfection

Plasmid pAMPKα1 (catalog no. 27297), vector (catalog no. 13031) and p*Prdm16* (catalog no. 15503) were obtained from Addgene (Cambridge, MA). Plasmid transfection was performed using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions. CRISPR/Cas9 plasmids were constructed by Genscript (Piscataway, NJ) (gRNA info: *Prkaa1* sequence: GTGGGCAAGCACGAGTTGAC; *Idh2*: ATGTTCCGGATCGTTCCGTT; scrambled sequence: AGTCATGGAACGCGTAGCGG).

Flow Cytometry

Primary BSVs were freshly prepared and blocked in anti-mouse CD16/CD32 antibody (101320, Biolegend, San Diego, CA) and then stained with anti-mouse CD45 (103111, Biolegend) and anti-mouse Sca-1 (45-5981, eBioscience). Brown Sca-1^+/$CD45^-$/Lin- progenitor cells were sorted on FACSaria (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (Treestar Inc., San Carlos, CA). Gates were made based on fluorescence minus one (FMO) control.

RNA extraction and Real-time PCR

Total RNA was isolated by TRIzol (Invitrogen, Grand Island, NY) followed by purification using Qiagen RNA columns (Qiagen, Valencia, CA). Extracted mRNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and used for real-time quantitative PCR. Relative expression was determined by real-time PCR (Bio-Rad IQ5) using iQ™ SYBR® Green Supermix (Bio-Rad). Relative expression of target genes was normalization to the 18S rRNA gene (Maunakea et al., 2010). Primer sequences and the amplicon sizes are listed in **Table S1**.

Nuclear extraction and TET activity assay

NE-PER™ Nuclear and Cytoplasmic Extraction Reagents was used for isolating nuclei (Thermo Scientific, Waltham, MA). TET activity was analyzed using Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit purchased from EpiGenTEK (Farmingdale, NY).

Immunoblotting analyses

Protein was isolated from cultured cells or tissues, and protein concentration of lysates was determined by the Bradford method (Bio-Rad). Primary antibodies including total AMPK (2532), p-AMPK (2535), pyruvate dehydrogenase (2784), cytochrome C (11940), β-tubulin were purchased from Cell Signaling Technology (Danvers, MA). The FABP4 (sc-18661) and UCP1 (sc-28766) antibodies were purchased from Santa Cruz Biotechnology. Antibodies against PRKAA1 (bs-1115r) and PRKAA2 (bs-2771r) were purchased from Antibodies-Online Inc. (Atlanta, GA). PRDM16 (PA5-20872) antibody was from Pierce (Thermo Scientific, Waltham, MA). RDye 800CW goat anti-rabbit secondary antibody (1:10,000) and IRDye 680 goat anti-mouse secondary antibody (1:20,000) were purchased from LI-COR Biosciences (Lincoln, NE). The target proteins were detected using an Odyssey infrared imaging systerm (LI-COR Biosiences).

Chromatin immunoprecipitation (ChIP)-PCR assay

Cells were trypsinized and homogenized in 1% formaldehyde and incubated for 10 min. After centrifugation, the pellet was washed two times using PBS. After washing, the pellet was resuspended in PBS containing 125 mmol/L glycine, then lysed in a cold lysis buffer (1% SDS, 10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L, NaCl, 3 mmol/L MgCl2, and 0.5% NP-40) containing 1/100 volume protease inhibitor cocktail (Thermo Fisher Scientific), sonicated, and centrifuged. The supernatant was precleaned with pre-blocked ChIP-grade Pierce™ magnetic protein A/G (Thermo Scientific, Waltham, MA) and then rotating with an antibody against H3K27me3 (9733), or normal rabbit IgG (2729) (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Then, the antibody-chromatin complex was precipitated with magnetic beads, further treated with RNaseA and then proteinase K for 2 h. Immunoprecipitated and input DNA were purified with ChIP DNA Clean & Concentrator (Zymo Research, Irvine, CA) and used as templates for PCR using the primers listed in **Table S1**. Relative enrichment folds of detected proteins were determined after normalization to input (Harms et al., 2014; Harms et al., 2015).

Hydroxymethyl-DNA and methylcytosine immunoprecipitation

Genomic DNA was isolated from cultured cells using the DNeasy® Blood & Tissue kit (Qiagen). DNA (10 µg) was diluted in 300 µl TE buffer and sonicated. DNA was denatured for 10 min at 95°C and immediately cooled on the ice. Then 5× IP buffer (50 mM Na-phosphate, pH 7; 0.7 M NaCl, 0.25% Triton X-100) was diluted with the denatured DNA solution to 1×concentration. 5-Hydroxymethylcytosine (A4001) or 5-methylcytosine antibody (A3001) (Zymo

Research, Irvine, CA) was added into denatured DNA with rotation overnight at 4°C. The DNA-antibody complex was pulled down with pre-blocked Pierce™ magnetic protein A/G (Thermo Scientific, Waltham, MA). The captured beads were washed three times with 1× IP buffer and re-suspended in 250 µl digestion buffer (50 mM Tris HCl, pH 8; 10 mM EDTA; 0.5% SDS). The solution containing DNA was digested by proteinase K and RNAase. DNA was purified using ChIP DNA Clean & Concentrator (Zymo Research, Irvine, CA) and immunoprecipitated DNA was used for real-time PCR (Bio-Rad IQ5) using Qprecise Green® (Earthox, Millbrae, CA). Relative enrichment folds of detected regions were determined after normalization to input (Blaschke et al., 2013) and primers listed in **Table S1**.

HPLC analysis

ATP and AMP were analyzed by Shimadzu HPLC series (Shimadzu, Japan) equipped with CBM-20A communications Bus Modules, DGU-20A 5R degasser, SIL-20ACHT auto-sampler, LC-20AD UFLC binary gradient pumps, CTO-20A column oven, and Nexera X2 Diode Array Detector (DAD). The sample separation was performed using a Luna C18 (2) column (150 x 3 mm i.d.; 3 µm particle size) (Phenomenex Inc, Torrance, CA, USA). The mobile phase consisted of 60 mM K_2HPO_4 and 40mM KH_2PO_4 (elute A) and methanol (elute B). The contents of ATP and AMP in samples were calculated with corresponding calibration curves by peak areas.

Metabolomic analyses of BAT and BAT derived stem cells

Cells or BAT were suspended in 300 µl extraction solvent (methanol-chloroform-water, 5:2:2) and homogenized. After adding 700 µl extraction solvent (as above) and 1.5 µg of the surrogate standard ribitol, the extract was centrifuged, and the supernatant was dried and suspended in 10 µl *O*-methoxylamine hydrochloride (20 mg ml^{-1} in pyridine, both from Sigma). Subsequently, samples were derivatized with 45 µl of MSTFA with 1% TMCS (Thermo-Pierce cat.-no.TS-48915). Gas chromatography-mass spectroscopy analysis was performed using a Pegasus 4D time-of-flight mass spectrometer (LECO) equipped with a Gerstel MPS2 autosampler and an Agilent 7890A oven. The derivatization products were separated on a 30 m, 0.25 mm i.d., 0.25 µm df Rxi-5Sil® column (Restek) with an IntegraGuard® pre-column using ultrapure He at a constant flow of 1ml min^{-1} as carrier gas. The linear thermal gradient started with a one-minute hold at 50 °C, followed by a ramp to 330 °C at 20 °C min^{-1}. The final temperature was held for 5 min prior to returning to initial conditions. Mass spectra were collected at 17 spectra s^{-1}. Peak identification was conducted using Fiehn primary metabolite library (Kind et al., 2009). The identities of αKG, citric, fumaric, and malic acids were additionally confirmed using authentic standards (all from Sigma). Peak alignment and relative quantification were carried out using the Statistical Compare feature of the ChromaTOF software (LECO) and the surrogate standard ribitol was used for normalization. Absolute quantification was based on standard curves generated using authentic standards. The technical error of the extraction procedure was tested with a set of cells from identical tissue and was <10% RSD.

Immunocytochemical staining

Cells grown on coverslips or multiple-well plates were incubated in cold methanol for 10 min, and permeabilized with PBS containing 0.25% Trition X-100 for 10 min. After blocking 1 h with 1% BSA, cells were incubated with 5hmC (1:200) (Zymo Research, Irvine, CA) or UCP1 antibody (1:200) (Santa Cruz, CA, sc-28766) at 4°C overnight. After washing for 3 times, fluorescent secondary antibody (1:1,000) was then added for 1 h. Fluorescence was examined by using an EVOS® Cell Imaging Systems (Mill Creek, WA).

Supplemental References

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