Supplemental Information

Rictor/mTORC2 Loss in the *Myf5*-lineage Reprograms Brown Fat Metabolism and Protects Mice against Obesity and Metabolic Disease

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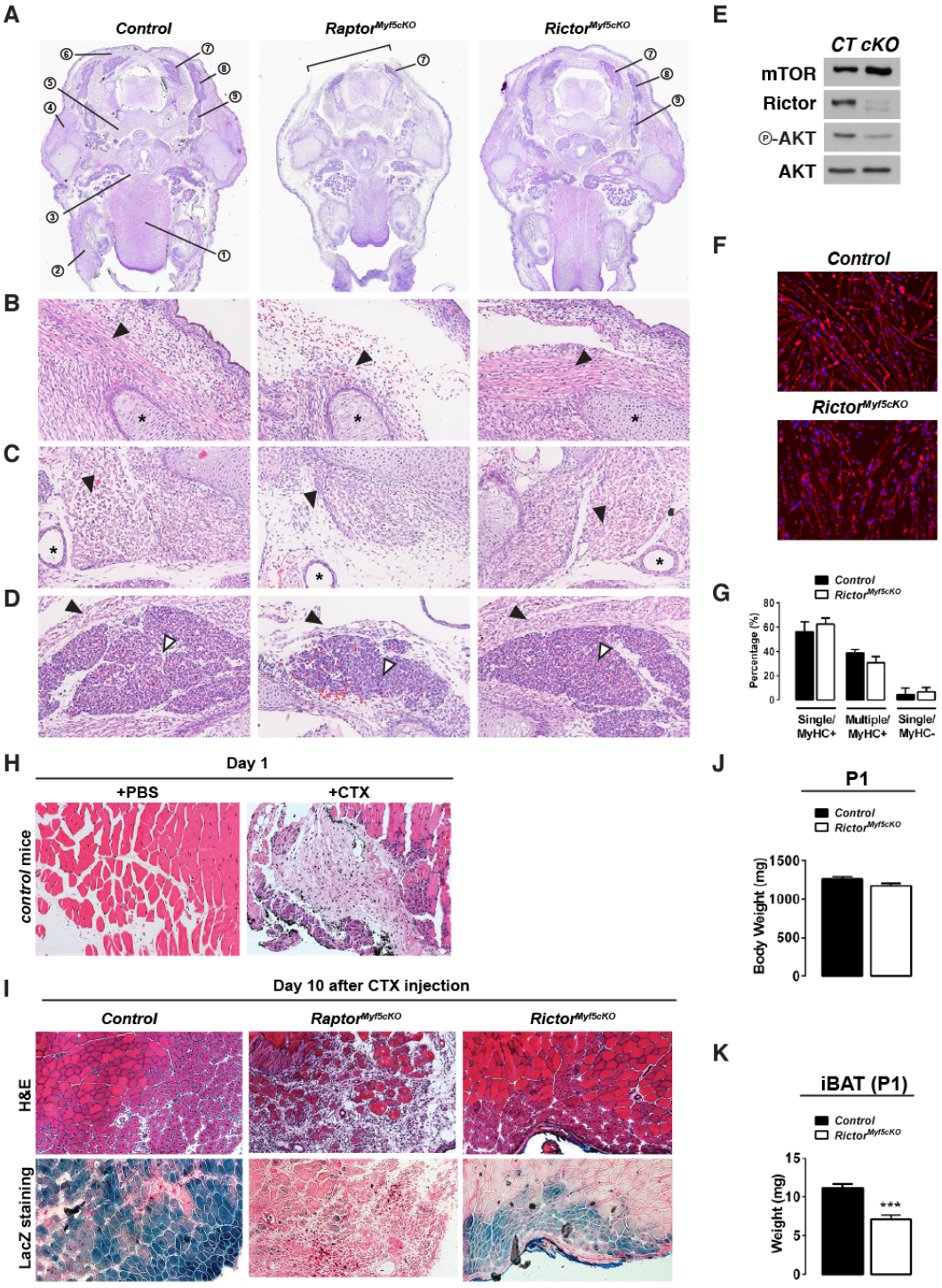


Figure S1, Related to Figure 1

(A) Transverse sections of E16.5 embryos. Tongue (1), masseter (2), sternohyoid and hyoglossus (3), supraspinatus (4), prevertebral (5), and trapezius muscles (7), and cervical BAT (7), interscapular BAT (8), and subscapular BAT precursors (9) are indicated. Bracket

marks region of hind neck fragility. **(B)** Enlarged image of supraspinatus muscle (arrowhead). Ossifying cartilage of the scapula marked with (*). **(C)** Enlarged image of prevertebral muscles of the neck (arrowhead). Carotid artery marked with (*). **(D)** Enlarged image of trapezius muscle (closed arrowhead) and cervical BAT precursors (open arrowhead). **(E)** Western blots of satellite cell lysates from control (*CT*) and *Rictor*^{Myf5cKO} conditional knockout (*cKO*) mice. **(F)** Differentiated satellite cells stained with myosin heavy chain antibody. **(G)** Quantification of nuclei number in individual differentiated satellite cells. **(H)** H&E images of tibialis anterior (TA) muscle 1 day after PBS or cardiotoxin injection in control mice (see also supplementary methods). **(I)** Mice deleted for *Raptor* or *Rictor* specifically in satellite cells with *Pax7*-CreER were subjected to an acute cardiotoxin injury assay. Mice also carried the *Rosa26-LacZ* reporter to follow the deleted cells. H&E images and corresponding images for LacZ staining of TA muscle 10 days after cardiotoxin injection. Regenerated muscle cells in the control and *Rictor* KO are indicated by the centrally localized nuclei in H&E stained sections. No regenerated cells are detectable in the *Raptor* KO. **(J-K)** Total body weight (J) and average iBAT weight (K) at postnatal day 1 (n=6; bars represent mean ± SEM; t-test; ***$p<0.001$).

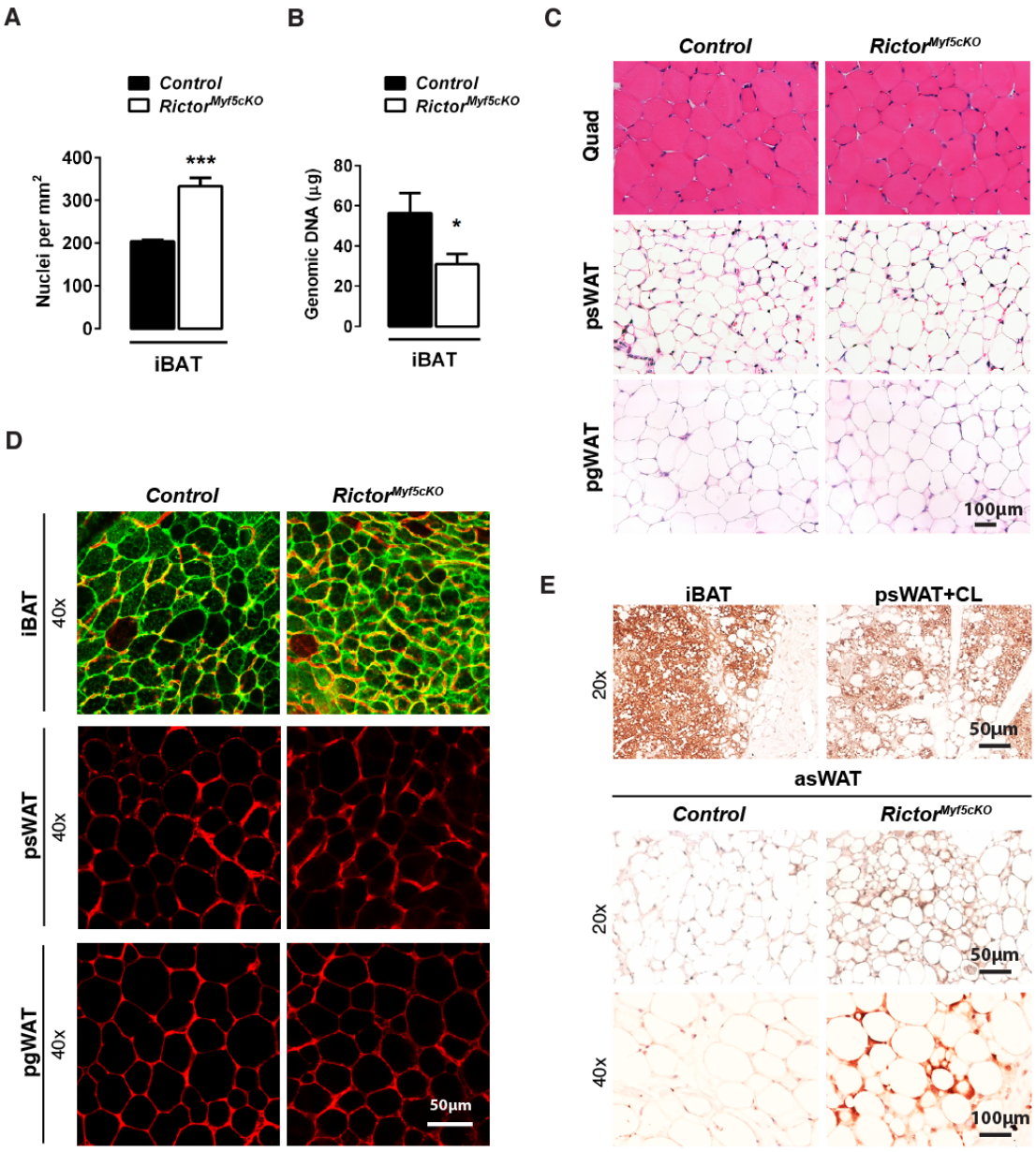


Figure S2, related to Figure 2.

(A) Nuclei density per mm^2 of iBAT (6-wks) (n=4; bars represent mean ± SEM; t-test; ***p<0.001). **(B)** Qunatification of genomic DNA from iBAT (6-wks) (n=8; bars represent mean ± SEM; t-test; *p<0.05). **(C)** H&E stains (40x) of the quadricep (Quad) muscle and posterior subcutaneous (psWAT) and perigonadal (pgWAT) white adipose tissue (6-wks). **(D)** Representative images of mTFP and mGFP labeled iBAT, psWAT and pgWAT adipocytes. Note adipocytes are homogenously mGFP$^+$ and smaller in the iBAT consistent with homogeneous *Rictor* loss in this tissue. **(E)** *Top*—UCP1 immunohistochemistry stains of iBAT and CL-316243 treated psWAT (20x). *Botton*—asWAT (20x and 40x) from control and *Rictor^{Myf5cKO}* mice.

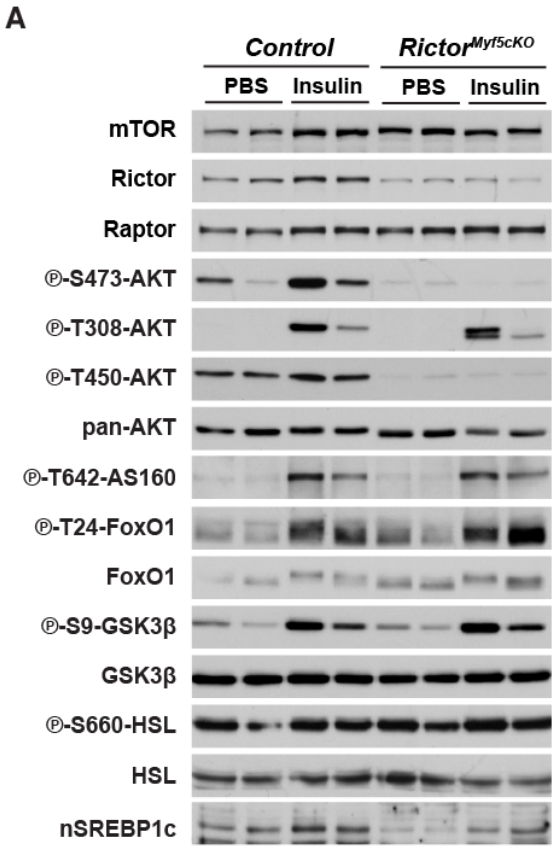
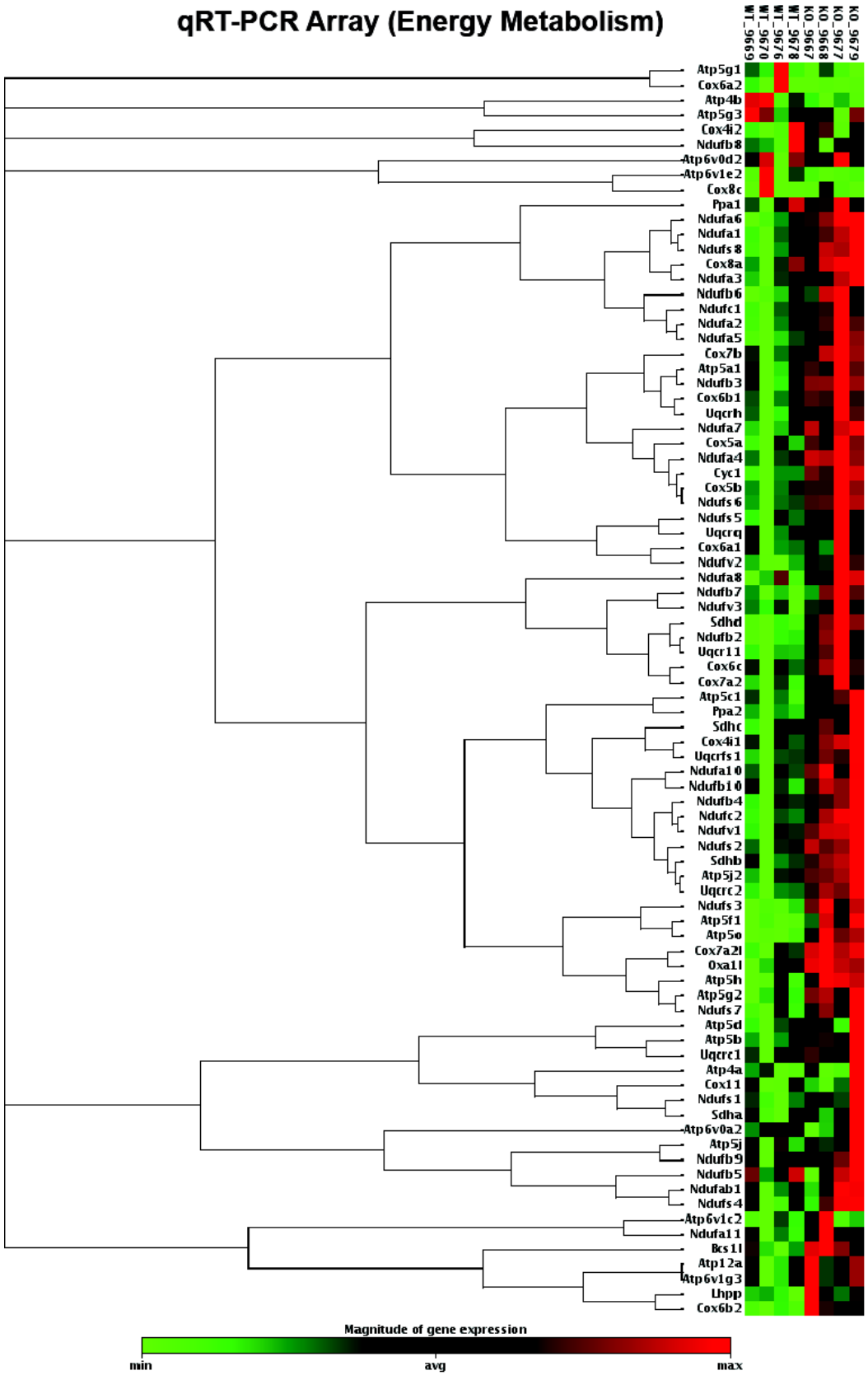


Figure S3, related to Figure 3.

(A) Western blots of the indicated total and phospho-proteins using lystates prepared from the iBAT of 8-week-old mice. Overnight fasted mice were *i.p*. injected with PBS or 150U/Kg insulin and tissues were collected 15 minutes post injection.

A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Cox6a2
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Atp5g3
Cox4i2
Ndufb8
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Atp6v1e2
Cox8c
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Magnitude of gene expression
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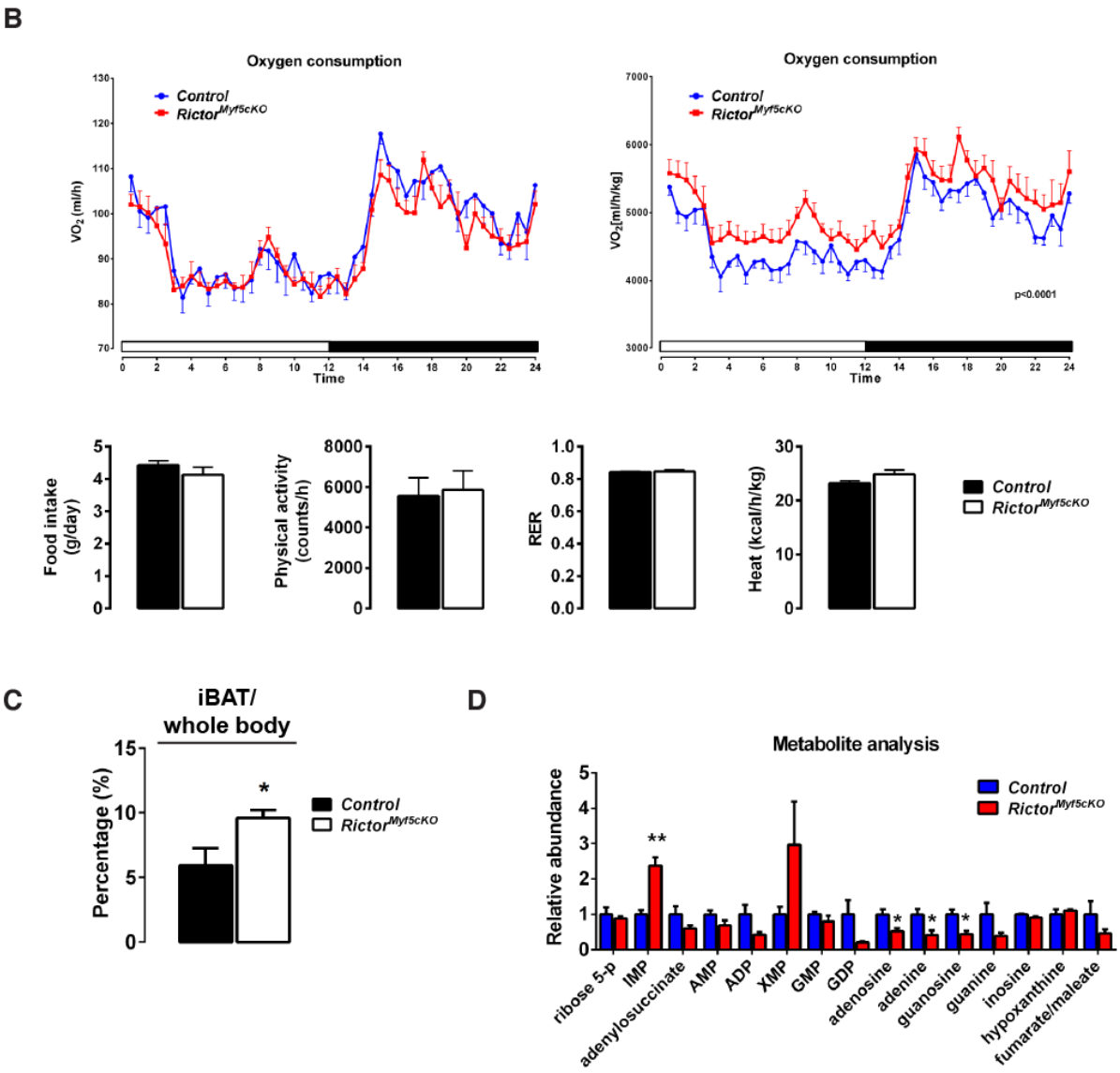

Figure S4, related to Figure 4.

(A) Clustering heat map for mitochondrial genes involved in energy metabolism qRT-PCR array (n=4). **(B)** Metabolic cage analysis of 6wk-old mice under normal housing temperature (22°C): *Top Left*—whole body oxygen consumption; *Top right*—whole body oxygen consumption normalized to body weight; *Botton*—food intake, physical activity, respiratory exchange ratio (RER) and energy expenditure. (n=6) **(C)** Glucose uptake by ^{18}FDG PET-CT (n=6; bars represent mean ± SEM; t-test; *p<0.05). **(D)** Metabolite profiling was performed on 6-week control and *Rictor^{Myf5cKO}* iBAT. Note the high levels of IMP, a deamination product of AMP. AMP is formed by the adenylate kinase reaction, which produces ATP (2ADP = AMP + ATP). During metabolic stress or following treatment with chemical uncouplers, AMP is deaminated to IMP to ensure ongoing adenylate kinase activity and ATP production in order to maintain energy balance (Balcke et al., 2011).

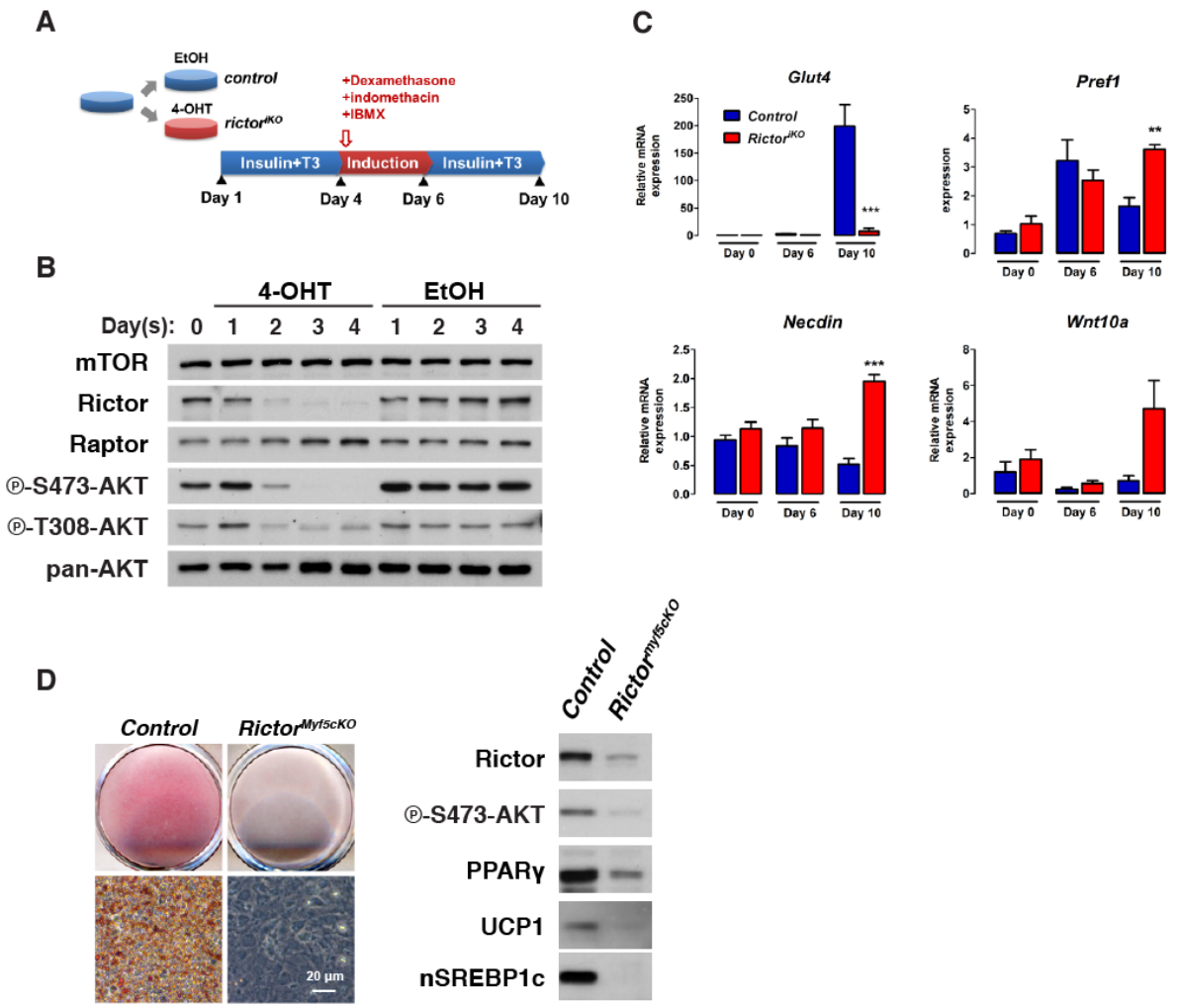

Figure S5, related to Figure 5.

(A) Inducible knockout differentiation protocol for comparing *Rictor*^{iKO} to isogenic control cells. Brown adipocyte precursors (bAPCs) were split from the same original dish into two dishes, one of which received vehicle (EtOH), the other 4-hydroxy-tamoxifem (4-OHT). After 3 days of treatment to induce deletion, cells were passed one time and then differentiated according to a standard 10-day brown adipocyte induction protocol (described in Experimental Procedures). **(B)** Western immunoblots showing time course following induced *Rictor* deletion in bAPCs with 4-OHT compared to vehicle (EtOH) treated isogenic controls. **(C)** qRT-PCR of mRNA levels for the indicated differentiation-related genes (n=3; bars represent mean ± SEM; t-test; *p<0.05, ***p<0.001). **(D)** *Left*—Oil Red O staining of control and *Rictor*^{Myf5cKO} bAPCs after differentiation. *Right*—Western immunoblots showing indicated differentiation markers.

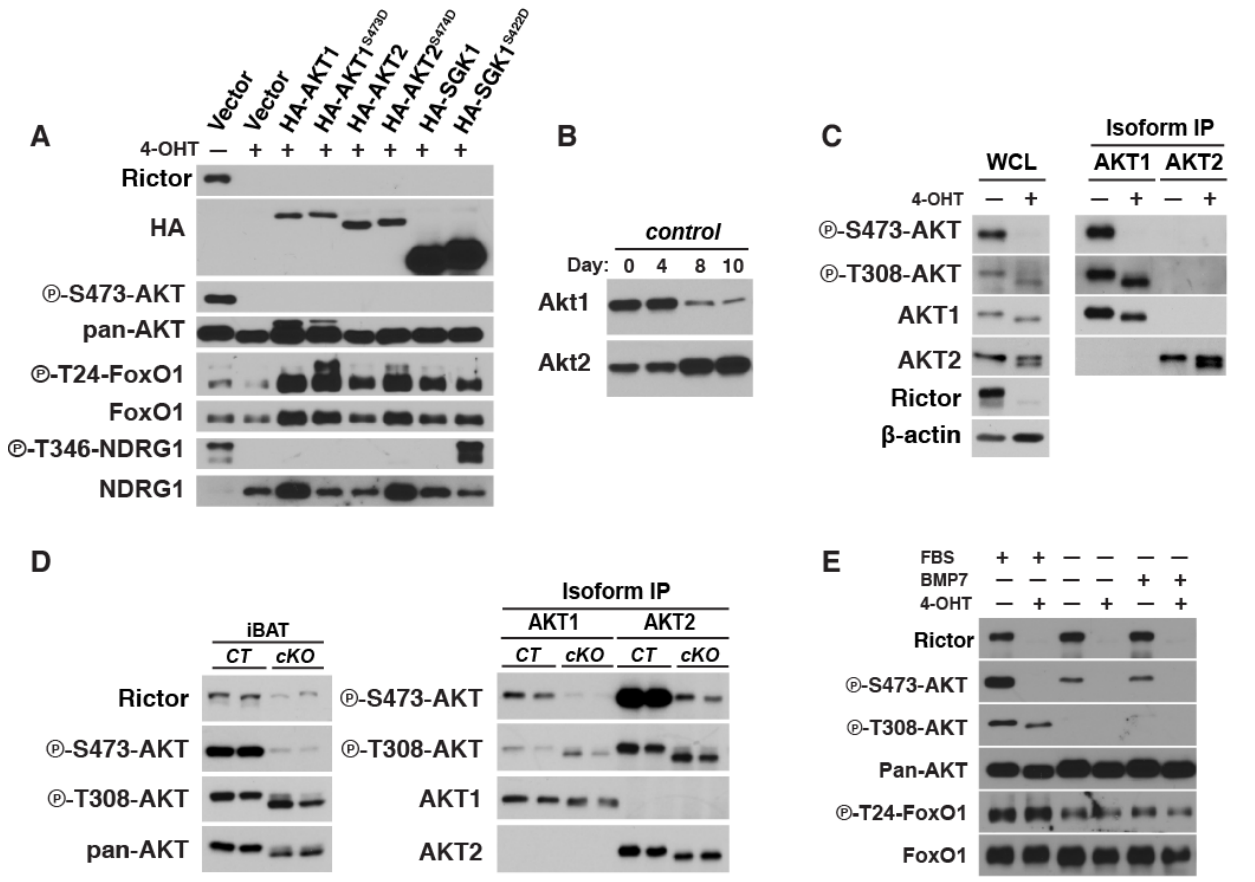

Figure S6, related to Figure 6.

(A) Western immunoblots of undifferentiated control and *Rictor*^{iKO} bAPCs stably expressing the indicated recombinant constructs. Cells were treated with fresh culture media before harvesting. **(B)** Western immunoblots of AKT1 and AKT2 protein expression at the indicated days during differentiation of wild type bAPCs. **(C)** Western immunoblots of lysates generated from AKT isoform-specific immunoprecipitation experiments using control or *Rictor*^{iKO} undifferentiated bAPCs. Immunoblots of the whole cell lystates (WCL) are shown to the left. **(D)** Western immunoblots of lysates generated from AKT isoform-specific immunoprecipitation experiments using iBAT dissected from 6-week-old control and *Rictor*^{Myf5cKO} mice. **(E)** Western immunoblots of lysates prepared from undifferentiated control and *Rictor*^{iKO} cells. Cells were serum deprived for 3 hours, then stimulated with FBS or BMP7 (3.2nM) for 15 minutes.

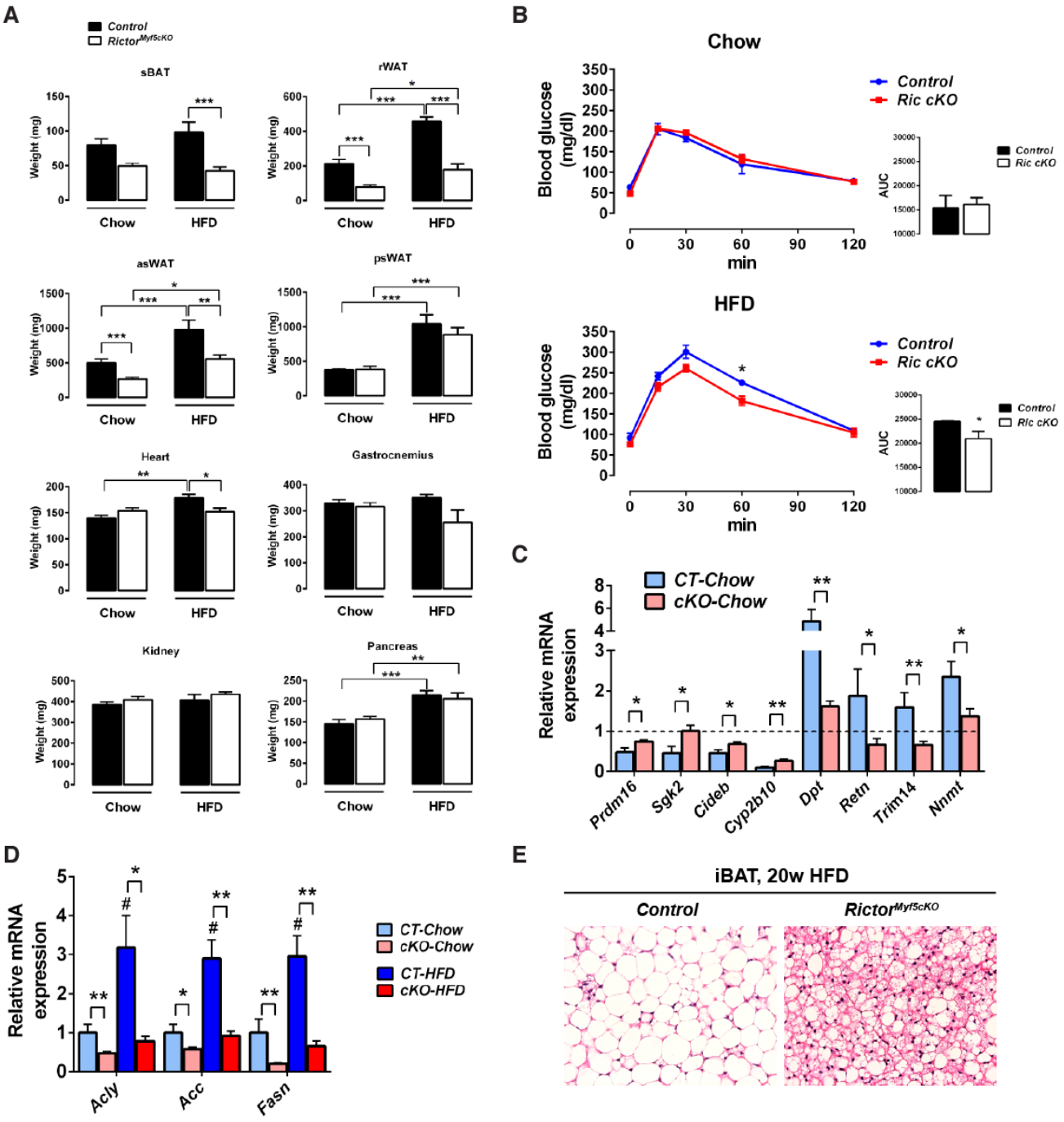

Figure S7, related to Figure 7.

(A) Mass (mg) of the indicated tissues collected from control and *Rictor*^{Myf5cKO} mice living at thermoneutrality (30°C) following 12-weeks of eating chow or HFD. (n=8 for control and n=12 for KO in chow; n=10 for both genotypes in HFD; bars represent mean ± SEM; two-way ANOVA; *p<0.05, ***p<0.001) **(B)** Glucose tolerance test of control and *Rictor*^{Myf5cKO} mice on chow (top) or HFD (bottom) living at thermoneutrality. The test was performed

during the 11th week of the 12-week experiment. **(C)** qRT-PCR of the indicated brown and white fat genes in iBAT from control and *Rictor*^{Myf5cKO} mice eating chow diet and living at thermoneutrality (n=8 for control and n=12 for KO in chow; bars represent mean ± SEM; two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001). The expression level of each gene is normalized to the corresponding gene level in iBAT from age-matched control mice eating chow but living at the standard housing temperature (22°C). **(D)** qRT-PCR of the indicated lipogenesis genes in iBAT from chow or HFD mice (n=8 for control and n=12 for KO in chow; n=10 for both genotypes in HFD; bars represent mean ± SEM; two-way *ANOVA*; *p<0.05, **p<0.01, ***p<0.001; # indicates significant difference over the control chow group). **(E)** Representative H&E images (n=4) of control and KO mice fed with HFD at thermoneutrality for 20 weeks.

Supplemental Experimental Procedures

Antibodies and reagents

Rictor (Cat# 2140), mTOR (2983), pan-AKT (9272), GSK3β (9315), ACC (3676), ACLY (4332), NDRG1 (9408) and β-actin (4970), Insulin receptor β (3025) and anti-HA-tag (2367) antibodies were purchased from Cell Signaling Technologies. SREBP1 (sc-366), p70 S6K (sc-9027), UCP1 antibody (sc-6528), and HRP-conjugated secondary antibodies were from Santa Cruz biotechnology. All phosphorylation-specific antibodies: S473-AKT (4058), T308-AKT (4056), T24-FoxO1 (9464), S9-GSK3β (9323), T1462-TSC2 (3617) and T389-S6K (9234) were from Cell Signaling Technologies. 4-hydroxy-tamoxifen (4-OHT) was from Toronto Research Chemicals. Indomethacin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and all other reagents were from Sigma-Aldrich.

Plasmids

Full length AKT1 cDNA obtained by PCR amplification from pcDNA3-myr-HA-AKT1 (addgene #9008) was subcloned into the pCMV-HA vector. Full length AKT2 cDNA obtained by PCR amplification from pBabe-myr-HA-AKT2 (addgene #9018) was subcloned into the pCMV-HA vector. SGK1 cDNA (Thermo Scientific #MHS6278-202755905) was PCR amplified to obtain a truncated SGK sequence (SGK-delta-60N, N-terminal 1-60 amino acids deletion). Phosphomimetic constructs with indicated mutation were done by QuikChange Site-Directed Mutagenesis kit (Stratagene) with appropriate primers. All cDNA constructs were also transferred into pBabe-puro retroviral vector for stable expression.

Mice

Rictor floxed (*Rictor*$^{fl/fl}$) mice (Shiota et al., 2006) or *Raptor*$^{fl/fl}$ mice (Peterson et al., 2011) were crossed with *Myf5-Cre* (JAX #007893)(Tallquist et al., 2000), *Ubc-CreERT2* (JAX #007001)(Ruzankina et al., 2007) and *Pax7-CreER*^{T2} (JAX #012476)(Lepper et al., 2009), to make conditional or inducible knockout mice. *Akt1* and *Akt2* floxed mice (provided by Morris Birnbaum) were also crossed with *Myf5-Cre*. *Rosa26-mTmG* (JAX #007676) and *Rosa26–LSL-LacZ* (JAX #003474) were also obtained from Jackson laboratory. Male 129/C57B6 mice were used for all studies. Mice were kept on a daily 12 h light/dark cycle

and fed a normal chow diet (Prolab® Isopro® RMH 3000) from LabDiet ad libitum at 22°C (except thermoneutrality studies). All animal experiments were approved by the University of Massachusetts Medical school animal care and use committee.

Embryo analysis

Timed matings were performed and embryos were dissected at the indicated days. Embryos were fixed overnight in paraformaldehyde, paraffin embedded, and processed for histological analysis according to conventional methods.

Satellite cells isolation and in vitro differentiation

Adult muscle satellite cells were isolated according to (Sherwood et al., 2004). Limb muscle including triceps surae (TS), tibialis anterior (TA), quadriceps and triceps were dissected and minced from 6 to 8-wks mice. Isolated interstitial and myofiber-associated cells were passed through 70μm nylon mesh and centrifuged at 1200 rpm. Red blood cells were removed from preparations by incubation with RBC lysis buffer (0.15 M ammonium chloride, 0.01 M potassium bicarbonate) on ice for 3 minutes. Antibody staining was performed for 20 min on ice in Hank's balanced salt solution supplemented with 2% FCS and 2 mM EDTA. After staining cells were filtered through a 35-μm cell-strainer capped tube to ensure single cell suspension. Sorting was performed immediately after filtration using a FACS Aria II cell sorter equipped with FASCDiva software. Cells were initially selected by size and shape and only live (PI-, calcein blue+) singlets were gated for further analysis of surface markers. Finally an enriched pool of cells (Sca-1-, Mac1-, Ter119-, CD45-, CXCR4+ and β1 intergrin+) were purified and re-sorted with the same scheme described above to ensure the purity. Double sorted satellite cells were plated at $4x10^3$ cells/well in collagen/laminin coated 96-well plates. Cells were maintained in growth media (20% horse serum in F10 media, Invitrogen) and feed with 5ng bovine FGF daily for 5 days. For inducing muscle fiber formation, cells were first transferred into matrigel (BD Biosence)-coated chamber slides and grown in growth media with bFGF. 2~3 days later, cells were exposed to differentiation media (2% horse serum in F10 without bFGF). After 2 to 4 days myofiber can be observed and fixed with 4% paraformaldehyde. Myosin heavy chains and DAPI staining were performed as described in immunofluorescence section.

Muscle regeneration after cardiotoxin injury

To induce *Rictor* deletion in vivo, *Pax7-CreER*^{T2} mice were i.p. injected with 200μg/g of tamoxifen (dissolved in ethanol first then diluted in corn oil to 10mg/mL) for consecutive 4 days. One day later the mice were anesthetized with 12mg/kg xylazine and 60mg/kg ketamine and 30μL cardiotoxin (10μmol/L from *Naja nigricollis,* Calbiochem) was directly injected into tibialis anterior muscle. 30μL PBS was given in contralateral TA muscle as control. 1 day and 10 days post injury, TA muscle was removed and muscle regeneration was examined by H&E staining and LacZ staining.

LacZ staining

Adipose tissue depots were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS for 30 min at room temperature. The tissues were then washed 3 times for 15 min in wash buffer (PBS carrying 2 mM MgCl2 and 0.02% Igepal® CA-630). Staining was perform in wash buffer containing X-gal (1mg/mL), potassium ferricyanide (5 mM) and potassium ferrocyanide (5 mM) at room temperature for at least 16 h. Next, tissues were further fixed in fixing solution for at least 12 h at room temperature, transferred to ethanol for dehydratation, then sectioned at 5 μm thicknesses. Sections were counter-stained with nuclear fast red dehydrated and mounted using CitosealTM 60 (Thermo Scientific). Lean tissues were snap frozen in isopentane-liquid nitrogen in OCT. Sections (10 μm) were stained overnight (X-gal (1mg/mL), potassium ferricyanide (5 mM) and potassium ferrocyanide (5 mM), MgCl2 (2 mM) in PBS at 37°C and counter-stained with nuclear fast red, dehydrated and mounted.

Tissue harvest and histology

Adipose tissue depots notations are described in (Walden et al., 2011). Each tissue was carefully dissected to avoid contamination from surrounding tissue. Samples for RNA were first immersed in RNAlater (Invitrogen) and stored at -80°C; otherwise, they were frozen down immediately in liquid nitrogen. For histology, tissue pieces were fixed by 10% formalin. Embedding, sectioning and Hematoxylin & Eosin (HE) staining was done by the UMass Morphology Core.

Immunohistochemistry

Adipose tissue sections were subjected to UCP1 IHC according to (Cohen et al., 2014). Briefly, fat sections were hydrated and antigen retrieval was done by incubating the sections in citrate buffer at 90-95°C water for 20 min. After blocking, primary antibody (anti-Ucp1 antibody, Abcam #ab10983) was applied overnight at 4°C. Next day, SuperPicture 3rd Gen IHC Detection Kit (Novex) was used for detection.

Whole-mount confocal microscopy

Indicated brown and white adipose tissues were dissected from 6 week-old mice and were mounted with Fluoromount-G (Southern Biotech) as described in (Berry and Rodeheffer, 2013). Mounted samples were imaged on a LSM 5 Pascal (Zeiss) point scanner confocal system. 40x objective was used with oil immersion. Background fluorescence was offset by using wild-type tissues (no *mT/mG* allele). GFP was excited at 488 nm and detected from 515 to 565 nm and iBAT form *Myf5-cre;Rosa26mT/mG* mice was used as positive control for GFP signal. TdTomato was excited at 543 nm and detected from 575 to 640 nm and pgWAT from mT/mG mice (without Cre-driver) was used as positive control for TdTomato.

Nuclei number and cell size quantification

ImageJ was used to quantify nuclei number in iBAT and cell size (diameter) in rWAT and asWAT. For each individual sample, 4 to 6 images were taken and analyzed. Nuclei density was presented as nuclei number per mm^2.

Genomic DNA quantification

Total genomic DNA was extracted and purified by using DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instruction. Isolated genomic DNA was quantified by NanoDrop 2000 (Thermo Scientific) spectrophotometer.

Immunofluorescence

Frozen section of interscapular brown adipose tissues were thawed and then fixed with methanol for 15 min at room temperature. The fixed sections were washed with 1mL PBS twice and then were permeabilized and blocked with PBSAT buffer (PBS with 1% BSA and 0.5% Triton X-100) for 15 min twice. Primary antibody against mitochondria Cox IV (1:100 dilution, CST #4850) was added to sections for overnight incubation. Slides were washed three times with 1mL PBSAT and incubated with secondary antibody conjugated with Alexa-568 or Alexa-647 (1:1000 dilution, Invitrogen) for 4 hours. Intensive wash was applied to remove unstained antibodies. DAPI was used to stain nuclei for 5 min and washed away by PBS immediately. The slides were embedded with 5μL mounting media (Prolong Gold, Invitrogen).

Glucose uptake

6-week old mice (n=5 per each genotype) were i.p. injected with ^{18}F-FDG, 364-483 uCi in100 ul saline, and 30 min later the PET imaging was performed in anesthetized animals (1.2-2% isoflurane carried in oxygen) immobilized on a Minerve bed (Bioscan). Immediately after PET acquisition, each mouse was transferred to the NanoSPECT/CT (Bioscan), for the CT acquisition. The PET images were reconstructed without photon attenuation correction using the PETView program (Philips) with the fully 3D iterative reconstruction algorithm, giving a pixel size of 1 mm. The CT acquisition was performed at standard frame resolution, 45 kVp tube voltage and 500 ms of exposure time. The CT reconstruction was accomplished using In-VivoScope 1.37 software (Bioscan). The PET image DICOM files were transferred to the NanoSPECT/CT reconstruction workstation to provide the PET/CT fusion images. Volume-of-Interest (VOI) analysis of the PET acquisitions was accomplished with the InVivo- Scope 1.37 software.

Transmission electron microscopy

iBATs (n=3 for each group) were dissected from 5 week-old mice and subjected to electron microscopy study done by Core Electron Microscopy Facility, UMass medical school.

Metabolite profiling

Brown fat samples were homogenized in four volumes of water using a TissueLyser II (Qiagen) and profiles of polar metabolites were obtained using LC-MS. The polar metabolite profiling methods were developed using reference standards of each metabolite to determine chromatographic retention times and MS multiple reaction monitoring transitions, declustering potentials and collision energies. The LC-MS methods have been recently described (Townsend et al., 2013). Briefly, negative ionization mode data were acquired using an ACQUITY UPLC (Waters) coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX) running hydrophilic interaction chromatography (HILIC) method. A 30µL aliquot of each homogenate was extracted using 120 µL of 80% methanol (VWR) containing 0.05 ng/µL inosine-15N4, 0.05 ng/µL thymine-d4, and 0.1 ng/µL glycocholate-d4 as internal standards (Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000 x g, 4ºC) and the supernatants (10 µL) were injected directly onto a 150 x 2.0 mm Luna NH2 column (Phenomenex) that was eluted at a flow rate of 400 µL/min. Initial mobile phase conditions were 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide (Sigma-Aldrich) in water (VWR)) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol (VWR)) and the column was eluted using a 10 min linear gradient to 100% mobile phase A. The ion spray voltage was -4.5 kV and the source temperature was 500°C. Positive ionization mode data were acquired using an 1100 Series pump (Agilent) and an HTS PAL autosampler (Leap Technologies) coupled to a 4000 QTRAP triple quadrupole mass spectrometer (AB SCIEX). A 10µL aliquot of each homogenate was extracted using nine volumes of 74.9:24.9:0.2 (v/v/v) acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (0.2 ng/µL valine-d8, Isotec; and 0.2 ng/µL phenylalanine-d8 (Cambridge Isotope Laboratories)). Samples were centrifuged (10 min, 9,000 x g, 4ºC) and supernatants (10 µL) were injected onto a 150 x 2.1 mm Atlantis HILIC column (Waters). The column was eluted isocratically at a flow rate of 250 µL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. The ion spray voltage was 4.5 kV and the source temperature was 450°C.

MultiQuant 1.2 software (AB SCIEX) was used for automated peak integration and metabolite peaks were manually reviewed for quality of integration and compared against a known standard to confirm identity.

Cold challenge

Mice were first fasted for 16 hours and transferred into a 4°C cold room without any food in the cage. Rectal temperature was measured by rectal probe (RET-3, ThermoWorks) hourly for 6 hours. Mice were sacrificed and tissues were collected at the end of experiment. Fasted mice kept at 22°C were used as control for qRT-PCR.

Cell culture and retrovirus production

All cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin at a 37°C. Primary brown preadipocytes (bBPAs) were isolated from P1 neonates of *Ubc-creER^{T2};Rictor$^{fl/fl}$* mice, *Myf5-cre;Rictor$^{fl/fl}$*,*Myf5-cre;Akt1$^{fl/fl}$*, *Myf5-cre;Akt2$^{fl/fl}$* mice and control littermates according to (Fasshauer et al., 2001) and were immortalized with pBabe-SV40 Large T antigen and selected by puromycin resistance. For recombinant AKT and SGK construct expression, retroviruses were made by cotransfecting pBabe-puro plasmid harboring different *Akt* or *Sgk* cDNAs with pCL-Ampho in HEK-293T cells. 24h and 48h after transfection the viral supernatant was harvested and applied to MEFs for 12h. Cells stably expressing each construct were obtained after puromycin selection.

Differentiation

To generate *Rictor^{iKO}* cells *ubc-creER^{T2};Rictor$^{fl/fl}$* bAPCs were treated with two doses of 1μM 4-OHT for 3 days. For brown preadipocyte differentiation, BPAs were seeded at 4x10^4 cells/ml and allowed to proliferate to confluence over 3 days in differentiation media (20nM insulin, 1nM T_3). On the 4th day, cells were induced to differentiate by adding induction media (20nM insulin, 1nM T_3, 0.125mM indomethacin, 2μg/mL dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine (IBMX)) for 2 days; the medium was then changed every two days with fresh differentiation media until day 10. Differentiated bAPCs were fixed with PBS-buffered formalin and stained with Oil-Red-O dye.

Western blots

Cells were lysed in a buffer containing 50mM Hepes, pH 7.4, 40mM NaCl, 2mM EDTA, 1.5mM $NaVO_4$, 50mM NaF, 10mM sodium pyrophosphate, 10mM sodium β-glycerophosphate and 1% Triton X-100 typically 1 hour after the cells were replenished with fresh culture medium. Tissues were homogenized using a TissueLyser (Qiagen) in the same lysis buffer but additionally supplemented with 0.1% SDS, 1% sodium deoxycholate. An equal amount of total protein was loaded into acrylamide/bis-acrylamide gels and transferred to PVDF membranes for detection with the indicated antibodies. Briefly, membranes were incubated with primary antibodies in 5% milk/PBST or 5% BSA/PBST overnight. HRP-conjugated secondary antibodies were given for 1h. Western blots were developed by enhanced chemiluminescence (PerkinElmer) and detected by X-ray films.

Immunoprecipitation

AKT1-specific antibody (CST# 2967) and AKT2-specific antibody-conjugated beads (CST#4090) were used to purify each isoform. 500ug Cell lysates or tissue lysates were incubated with 1ul of antibodies at 4°C overnight. For AKT1 purification, protein-antibody complex was precipitated by 2 hrs incubation with protein G sepharose beads (Invitrogen). Samples were then boiled in 2x SDS sample buffer.

***Ex vivo* oxygen consumption**

Freshly isolated brown adipose tissues were rinsed with KHB buffer (111nM NaCl, 4.7mM KCl, 2mM $MgSO_4$, 1.2mM Na_2HPO_4, 0.5mM carnitine and 2.5mM glucose) and then cut into small pieces (5~10mg). After 5 times of washing with KHB buffer, each piece was placed into the center of one single well in a XF24 islet capture microplate (Seahorse Bioscience #101122-100) and covered by a provided screen. 450ul KHB buffer was loaded in each well and tissue metabolic rates were measured following program: 3 cycles for basal oxygen consumption rate (OCR) (2 min mix, 2 min wait, and 3 min measure), then injection of 50ul 100mM pyruvate, then followed by 3 cycles for pyruvate-stimulated OCR (3 min mix, 3 min wait, and 2 min measure). Each OCR value was obtained from five different

pieces of a tissue and from 3 repeated measurements. The final OCR values were the average of five independent experiments and normalized to genomic DNA content.

Gene expression analysis

Total RNA was isolated from cells or tissues using Qiazol (Invitrogen) and an RNeasy kit (Invitrogen). Equal amounts of RNA were retro-transcribed to cDNA using a High capacity cDNA reverse transcription kit (#4368813, Applied Biosystems). Quantitative RT-PCR was performed in 10μL reactions using a StepOnePlus real-time PCR machine from Applied Biosystems using SYBR Green PCR master mix (#4309156, Applied Biosystems) according to manufacturer instructions. Standard and melting curves were run in every plate for every gene to ensure efficiency and specificity of the reaction. *Tbp* expression was used as a normalization gene in all conventional RT-PCR experiments. Primer information is listed in the table below. Quantitative RT-PCR arrays for mitochondria (PAMM-087) and mitochondria energy metabolism (PAMM-008) were purchased from Qiagen. Interscapular brown fat pads were removed from 6-wks mice *ad libitum* and Rictor protein deletion in *Myf5-re;Rictor*$^{fl/fl}$ samples was confirmed by western blots before analyzing expression. Data analysis was performed on web-based software provided by the manufacturer.

Primer sequences for quantitative RT-PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Tbp	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
Prdm16	GACATTCCAATCCCACCAGA	CACCTCTGTATCCGTCAGCA
Ppargc1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
Pparγ	TCAGCTCTGTGGACCTCTCC	ACCCTTGCATCCTTCACAAG
C/ebpα	CAAGCCCAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
C/ebpβ	TCGGGACTTGATGCAATCC	AAACATCAACAACCCCGC
C/ebpδ	GCTTTGTGGTTGCTGTTGAA	ATCGACTTCAGCGCCTACA
Ucp1	CTGCCAGGACAGTACCCAAG	TCAGCTGTTCAAAGCACACA
DiO2	TGCGCTGTGTCTGGAACAG	CTGGAATTGGGAGCATCTTCA
Lpl	GGCCAGATTCATCAACTGGAT	GCTCCAAGGCTGTACCCTAAG
aP2	GATGCCTTTGTGGGAACCT	CTGTCGTCTGCGGTGATTT
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT

Dpt	CTGCCGCTATAGCAAGAGGT	TGGCTTGGGTACTCTGTTGTC
Srebf1a	TAGTCCGAAGCCGGGTGGGCGCCGG	GATGTCGTTCAAAACCGCTGTGTGTC
Srebf1c	AAGCAAATCACTGAAGGACCTGG	AAAGACAAGCTACTCTGGGAG
Srebf2	GGATCCTCCCAAAGAAGGAG	TTCCTCAGAACGCCAGACTT
Chrebp	CACTCAGGGAATACACGCCTAC	ATCTTGGTCTTAGGGTCTTCAGG
Chrebpα	CGACACTCACCCACCTCTTC	TTGTTCAGCCGGATCTTGTC
Chrebpβ	TCTGCAGATCGCGTGGAG	CTTGTCCCGGCATAGCAAC
Acly	CTCACACGGAAGCTCCATAA	ACGCCCTCATAGACACCATC
Acc	GGAGATGTACGCTGACCGAGAA	ACCCGACGCATGGTTTTCA
Fasn	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
Elvol6	TCAGCAAAGCACCCGAAC	AGCGACCATGTCTTTGTAGGAG
Scd1	CCCTGCGGATCTTCCTTATC	TGTGTTTCTGAGAACTTGTGGTG
Insig1	TGTGGTTCTCCCAGGTGACT	TAGCCACCATCTTCTCCTCC
Insig2	TGAAGCAGACCAATGTTTCAA	GGTGAACTGGGGGTCTCC
Tfam	GTCCATAGGCACCGTATTGC	CCCATGCTGGAAAAACACTT
Cpt1b	GGGCACCTCTGGGAGTTTGT	TTGGCTCACCCACACAGTGT
Necdin	CACTTCCTCTGCTGGTCTCC	ATCGCTGTCCTGCATCTCAC
Pref1	AGTACGAATGCTCCTGCACAC	CTGGCCCTCATCATCCAC
Wnt10a	CACCCGGCCATACTTCCT	CACTTACGCCGCATGTTCT

*Primer sequences for the brown and white fat marker genes *Sgk2, Cideb, Cyp2b10, Retn, Trim14* and *Nnmt* are described in (Harms et al., 2014).

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