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Supplemental Information

Adipose mTORC1 Suppresses Prostaglandin Signaling and Beige Adipogenesis via the CRTC2-COX-2 Pathway

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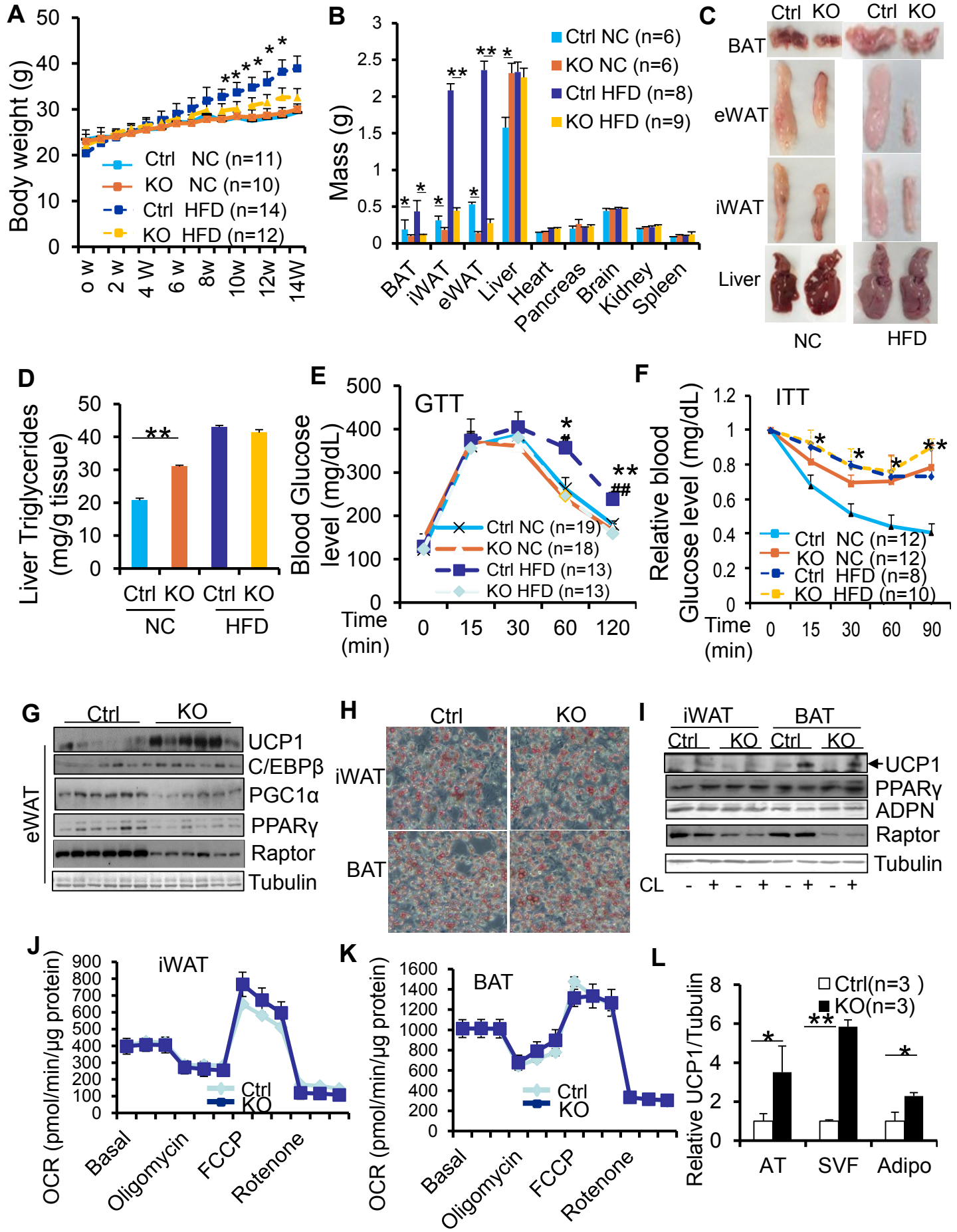
A
Body weight (g)
Ctrl NC (n=11)
KO NC (n=10)
Ctrl HFD (n=14)
KO HFD (n=12)
0 w
2 w
4 W
6 w
8w
10w
12w
14W
B
Mass (g)
Ctrl NC (n=6)
KO NC (n=6)
Ctrl HFD (n=8)
KO HFD (n=9)
BAT
iWAT
eWAT
Liver
Heart
Pancreas
Brain
Kidney
Spleen
C
Ctrl
KO
BAT
eWAT
iWAT
Liver
NC
HFD
D
Liver Triglycerides (mg/g tissue)
Ctrl
KO
NC
HFD
E
GTT
Blood Glucose level (mg/dL)
Ctrl NC (n=19)
KO NC (n=18)
Ctrl HFD (n=13)
KO HFD (n=13)
Time (min)
F
ITT
Relative blood Glucose level (mg/dL)
Ctrl NC (n=12)
KO NC (n=12)
Ctrl HFD (n=8)
KO HFD (n=10)
Time (min)
G
Ctrl
KO
eWAT
UCP1
C/EBPβ
PGC1α
PPARγ
Raptor
Tubulin
H
Ctrl
KO
iWAT
BAT
I
iWAT
BAT
Ctrl
KO
UCP1
PPARγ
ADPN
Raptor
Tubulin
CL
J
iWAT
OCR (pmol/min/μg protein)
Ctrl
KO
Basal
Oligomycin
FCCP
Rotenone
K
BAT
OCR (pmol/min/μg protein)
Ctrl
KO
Basal
Oligomycin
FCCP
Rotenone
L
Relative UCP1/Tubulin
Ctrl(n=3)
KO(n=3)
AT
SVF
Adipo

Fig. S1. Adipose-specific raptor deficient mice are resistant to diet-induced obesity and glucose intolerance, Related to Fig. 1. 6 week-old raptor KO and control mice were fed with normal chow (NC) or high fat diet (HFD) for 14 weeks. The following studies were performed using these animals. **A**. The body weights were monitored weekly during feeding with NC or HFD. **B**. Raptor deficiency decreased fat mass and protected against diet-induced adiposity, while increasing the mass of liver and pancreas under NC but not under HFD feeding condition. **C**. The representative images of BAT, iWAT, eWAT and liver in raptor KO and control mice fed with NC or HFD. **D**. Raptor deficiency upregulated triglyceride levels in the liver under normal chow feeding condition, but diminished the diet-induced increase of triglyceride levels in liver. Raptor deficient mice were insulin intolerant under NC-feeding condition, but abrogated diet-induced glucose intolerance (**E**) and insulin intolerance (**F**) despite insulin resistance under normal chow condition compared to control mice. **G**. Raptor deficiency upregulated expression levels of UCP1 and C/EBPβ, while suppressed adipogenic markers PGC1α, PPARγ and adiponectin in eWAT at room temperature. **H**. Oil red O staining of primary differentiated adipocytes from preadipocytes from iWAT and BAT of raptor KO and control mice. **I**. Basal and CL316243-induced expression of UCP1 and adipogenic markers PPARγ and adiponectin in raptor KO and control primary differentiated adipocytes. The oxygen consumption rate (OCR) of raptor KO and control primary differentiated adipocytes from preadipocytes of iWAT (**J**) and BAT (**K**) determined by Seahorse XF analyzer. **L**. The data for Fig. 1H were quantified and analyzed. The data in Figs. S1H and S1I are representative from three individual experiments. The data in Figs. S1A-1B, S1D, and S1J-1L are presented as the mean ± S.E.M. *$p<0.05$, **$p<0.01$, comparison between two groups as indicated. For Fig. S1A, *$p<0.05$ control mice with NC vs control mice with HFD. For Figs. S1E and S1F, *$p<0.05$, **$p<0.01$, control mice with NC vs control mice with HFD, and $^{\#}p<0.05$, $^{\#\#}p<0.01$, control mice with HFD vs raptor KO with HFD.

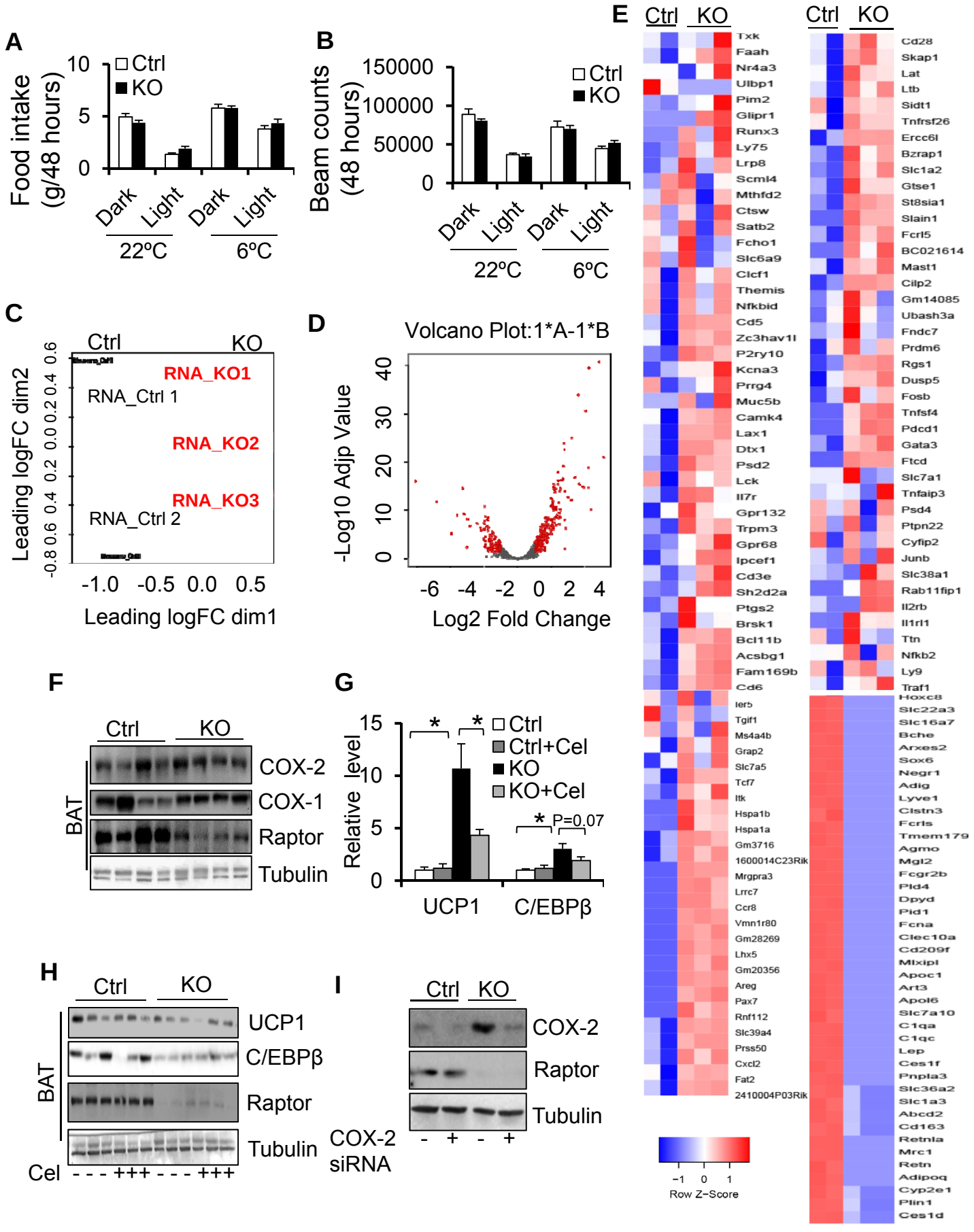
A
Food intake (g/48 hours)
Ctrl
KO
Dark
Light
Dark
Light
22ºC
6ºC
B
Beam counts (48 hours)
C
Leading logFC dim2
Leading logFC dim1
RNA_KO1
RNA_Ctrl 1
RNA_KO2
RNA_KO3
RNA_Ctrl 2
D
Volcano Plot:1*A-1*B
-Log10 Adjp Value
Log2 Fold Change
E
Row Z-Score
F
BAT
COX-2
COX-1
Raptor
Tubulin
G
Relative level
Ctrl+Cel
KO+Cel
P=0.07
UCP1
C/EBPβ
H
UCP1
C/EBPβ
Raptor
Tubulin
Cel
I
COX-2
Raptor
Tubulin
COX-2 siRNA

Fig. S2

Fig. S2. RNA sequencing of raptor KO and control iWAT, Related to Fig. 3 and Fig. 4. Food intake (**A**) and beam counts (**B**) of raptor KO and control mice at room temperature (22ºC) and under cold stress condition (6ºC). n=8/group in Figs. S2A and S2B. RNA seq Gene expression signatures of inguinal fat from 10 week-old male raptor KO and control mice. **C**. PCA of RNA-seq data from 20 ACC tumors (red) and 5 normal salivary gland samples (black). **D**. Volcano plot showing distribution of gene expression changes in ACC tumor samples, with genes showing >2-fold change and adjusted $P < 0.05$ shaded red. **E**. Heatmap summarizing differential gene expression in raptor KO iWAT versus control samples and illustrating gene expression signatures for raptor deficiency. Blue and red shading indicate downregulation and upregulation, respectively, as shown in the color key at upper right. n=2-3/ group in Figs. S2C-S2E. **F**. The expression levels of COX-2 and COX-1 in raptor KO and control BAT. n=4/group. **G**. Quantification of UCP1 and C/EBPβ expression in Fig. 4A. **H**. The levels of UCP1 and C/EBPβ were not significantly affected by celecoxib treatment in BAT of raptor KO mice. n=8/group in Figs. S2G and S2H. **I**. Introduction of COX-2 siRNA suppressed basal and raptor deficiency-induced COX-2 expression in primary adipocytes. The data in Fig. S2I was representative from three individual experiments. Data in Figs. S2A, S2B and S2G are presented as the mean± S.E.M. *p<0.05.

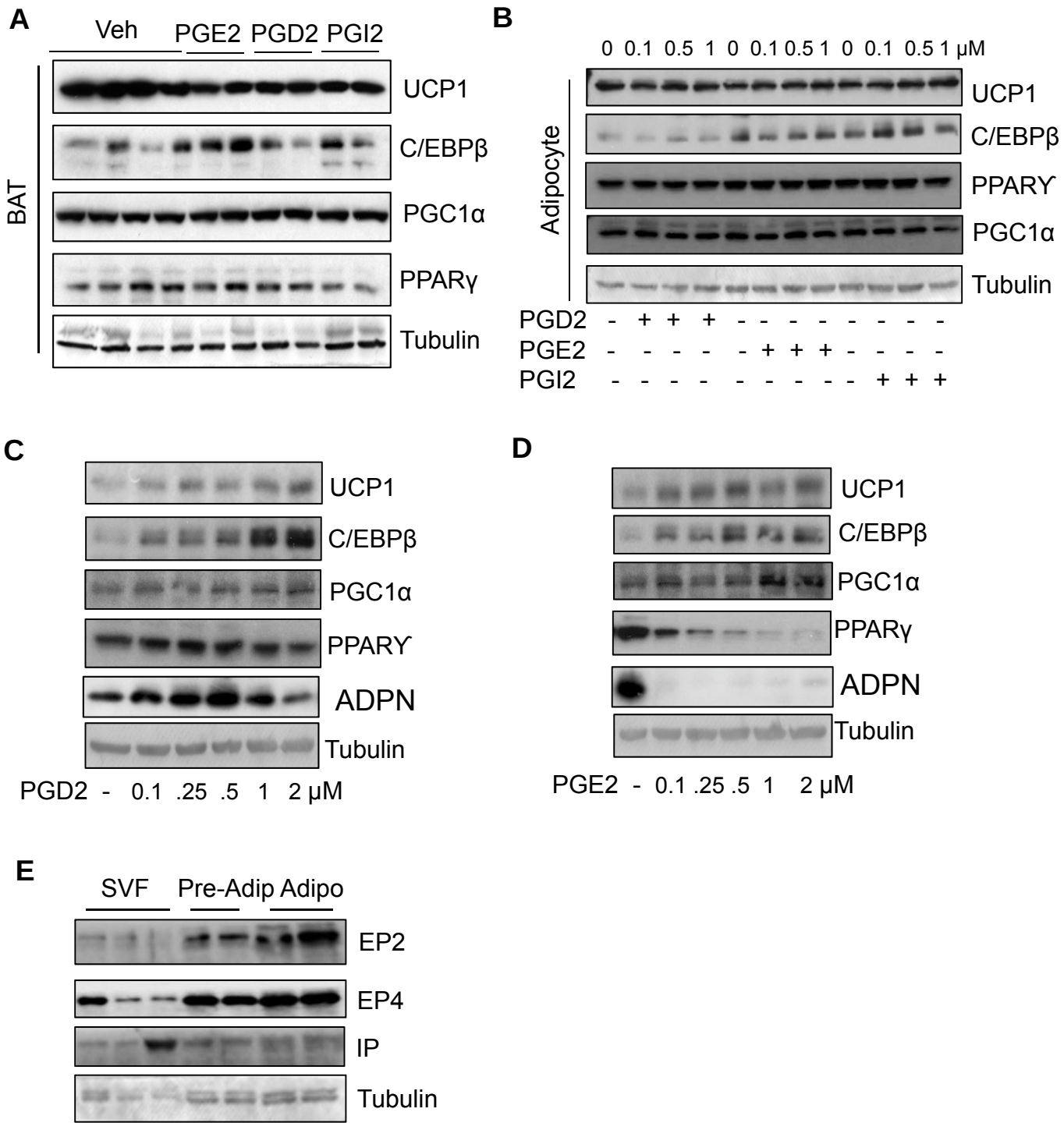
A
Veh
PGE2
PGD2
PGI2
BAT
UCP1
C/EBPβ
PGC1α
PPARγ
Tubulin
B
0 0.1 0.5 1 0 0.1 0.5 1 0 0.1 0.5 1 μM
Adipocyte
UCP1
C/EBPβ
PPARγ
PGC1α
Tubulin
PGD2 - + + + - - - - - - - -
PGE2 - - - - - + + + - - - -
PGI2 - - - - - - - - - + + +
C
UCP1
C/EBPβ
PGC1α
PPARγ
ADPN
Tubulin
PGD2 - 0.1 .25 .5 1 2 μM
D
UCP1
C/EBPβ
PGC1α
PPARγ
ADPN
Tubulin
PGE2 - 0.1 .25 .5 1 2 μM
E
SVF
Pre-Adip
Adipo
EP2
EP4
IP
Tubulin

Fig. S3

Fig. S3. Prostaglandin treatment induced beige adipogenesis, Related to Fig. 5. A. Administration of PGE2, PGD2 or PGI2 for 2 days had no significant effect on the expression levels of UCP1, C/EBPβ, PGC1α and PPARγ in BAT. **B**. Treatment of PGE2, PGD2 or PGI2 had no significant effect on the expression levels of UCP1, C/EBPβ, PGC1α and PPARγ in primary differentiated adipocytes. The expression levels of UCP1 and C/EBPβ but not PPARγ and PGC1α were induced by PGD2 (**C**) or PGE2 (**D**) treatment at the different dose during differentiation of primary preadipocytes. **E**. The expression of PGI2 receptor (IP) and PGE2 receptors EP2 and EP4 in primary preadipocyte, adipocyte and SVF.

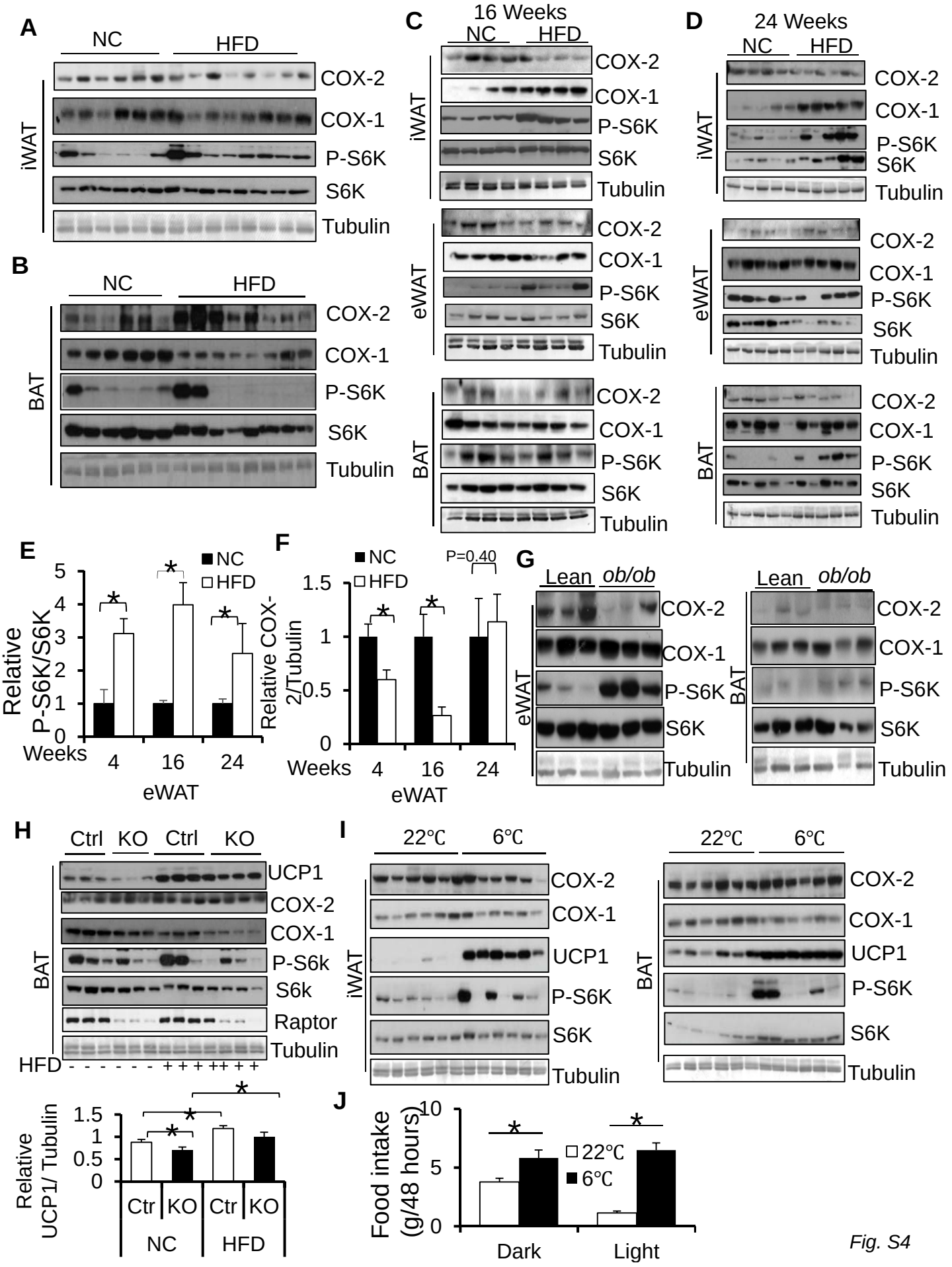
A
NC
HFD
iWAT
COX-2
COX-1
P-S6K
S6K
Tubulin
B
BAT
C
16 Weeks
D
24 Weeks
eWAT
E
Relative P-S6K/S6K
Weeks
4
16
24
eWAT
F
P=0.40
Relative COX-2/Tubulin
G
Lean
ob/ob
H
Ctrl
KO
UCP1
Raptor
HFD
Relative UCP1/ Tubulin
I
22℃
6℃
J
Food intake (g/48 hours)
Dark
Light

Fig. S4

Fig. S4. Activation of mTORC1 or obesity suppressed COX-2 expression *in vivo* and *in vitro, Related to Fig. 6*. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in eWAT (**A**) and BAT (**B**) after 4 week-HFD feeding. n=6-7/group. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in iWAT, eWAT and BAT after 6-week-old male C57BL/6 mice were fed with NC or HFD for 16 (**C**) or 24 weeks (**D**). n=4/ group in Figs. S4C and S4D. The data in Figs. S4C (**E**) and S4D (**F**) were quantified and analyzed. **G**. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in eWAT and BAT of *ob/ob* and lean mice. n=5/group. **H**. Raptor deficiency had no significant effect on the expression of COX-2 and HFD-induced UCP1 in BAT *in vivo*. n=6-7/group. **I**. Cold exposure for 48 hours had no significant effect on the expression of COX-2 and COX-1 despite increased activity of mTORC1 in iWAT and BAT. n=6/group. **J**. food intake was significantly increased by cold exposure for 48 hours. n=6/group. The data in Figs. S4E, S4F S4H, and S4J are presented as the mean ± S.E.M. *$p<0.05$.

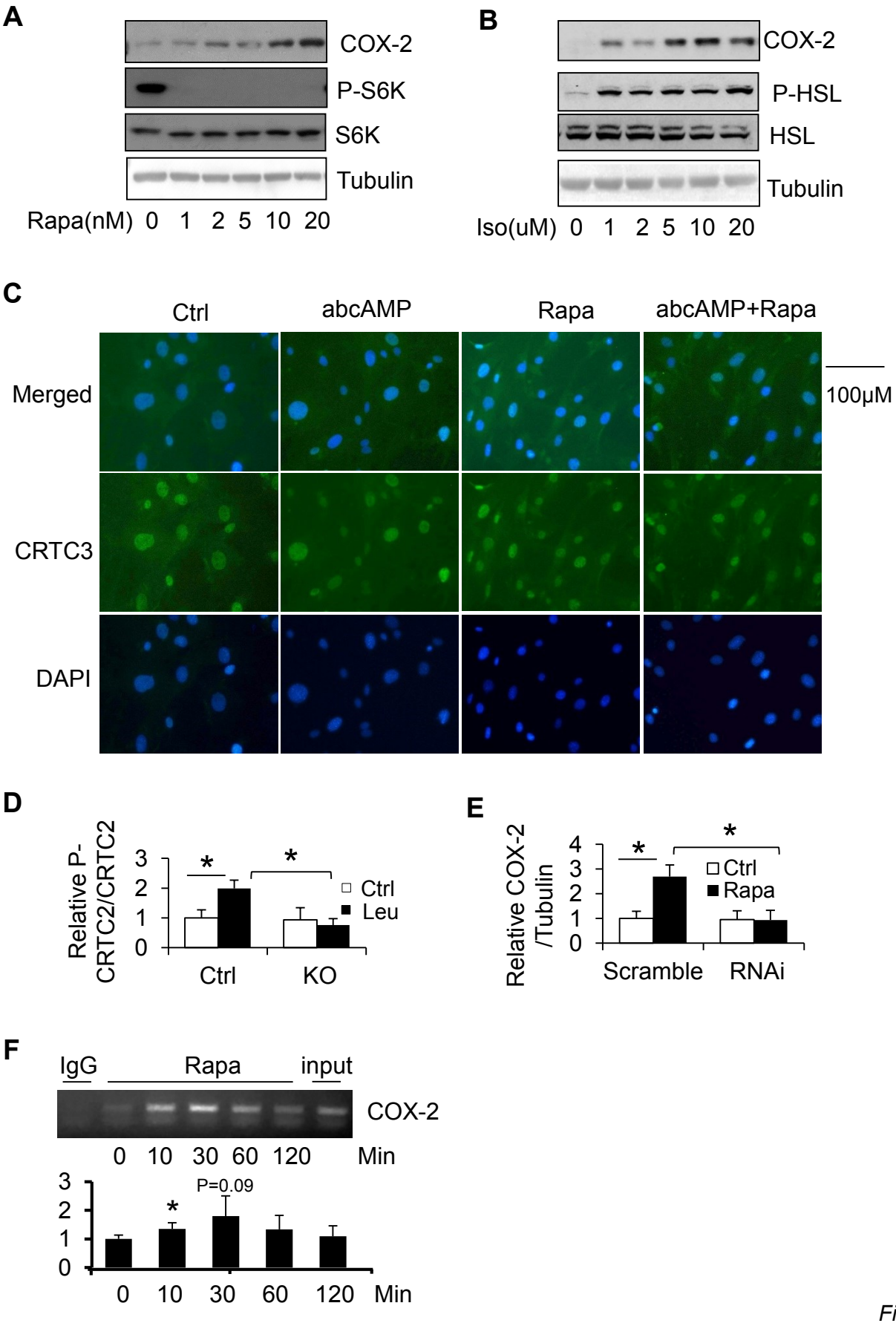
A
COX-2
P-S6K
S6K
Tubulin
Rapa(nM) 0 1 2 5 10 20
B
COX-2
P-HSL
HSL
Tubulin
Iso(uM) 0 1 2 5 10 20
C
Ctrl
abcAMP
Rapa
abcAMP+Rapa
Merged
CRTC3
DAPI
100μM
D
Relative P-CRTC2/CRTC2
Ctrl
Leu
Ctrl
KO
E
Relative COX-2 /Tubulin
Ctrl
Rapa
Scramble
RNAi
F
IgG
Rapa
input
COX-2
0 10 30 60 120 Min
P=0.09
0 10 30 60 120 Min

Fig. S5

Fig. S5. mTORC1 suppresses COX-2 in CRTC2-dependent manner, Related to Fig. 7. Expression of COX-2 was induced by the treatment of rapamycin (**A**) or the treatment of isoproterenol (**B**) in dose-dependent manner in preadipocytes. **C**. Immunofluorescence staining of CRTC3 after treatment of 100 μM cAMP homolog Dibutyryl cyclic AMP, 10 nM rapamycin or co-treatment in primary preadipocytes. **D**. Quantification of phosphorylation levels of CRTC2 at Ser^{136} and normalization with CRTC2 in Fig. 7F. **E**. Quantification of COX-2 levels in Fig. 7G. **F**. Treatment of rapamycin increased CREB binding to the *cox*-2 promoter in a time-dependent manner in 3T3-L1 preadipocytes. The binding of CREB to the *cox-2* promoter was determined using CHIP assay. Figs. S5A-S5C are the representatives from three individual experiments. The data in Figs. S5D-S5F are presented as the mean ± S.E.M. *$p<0.05$.

Supplemental information

Methods

Materials Antibodies against PKA substrate phosphorylation, Flag (DYKDDDK tag), raptor, S6K, phospho-S6K at Thr389, COX-1, COX-2 (catalog #12282, anti-human) and PPARγ were from Cell Signaling Technology (Danvers, MA). Anti-PGC1α and tyrsosine hydroxylase (TyrH) (catalog #2950738) were purchased from Millipore. Anti-C/EBPβ was from Santa Cruz. Anti-CRTC2 from Invitrogen was used for staining and anti-CRTC2 from Calbiochem was used for Western blot analysis. The phosphor-CRTC2 at S136 antibody as well as plasmids encoding wild-type and S136A/D mutant CRTC2 and the control vector were kindly provided by Dr. Yiguo Wang at Tsinghua University, China. PGD2, PGE2, PGI2, Celecoxib, CAY10441 and antibodies to COX-2 （catalog #160106， anti-mouse）, EP4, EP2, and IP were obtained from Cayman Chemical Company (Ann Arbor, MI). Polyclonal antibodies to adiponectin were a gift from Dr. Feng Liu at UTHSA as described previously (Liu et al., 2008). The anti-UCP1 and anti-CRTC3, and cAMP Assay Kit were purchased from Abcam. Dibutyryl cyclic AMP (dbcAMP), CL316,243, leucine, Rapamycin, H89, the medium without arginine, leucine and lysine and control medium were from Sigma-Aldrich.

Administration of PGD2, PGE2, PGI2, Celecoxib and CAY10441 PGE2, PGD2 and PGI2 were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in vehicle solution containing 5% Tween-80 and 5% PEG-400 in PBS to reach the concentration of 100 μg/ml. 2 month-old male C57/BL/6 mice were administered with PGD2, PGE2, PGI2 or vehicle through subcutaneous injection at 100 μg/kg once a day for 2 days as described previously (Madsen et al., 2010). The mice were then euthanized and fat tissues were collected for tissue respiration, Western blot analysis and RT-PCR. Celecoxib or CAY10441 was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in vehicle solution containing 5% Tween-80 and 5% PEG-400 in PBS. 2-month-old raptor KO or control mice were administered with celecoxib (15 mg/kg), CAY10441 (2 mg/kg) or vehicle by intraperitoneal (i.p.) injection once a day for 10 days as the previous studies described (Fan et al., 2013; Vegiopoulos et al., 2010). The mice were then euthanized and adipose tissue samples were collected for H&E staining, tissue respiration, RT-PCR and Western blot analyses.

Food intake, body weight, and body composition Mouse food intake and body weight were measured weekly for 14 weeks. To check body composition, mice were anesthetized by i.p. injection with avertin (120 mg/kg animal body weight). Bone mineral density, fat mass, lean mass, and percentage of fat were determined using dual-energy X-ray absorptiometry (DEXA) (GE Medical Systems, Madison, WI).

Hematoxylin and eosin, Oil Red O staining and immunohistochemical analysis For hematoxylin and eosin (H&E) staining, adipose tissues were fixed with 4% formalin for 48 h and embedded in paraffin. Tissue sections (6-μm thick) were stained with H&E according to standard protocols and analyzed using the NIH Image J software. Immunohistochemistry was performed as described in our previous study (Liu et al., 2014). For the analysis of adipocyte size in adipose tissue, stained tissues were visualized with NanoZoomer Slide Scanner. Five sections per animal were stained. Eight representative images per section were used to determine adipocyte size (Liu et al., 2014). Size of adipocytes were determined with ImageJ Analysis of H&E stained tissues (Zhao et al., 2018). For Oil Red O staining, differentiated primary adipocytes were fixed with

10% formalin for 5 min and stained with Oil Red O in 60% isopropanol for 20 min (Liu et al., 2012).

Energy expenditure The mice were individually housed in a Promethion Metabolic Phenotyping System (Sable Systems International) coupled with a temperature-control chamber. Oxygen consumption (VO2), carbon dioxide release (VCO2), food and water intake, and the activity of each animal (single-housed in 8 cages) were monitored at room temperature (22°C) for 72 hours followed with cold stress (6°C) for 48 hours. The data were analyzed using the software of ExpeData associated with the system and oxygen consumption was normalized by body weight as described in our previous study (Luo et al., 2017).

Primary culture and differentiation of adipocytes Primary stromal vascular fractions from interscapular brown and inguinal fat depots of 3-week-old raptor knockout, control or C57BL/6 wild type mice were isolated, cultured and differentiated into adipocytes according to the procedure as described previously (Liu et al., 2014). For prostaglandin treatment, primary preadipocytes from inguinal fat were differentiated and treated with PGD2, PDE2 or PGI2 for 4 days during the differentiation. On the day 5, cells were harvested for Western blot analysis or RT-PCR. To generate CRTC2-suppressed primary preadipocytes, one of two CRTC2 small interfering RNA (siRNA) constructs (ThermoFisher, catalog 4390771, ID s92672 and ID s92673) was transfected into preadipocytes using a NEON transfection system as described in our previous study (Liu et al., 2014). One of two control siRNA constructs, including Silencer® Select GAPDH Positive Control siRNA (ThermoFisher, 4390849) and Silencer™ Select Negative Control No. 1 siRNA (ThermoFisher, 4390843), was transfected as a control. A similar approach was used to generate COX-2-suppressed and control primary adipocytes using COX-2 small interfering RNA (siRNA) constructs (ThermoFisher, catalog AM16708, with ID 151322 and ID 151322) and control constructs respectively. On day 5 of adipocyte differentiation, COX-2 expression was suppressed using RNAi. The medium was changed to fresh differentiation medium and adipocytes were cultured for another 24 hours. The collected media was used for the differentiation of primary preadipocytes isolated from the iWAT of C57BL/6 animals. For treatment using the PGI2 antagonist, the media was collected from raptor KO and control differentiated primary adipocytes and used for the differentiation of primary adipocytes in the presence of 3 μM CAY10441, a PGI2 receptor antagonist. For the overexpression of CRTC2, a plasmid encoding wild-type, or mutant CRTC2 of S136A or S136D, or empty vector was transfected into 3T3-L1 preadipocytes using a NEON transfection system.

Chromatin Immunoprecipitation (CHIP) assay 3T3-L1 preadipocytes or CRTC2-overexpressed preadipocytes were treated with or without 5 nM Rapamycin for 10, 30, 60 or 120 min, and then fixed in 1% formaldehyde for 10 min at 37℃. Cells were washed twice with cold PBS containing protease inhibitor cocktail, and subsequently lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1). Extracts were sonicated until DNA fragments of 200∼1000 bp were achieved. Sonicated solution was diluted in ChIP dilution buffer (TE buffer, 1.1% Triton X-100, 167 mM NaCl, 1.1x cocktail). The immuno-complexes were precipitated using an anti-CREB (Millipore) antibody or IgG as the control. Precipitated complexes were reverse cross-linked at 65℃. The amount of precipitated DNA was detected by semi-quantitative PCR using the primers 5'-CAGAGAGGGGGAAAAGTTGG-3 and 5'-GAGCAGAGTCCTGACTGACTC-3'.

Luciferase assay The luciferase construct of the *cox-2* promoter was generated by subcloning Xhol-HidIII fragment (-74 to -727) of the *cox-2* promoter into pGL3-basic with the primers 5'-

CCCTCGAGAACCCGGAGGGTAGTTCCAT-3' and 5'-CCAAGCTTGAGCAGA GTCCTGA-CTGACTC-3'. 3T3-L1 preadipocytes were grown in a 24-well plate to 70% confluency. The cox-*2* promoter luciferase construct was co-transfected with pSV-β-Galactosidase Control Vector (PROMEGA, Madison, WI USA) in combination with pcDNA 3.1, pcDNA/CRTC2, pcDNA/CRTC2-S136A, or pcDNA/CRTC2-S136D. 24 hours post transfection, cells were treated with or without 5 nM Rapamycin for 4 hours and then lysed for the measurement of the luciferase with the Luciferase Assay Kit (PROMEGA) and β-galactosidase activities using a Turner TD20/20 luminometer.

Seahorse assays and tissue respiration Primary preadipocytes from brown or beige fat were cultured and differentiated into adipocytes as described previously (Liu et al., 2014). The differentiated adipocytes were then cultured in a plate with XF Assay Media from SeaHorse Bioscience (Billerica, MA) containing 4500 mg/L glucose free of CO2 and incubated at 37°C overnight. SeaHorse XF24 Extracellular Analyzer injection ports were loaded with oligomycin at a final concentration of 1.0 μM for 24 minutes followed by the addition of carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP) at a final concentration of 1.25 μM for 24 minutes. Post FCCP measurements, rotenone was added in for another 24 minutes to achieve a 1.0 μM final concentration. Oxygen consumption (OCR) was measured and normalized by the amount of protein in each well.

Analysis of adipose tissue respiration was performed using a Clark electrode (Strathkelvin Instruments) as previous studies described with minor modifications (Cohen et al., 2014; Kir et al., 2014). Tissues (10-15 mg) were minced and placed in respiration buffer (Dulbecco's phosphate-buffered saline, 2% BSA, 25mM glucose and 1mM pyruvate). For each adipose depot, three separate pieces of tissue of equivalent size were analyzed, and O2 consumption was normalized to tissue weight.

Measurement of cAMP, PGD2, PGE2 and PGI2 The intracellular cAMP level in preadiopocytes was determined by cAMP ELISA kit as described previously (Ohyama et al., 2015; Xu et al., 2013). For the measurement of PGD2, PGE2 and PGI2, the sample preparation for PGs release was performed following the previous study (Vegiopoulos et al., 2010). In brief, 3-month old mice were sacrificed and WAT was dissected. Approximately 50 mg pieces of tissue were plated in duplicate in 0.5 ml medium (Hank's basic plus CaCl2 1.28 mM, MgSO4 1.2 mM, NaHCO3 25 mM, HEPES 15 mM, BSA 0.5 %, D-glucose 5 mM, Penicillin/Streptomycin 1 %) and incubated at 37°C, 5% CO2 for 8 hours. Conditioned media was centrifuged at 5 krpm, for 5 minutes at 4°C and the supernatants were snap-frozen. To measure secreted PGs in primary adipocytes, preadipocytes from iWAT were cultured and differentiated into adipocytes. Differentiated adipocytes were starved for 4 h in 0.4% BSA/DMEM, washed with PBS, and incubated with fresh DMEM containing 1% BSA overnight. Culture media was centrifuged at 10,000 g for 10 min, and the supernatants were collected for the assays of PGs. The levels of PGD2, PGE2 or PGI2 were determined using their enzyme immunoassay kits according to the manufacturer's protocol (Caymann, Ann Arbor, MI, USA). Values were normalized to wet tissue weight or total protein level in the cells.

Real-time PCR Mouse tissue samples were homogenized and the total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen). RNA (1 μg) was reverse-transcribed using cDNA kit (Qiagen). PCR amplification was detected using SYBR Green PCR master mixture on a Roche 480 Real-time PCR system (Roche). Primer sequences are listed in Table 1. Each primer pair amplifies products spanning several exons, distinguishing spliced mRNA from genomic

DNA contamination. The reaction yielded about 200 bp of PCR products for specified genes and internal control genes, including β-tubulin and β-actin. The relative expression of target genes was normalized to GAPDH.

Immunoprecipitation For overexpression of CRTC2, a plasmid encoding wild-type, empty vector or mutant CRTC2 of S136A or S136D was transfected into 3T3-L1 preadipocytes using a NEON transfection system. Cells were treated with or without 5 nM Rapamycin for 30 minutes. Protein A beads (GE Healthcare) were pre-incubated with 1.5 μg IgG or anti-CREB for 2 hours at room temperature, and then washed with WGB buffer before incubation with cell lysate at 4ºC for overnight followed by Western blot as described in our previous study(Liu et al., 2008).

Western blot The general procedures were used for Western blot as described in our previous study (Liu et al., 2014). Quantification of protein levels was performed by analyzing Western blots using the Scion Image Alpha 4.0.3.2 program (Scion Corp.). The protein level was normalized for the amount of protein loaded in each experiment and the relative change in protein level was expressed as percentage of control protein levels. The relative level of each control sample was arbitrarily set at 1.0 in the cell and in vivo studies.

Immunofluorescence staining Treated preadipocytes in 96 well plates were fixed in 4% paraformaldehyde for 5 min, and then permeabilized with 0.1% saponin in 3% bovine serum albumin (BSA) for 30 min followed by incubation with anti-CRTC2 or CRTC3 for 2 hours and secondary antibodies for 1 hour. Cell Nuclei were stained by DAPI (blue). Confocal images were taken using EVOS FL Cell Imaging System in the Department of Biochemistry and Molecular Biology at UNMHSC.

Leucine treatment 3T3-L1 preadipocytes were maintained with DMEM-high glucose (ATCC) containing 10% bovine serum. The medium was changed into DMEM-low glucose without arginine, leucine, and lysine (D9443, Sigma). 4 hours post medium change, starved cells were treated with and without 100 mM leucine at different time points as indicated in the figures.

Adipocyte size analysis H&E staining was performed on tissues. Stained tissues were visualized with NanoZoomer Slide Scanner. Five sections per animal were stained. Eight representative images per section were used to determine adipocyte size. Size of adipocytes were determined with ImageJ Analysis of H&E stained tissues (Zhao et al., 2018).

Supplemental References

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