SIRT1 deacetylase in POMC neurons is required for homeostatic defenses against diet-induced obesity

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Running title: Metabolic actions of SIRT1 in POMC neurons

Supplemental Data

Supplemental Figure and Table Legends

Supplemental Figure 1

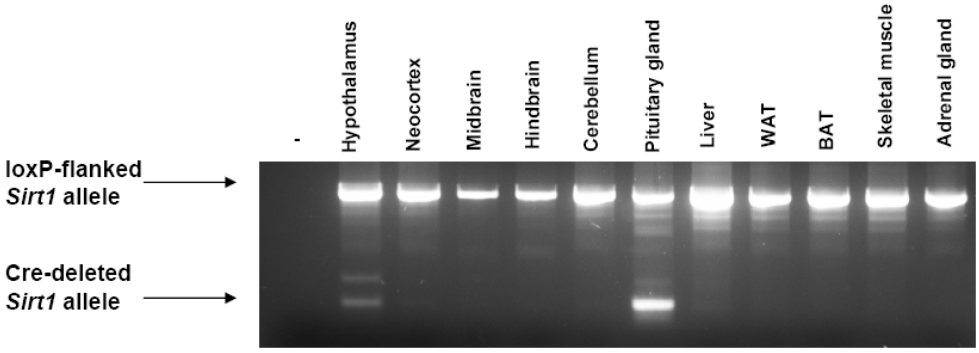

Figure S1, related to Figure 1. Deletion of SIRT1 is restricted to tissues containing POMC cells. PCR genotyping analysis of several tissues from *Pomc*-Cre, *Sirt1*$^{loxP/loxP}$ mice was performed using primers designed against sequences upstream and downstream of the loxP-flanked *Sirt1* exon 4. The use of these primers allowed discrimination between the loxP-flanked (upper band) and the Cre-deleted (lower band) *Sirt1* alleles. WAT and BAT are acronyms for white and brown adipose tissue, respectively. - = Same PCR mix but without DNA template.

Supplemental Figure 2

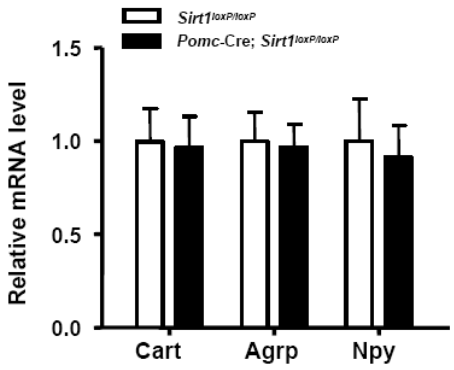

Figure S2, related to Figure 3. Normal expression of hypothalamic neuropeptides in mice lacking SIRT1 in POMC neurons. Hypothalamic *Cart, Npy and Agrp* mRNA level in 28-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ females fed on a high calorie diet for 20 weeks (n=9-11). mRNA levels were normalized to *β-actin* mRNA contents. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test and no differences were noted between groups.

Supplemental Figure 3

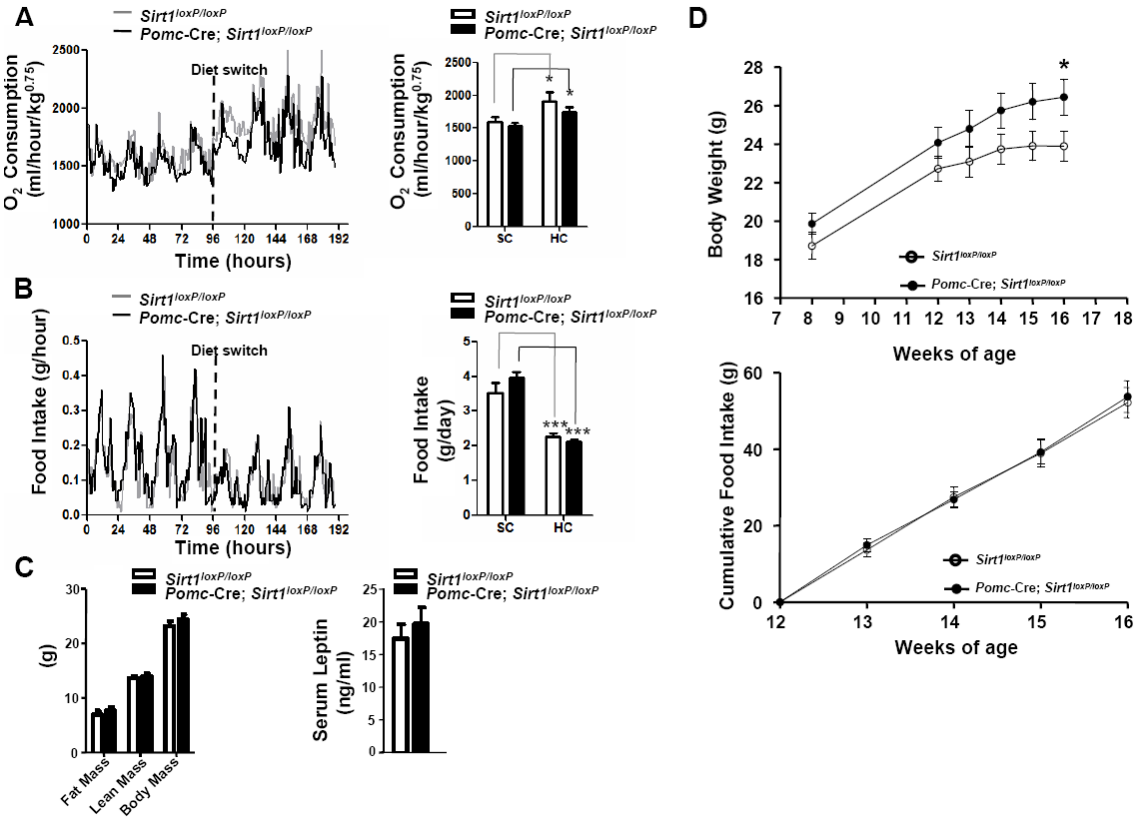

Figure S3, related to Figure 5. Normal acute metabolic adaptations to high-calorie diet in mice lacking SIRT1 in POMC neurons. (**A**) O_2 consumption, and (**B**) food intake were measured during the transition from standard chow (SC) to high-calorie (HC) diet in 8-week-old *Sirt1*$^{loxP/loxP}$ (n=10) and *Pomc*-Cre, *Sirt1*$^{loxP/loxP}$ (n=11) females. Data were collected using the Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS). Time 0 represents 6 hours after the beginning of the light cycle in a 12-hour dark/light cycle environment. Both genotypes similarly increased average O_2 consumption and reduced average food intake during the 4 days on the HC diet compared to the 4 days on the SC diet. (**C**) Fat, lean and total body mass and leptinemia in 12-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre, *Sirt1*$^{loxP/loxP}$ females fed on a high-calorie diet for 4 weeks (n=10-12). (**D**) Body weight curves of another cohort of females that were fed on a

SC diet up to 8 weeks of age and then switched and maintained on a HC diet (n=12-17). Cumulative food intake was measured from week-of-age 12 onward in individual mice. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test. *P<0.05, ***P<0.001.

Supplemental Figure 4

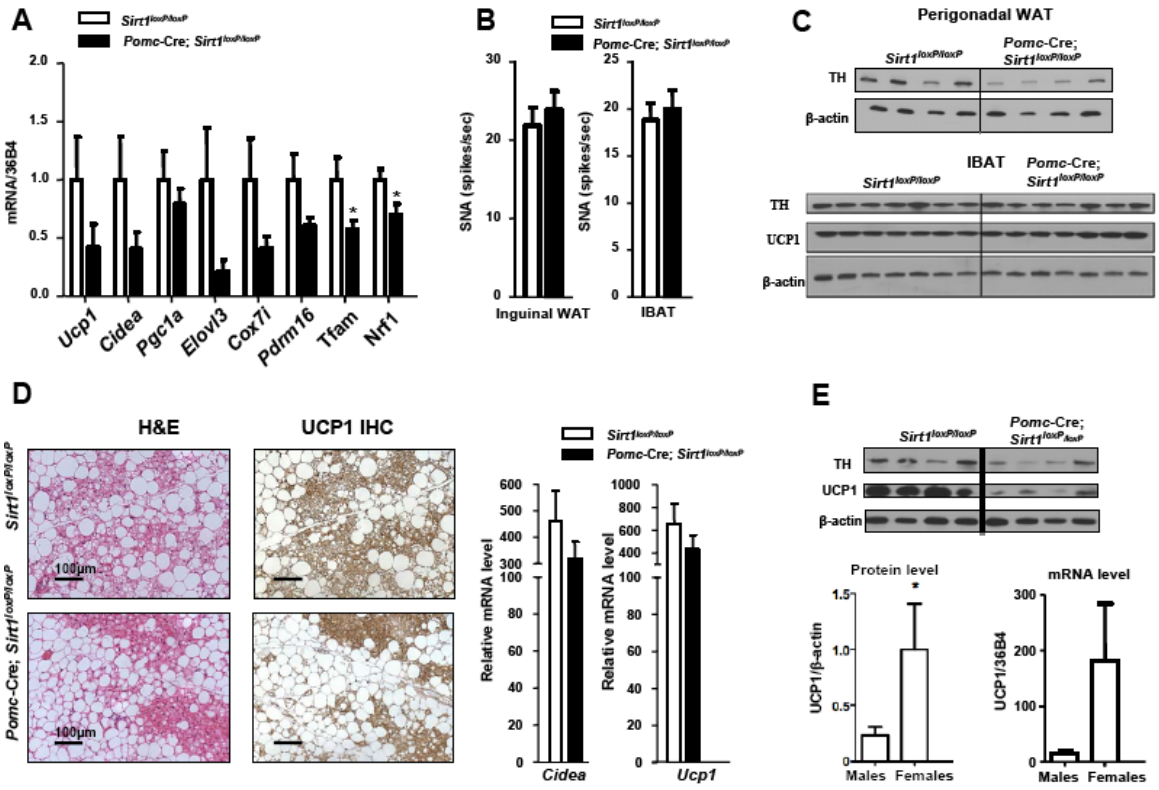

Figure S4, related to Figure 6. BAT-like remodeling of perigonadal fat in *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ HC-fed mice. (**A**) mRNA levels of genes for mitochondrial biogenesis/function and BAT-specific genes in perigonadal WAT in 12-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre, *Sirt1*$^{loxP/loxP}$ females fed on a high-calorie diet for 4 weeks (n=10-12). Individual mRNA levels were normalized to *36B4* mRNA contents. (**B**) Quantification of sympathetic nerve activity (SNA) in inguinal WAT and interscapular

BAT (IBAT) of 12-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ females fed on a HC diet for 4 weeks (n=7). (**C**) TH, UCP1, and β-actin (used as loading control) protein levels were assessed in fat depots of 28-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ females fed on a HC diet for 20 weeks by western blot; representative results are shown. Note that UCP1/β-actin and TH//β-actin are normal in IBAT whereas TH//β-actin is reduced in perigonadal WAT of mutant mice ($P<0.01$; n=7). (**D**) Representative photomicrographs of paraffin-embedded perigonadal WAT sections stained with hematoxylin and eosin (H&E) or treated for UCP1 immunohistochemistry (IHC) and *Ucp1* and *Cidea* mRNA levels in perigonadal WAT of 8-month-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ HC-fed females after one week of treatment with CL316,243 (n=8). Dark-brown staining represents UCP1-expressing brown adipocytes. Scale bar = 100 μm. (**E**) UCP1, TH and β-actin (used as loading control) protein levels were assessed in perigonadal WAT of 28-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ males fed on a HC diet for 20 weeks by western blot; representative results are shown. Note that UCP1/β-actin and TH//β-actin are reduced in perigonadal WAT of mutant mice ($P<0.05$; n=7); and UCP1 protein and mRNA levels in perigonadal WAT of 28-week-old *Sirt1*$^{loxP/loxP}$ males and females fed on a HC diet for 20 weeks. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test. *$P<0.05$.

Supplemental Figure 5

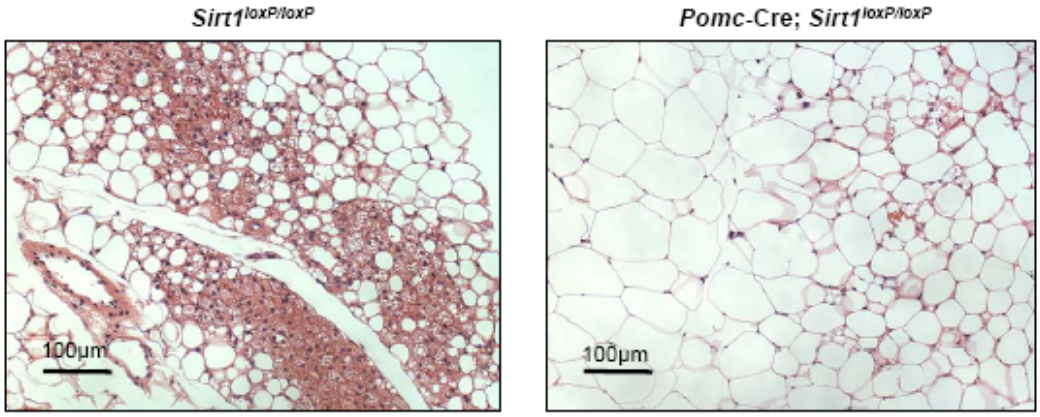

Figure S5, relative to Figure 7. Leptin-induced BAT-like remodeling of perigonadal fat is impaired in *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ mice. Representative photomicrographs of paraffin-embedded perigonadal WAT sections stained with hematoxylin and eosin. Tissues were collected from 16-week-old *Sirt1*$^{loxP/loxP}$ and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ HC-fed females that were treated with intracerebroventricular infusion of leptin for 10 days. Scale bar = 100 μm.

Supplemental Table 1. Body length and serum composition of mice lacking SIRT1 in POMC neurons

			Sirt1$^{lox/lox}$	*Pomc-Cre;Sirt1*$^{lox/lox}$	
	Age (weeks)	ZT	Data		P value
Snout-anus length (cm)					
Males	16		9.68 ± 0.10 (12)	9.70 ± 0.10 (19)	0.91
Females	16		9.03 ± 0.07 (15)	9.07 ± 0.07 (15)	0.61
Insulin (ng/mL)					
Males	28	7	4.68 ± 1.12 (11)	3.22 ± 0.72 (12)	0.6
Males Fasted	28	3	1.64 ± 0.40 (11)	1.48 ± 0.31 (13)	0.75
Females	28	7	0.61 ± 0.26 (6)	0.69 ± 0.18 (8)	0.8
Females Fasted	28	3	0.43 ± 0.09 (9)	0.52 ± 0.15 (11)	0.63
Glycemia (mg/dL)					
Males	28	7	152.54 ± 4.42 (11)	144.77 ± 4.83 (13)	0.25
Males Fasted	28	3	111.54 ± 5.63 (11)	108.86 ± 4.58 (14)	0.71
Females	28	7	110.92 ± 3.81 (14)	119.53 ± 3.61 (19)	0.13
Females Fasted	28	3	106.85 ± 3.66 (14)	101.83 ± 3.63 (18)	0.34
Triglycerides (mg/dL)					
Males	28	7	159.87 ± 4.36 (11)	161.75 ± 8.54 (14)	0.86
Females	28	7	110.51 ± 6.43 (9)	122.01 ± 9.32 (11)	0.35
NEFA (mEq/L)					
Males Fasted	28	3	1.19 ± 0.05 (15)	1.10 ± 0.05 (20)	0.21
Females Fasted	28	3	0.97 ± 0.07 (14)	1.01 ± 0.06 (19)	0.72
Corticosterone (ng/mL)					
Females	12	1	34.39 ± 9.51 (10)	55.23 ± 10.87 (12)	0.17
		7	50.32 ± 12.30 (10)	79.66 ± 13.44 (12)	0.13
		11	206.85 ± 32.75 (10)	246.4 ± 20.97 (12)	0.31

Mice were fed on a standard diet up to 8 weeks of age and then switched and maintained on a high calories diet. All data represent the mean ± s.e.m. The number of mice in each group is shown in parentheses. Fasted parameters were assessed in mice that were fasted for 14h. ZT = Zeitberger time. Statistical analyses were done using two-tailed unpaired Student's t test and P values are presented.

Supplemental Table 1, relative to Figure 4. Body length and serum composition of mice lacking SIRT1 in POMC neurons. Mice were fed on a standard diet up to 8 weeks of age and then switched and maintained on a high calories diet. All data represent the mean ± s.e.m. The number of mice in each group is shown in parentheses. Fasted parameters were assessed in mice that were fasted for 14h. ZT = Zeitberger time. Statistical analyses were done using two-tailed unpaired Student's t test and P values are presented.

Supplemental Experimental Procedures

Immunohistochemistry analyses

β–endorphin immunohistochemistry was performed on brain sections from *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ and *Sirt1*$^{loxP/loxP}$ mice. These mice were generated as described above. SIRT1/GFP co-immunohistochemistry was performed on brain sections from *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$; Z/EG and *Pomc*-Cre; Z/EG mice. To generate the former, breeding pairs were set up between mice carrying the Cre-conditional, GFP reporter allele (i.e.: Z/EG mice (Jackson labs, stock#003920); (Novak et al., 2000)) and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$ mice. The resulting *Pomc*-Cre; *Sirt1*$^{loxP/+}$; Z/EG and *Sirt1*$^{loxP/+}$; Z/EG mice were then bred together and *Pomc*-Cre; *Sirt1*$^{loxP/loxP}$; Z/EG mice obtained. *Pomc*-Cre; Z/EG mice were generated by breeding *Pomc*-Cre mice with Z/EG mice. Mice were perfused with 10% neutral buffered formalin, brains sectioned on a microtome and β–endorphin immunohistochemistry was performed as follows. All steps were performed on free-floating mouse brain sections in cell strainers at room temperature on an orbital shaker at ~50 rpm. Sections stored in PBS (27.6g Sodium Phosphate, dibasic + 160g Sodium Chloride in 20L distilled H_2O at pH 7.4)-Azide (0.8g of Sodium Azide in 4 lites of PBS) were first washed in PBS 3 times for 10 minutes each. Sections were then incubated in 0.3% H_2O_2 for 30 minutes to block the endogenous peroxidase activity and subsequently rinsed in PBS 3 times for 10 minutes each. All sections were then placed in blocking solution of 3% normal donkey serum in PBT-Azide (2.5mL of Triton X-100 in 1000mL of PBS-Azide) for 2 hours. The sections were then transferred into 1:20,000 rabbit anti-β–endorphin primary antisera (Phoenix Pharmaceuticals Inc.; Code H-022-33, Lot# 00569) with 3% normal donkey serum in PBT-Azide overnight. The sections were then

rinsed with PBS 6 times for 10 minutes each. Next, sections were transferred into 1:1,000 biotin-conjugated donkey anti-rabbit secondary antisera (Jackson Immuno Research; Code 711-065-152; Lot# 81161) with 3% normal donkey serum in PBT for 2 hours and subsequently rinsed in PBS 3 times for 10 minutes each. Avidin Biotin Complex (Vectastain Elite PK-6100 ABC kit, Vector Laboratories) solution was prepared (1:500 in PBS) 30 minutes prior to incubating the sections in it for 1 hour and subsequently rinsed in PBS 2 times for 10 minutes each. The sections were then incubated in 0.04% 3,3'-Diaminobenzidine and 0.01% H_2O_2 in PBS for 8 minutes. Finally, the sections were washed in PBS 2 times for 10 minutes each and mounted on gelatin coated glass slides for visualization. SIRT1/GFP co-immunohistochemistry was performed as above. After washing and H_2O_2 treatments sections were incubated overnight at 4^oC into 1:1,000 rabbit anti-SIRT1 primary antisera (a generous gift of Dr. Yoshiyuki Horio) in PBT-azide containig 3% (w/v) normal donkey serum. Sections were then rinsed with PBS 6 times for 10 minutes each and then transferred for 2 hours into 1:1,000 biotin-conjugated donkey anti-rabbit secondary antisera (Jackson Immuno Research; 711-065-152) in PBT containing 3% (w/v) normal donkey serum and subsequently rinsed in PBS 3 times for 10 minutes each. Avidin Biotin Complex (Vectastain Elite PK-6100 ABC kit, Vector Laboratories) solution was prepared (1:500 in PBS) 30 minutes prior to incubating the sections in it for 1 hour and subsequently rinsed in PBS 2 times for 10 minutes each. Sections were then incubated in 0.04% 3,3'-Diaminobenzidine and 0.01% H_2O_2 in PBS for 8 minutes. After three washes in PBS, sections were blocked two hours in PBT containing 3% (w/v) normal goat serum and incubated four hours in 1:5000 chicken-Anti-GFP IgG (Aves Lab Inc; GFP-1020) in PBT containing 3% (w/v) normal goat

serum. Finally, sections were washed three times, incubated at one hour in 1:200 Alexa-488-Goat-anti-chicken antibody (Invitrogen; AA11039), rinsed 3 times in PBS and mounted on gelatin coated glass slides for visualization. SIRT1 and GFP double labeled neurons were counted in 3 arcuate nucleus sections per mouse (n=2). Hematoxylin and eosin (H&E) staining and UCP1 immunohistochemistry were performed using paraffin-embedded adipose tissue sections as previously described (Plum et al., 2007).

Body composition, oxygen consumption and locomotor activity analyses

Body fat and lean mass were determined using the EchoMRI-100 system (Echo Medical Systems, Houston, TX, USA). For *in vivo* scans, mice were anesthetized by 1% isoflurane inhalation and positioned in the mouse bed. The whole body of each mouse was scanned at an isotropic voxel size of 93μm (80 kV, 450 μA and 100 ms integration time) using the eXplore Locus micro-CT scanner (GE Health Care). The legs and head were not scanned. Selection of the scan energy and voxel size (scanning increment) was based on optimizing the requirements of scanning time and tissue detail, and to minimize exposure to radiation. Based on the scan parameters, the estimated radiation exposure was 4 rad (0.04 Gy) for each scan. Three dimensional images were reconstructed from two-dimensional gray-scale image slices and visualized using Microview Software (GE Medical System). Density values for soft tissue and bone were calibrated from a phantom (GE Health Care) containing air bubble, water and hydroxyl apatite rod. The region of interest (ROI) for each animal was defined based on skeletal landmarks from the gray-scale images. Metabolic rate and physical activity were measured using a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH, USA).

Mice were acclimated in the monitoring chambers for 3 days, and then data were collected for 4 days. These instruments were available at the UTSW Mouse Metabolic Phenotyping Core facility.

Blood composition

Fed hormones/metabolites levels were determined by collecting tail blood from mice that were without food for 3 hours. Fasted hormones/metabolites levels were assessed in mice provided only with water *ad libitum* and without food for the indicated period. Time at day at which blood was collected was the same between groups. Tail vein blood was assayed for glucose levels using a standard glucometer (Fisher Scientific, Morris Plains, NJ). Serum was collected by centrifugation and assayed for leptin (Crystal Chem. Inc., Downers Grove, IL), insulin (Crystal Chem. Inc.), and corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA) levels using commercially available kits.

Quantification of POMC-derived peptides

The contents of hypothalamic POMC-derived peptides were determined as previously described (Perello et al., 2007). In brief, mice were sacrificed by decapitation. Brains were removed from the skull, and hypothalamic arcuate nucleus (ARC)-enriched sections were dissected under a stereomicroscope using the median eminence as a reference. ARC samples were collected in 600 ml of 2 N acetic acid freshly supplemented with a protease inhibitor mix (containing AEBSF, pepstatin A, E64, bestatin, leupeptin, and aprotin) and immediately frozen on dry ice. To perform the cell disruption, samples were heated at 95°C for 15 min, homogenized using Dounce homogenizers and, then, sonicated for 5

min. Finally, samples were centrifuged at 15000 rpm at 4°C for 30 min and supernatants were collected. Protein concentration in supernatants was determined by the Bradford assay (Coomassie Protein Assay Reagent). The adequate volumes of supernatants were evaporated using an ATR speed vacuum system and then reconstituted in 1 mL of 0.1% trifluoracetic acid (TFA) solution. ARC extracts containing 20 mg of total protein for each sample were injected in HPLC gradient system for α-MSH assay. For ACTH assay, 250 mg of total protein were generated by pooling 2-3 ARC samples and also injected in HPLC gradient system. A Varian ProStar Gradient HPLC System equipped with a C18 reverse phase column (Microsorb MV 300-5) was used to fractionate the samples. For peptides elution, a linear gradient was used from 20 to 40% of B in 20 min using the following mobile phases: (A) 0% acetonitrile/0.1% TFA and (B) 100% acetonitrile/0.1% TFA. The flow rate was 1.5 mL/min and there were equilibration times used on either side of the gradient. Fractions (0.75 mL) were collected over the entire 20 minutes gradient. Fractions were then evaporated using a -110°C speed vacuum system and then reconstituted in the buffer used in the radioimmunoassay (RIA). Synthetic peptides were injected on the HPLC to determine retention times. The RIA used for α-MSH and ACTH-derived peptides were developed in our laboratory using commercially available peptides and primary antibodies. Each purified peptide was iodinated with 125I using the Chloramine T oxidation-reduction method, purified by HPLC, and used as tracer. The α-MSH RIA was performed using anti- α-MSH antiserum (1:20,000), and 5000 cpm of 125I des-α-MSH tracer. The sensitivity of the assays was approximately 6.5 pg/tube, and the intra- and inter-assay variability were approximately 5-7% and 10-11%, respectively. The ACTH RIA was also performed using the anti-ACTH antiserum (1:30,000) and 5000

cpm of 125I-ACTH tracer. The sensitivity of the assays was approximately 10.0 pg/tube, and the intra- and inter-assay variability were approximately 5-7% and 10-11%, respectively.

mRNA and protein contents

Fed *ad libitum* mice were sacrificed, tissues quickly removed, snap-frozen in liquid nitrogen and subsequently stored at – 80ºC. RNAs were extracted using Trizol reagent (Invitrogen). Complementary DNA was generated by Superscript II (Invitrogen) and used with SYBR Green PCR master mix (Applied Biosystem, Foster City, CA, USA) for quantitative real time PCR (q-RTPCR) analysis. mRNA contents were normalized to *β-actin* and/or *36B4* mRNA levels. Sequences of deoxy-oligonucleotides primers used are outlined here: *Ucp1* 5'GAGGTGTGGCAGTGTTCATTG and 5'GGCTTGCATTCTGACCTTCA; *Cidea* 5'GGTTCAAGGCCGTGTTAAGG and 5'CGTCATCTGTGCAGCATAGG; *Pomc* 5'GAGGCCACTGAACATCTTTGTC and 5'GCAGAGGCAAACAAGATTGG; *36B4* 5'CACTGGTCTAGGACCCGAGAAG and 5'GGTGCCTCTGAAGATTTTCG; *β-actin* 5'CATCGTGGGCCGCTCTA and 5'CACCCACATAGGAGTCCTTCTG. *Elovl3*, *Cox7i*, *Pdrm16* and other primers are as previously described (Balthasar et al., 2004; Seale et al., 2008). All assays were performed using an Applied Biosystems Prism 7900HT sequence detection system. For each mRNA assessment, q-RTPCR analyses were repeated at least 3 times. Proteins were extracted by homogenizing samples in lysis buffer (Tris 20mM, EDTA 5mM, NP40 1% (v/v), protease inhibitors (P2714-1BTL from Sigma, St. Louis, MO, USA), then resolved by SDS-PAGE and finally transferred to a nitrocellulose membrane by electroblotting.

Proteins were detected using commercially available antisera (UCP1 and β-actin: Abcam, Cambridge, MA, USA; TH: Millipore, Billerica, MA, USA).

Recording of Sympathetic Nerve Activity

Mice were anesthetized with an IP injection of a ketamine (91 mg/kg)/xylazine (9.1 mg/kg) cocktail mixture. A PE-50 tubing was inserted into the trachea for spontaneous exchange of O_2-enriched air. Both the right jugular vein and the left carotid artery were each cannulated with a tapered micro-renathane tubing (MRE-040, Braintree Scientific, Braintree, MA) for intravenous infusion of α-chloralose (initial dose of 12 mg/kg initially, then a sustaining dose of 6.25 mg/kg/hr) and for the continuous measurement of arterial blood pressure, respectively. Rectal body temperature was strictly maintained at 37.5 C with the assistance of a heat lamp and a surgical heating pad. A small incision was made in the lower pelvic area to access the perigonadal fat depot. Brown adipose tissue sympathetic nerve (BAT) was identified in the intra-scapular region through a ventral incision made at the nape of the neck. The inguinal fat pad was accessed through a small incision made on the right flank near the hind limb. The nerve subserving each fat pad was carefully dissected, isolated and was placed on bipolar 36-gauge platinum-iridium wire electrodes and then encased with silicone gel (Kwik-Sil; World Precision Instruments, Inc. Sarasota, FL). Sympathetic nerve activities were recorded for 30 minutes. Inguinal and interscapular SNA were recorded from a different cohort of age-matched, HC-fed mice. The nerve signal was amplified 10^5 times with a Grass P5 pre-amplifier and filtered at a low and high frequency cutoff of 100 Hz and 100 Hz. The amplified, filtered nerve signal was directed to 1) a speaker system and to an oscilloscope

(model 54501A, Hewlett-Packard Co., Palo Alto, CA) for auditory and visual monitoring of the nerve activity, 2) to a resetting voltage integrator (model B600C University of Iowa Bioengineering, Iowa City, IA) that sums the total voltage output in units of 1V*sec before resetting to zero and finally 3) to the MacLab analogue-digital converter (Model 8S, AD Instruments, Castle Hill, New South Wales, Australia) where the placement of the cursor (for counting the number of spikes per second) was made just above background noise of the amplified, filtered neurogram. After death, background noise was subtracted from both the resetting voltage integrator and the frequency histogram.

Central leptin administration

Mice were fed *ad libitum* on a regular chow diet up to 8-week of age and then were switched and maintained on the high-caloric diet (Catalog D12331 from Research Diet, New Brunswick, NJ, USA). After 8 weeks on this diet, mice underwent the following surgical procedure. A cannula was positioned stereotaxically into the cerebral lateral ventricles (-0.34mm from bregma; ±1mm lateral; -2.3mm from skull) and a small osmotic minipump (model 1004, Alzet, Cupertino, CA, USA) implanted subcutaneously was attached via a catheter to the cannula for intracerebroventricular infusion. Leptin was delivered at the dose of 14 ng/Kg of body weight per minute as previously described (Plum et al., 2007). After surgical implantation of the cannula and the minipump, mice were maintained on the same high-caloric diet until they were sacrificed.

Hypothalamic organotypic slice culture

To directly test the effects of SIRT1 deletion on the PI3K signaling in POMC neurons, we introduced the Cre-conditional FoxO1-GFP allele (Fukuda et al., 2008) in either *Pomc*-Cre or *Pomc*-Cre; *Sirt1*$^{loxP/lox}$ mice. Hypothalamic slices were made as previously described (Fukuda et al., 2008). Briefly, 8–11 day old mice were decapitated and their brains quickly removed. Hypothalamic tissues were blocked and sectioned at a thickness of 300 μm on a vibratome in chilled Gey's Balanced Salt Solution (Invitrogen) enriched with glucose (0.5%) and KCl (30 mM). Coronal slices containing the arcuate nucleus were then placed on Millicell-CM filters (Millipore, pore size 0.4 μm, diameter 30 mm), and then maintained at an air-media interface in MEM (Invitrogen) supplemented with heat-inactivated horse serum (25%, Invitrogen), Glucose (32 mM) and GlutaMAX (2 mM, Invitrogen). Cultures were typically maintained for 10 days in standard medium, which was replaced three times a week. After overnight incubation in low-serum (2.5%) MEM supplemented with GlutaMAX (2 mM), slices were used for hormonal challenges, immunohistochemistry for GFP, and imaged as previously described (Fukuda et al., 2008). Quantification analyses were also done as previously described (Fukuda et al., 2008).

Statistical analysis

Data sets were analyzed for statistical significance using PRISM (GraphPad, San Diego, CA) for a two-tail unpaired Student's t test when two groups were compared or one-way ANOVA (Tukey's post test) when three or more groups were compared.

Supplemental References

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