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Supplemental Information

A Diurnal Rhythm in Brown Adipose Tissue Causes Rapid Clearance and Combustion of Plasma Lipids at Wakening

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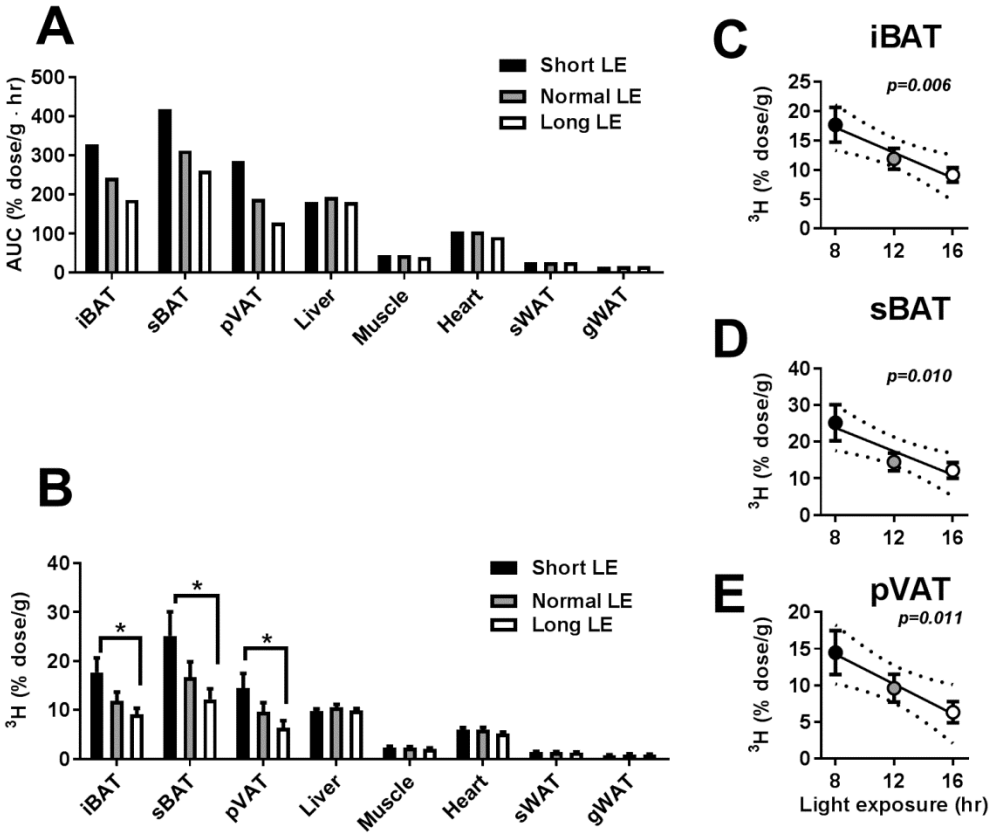

Figure S1. Overall diurnal TG-derived FA uptake by BAT adapts to daily light exposure. Related to Figure 1. Wild-type mice subjected to short LE, normal LE or long LE were injected with glycerol tri[^{3}H]oleate-labeled particles at 6 time points (n=4/group) and killed after 15 min of injection. AUC (A) and the average daily uptake of glycerol tri[^{3}H]oleate-derived activity were determined per organ (B). Correlations were made between light exposure duration in hours and uptake of [^{3}H]oleate by iBAT (C), sBAT (D) and pVAT (E). Data are presented as means ±SEM. * $p<0.05$ (compared to short LE, one-way ANOVA, Tukey's post-hoc test). Correlations were analyzed by linear regression (dotted line represents 95%-confidence band). Abbreviations: AUC = area under the curve, iBAT = interscapular brown adipose tissue; sBAT = subscapular brown adipose tissue; pVAT = perivascular adipose tissue, sWAT = subcutaneous white adipose tissue; gWAT = gonadal white adipose tissue.

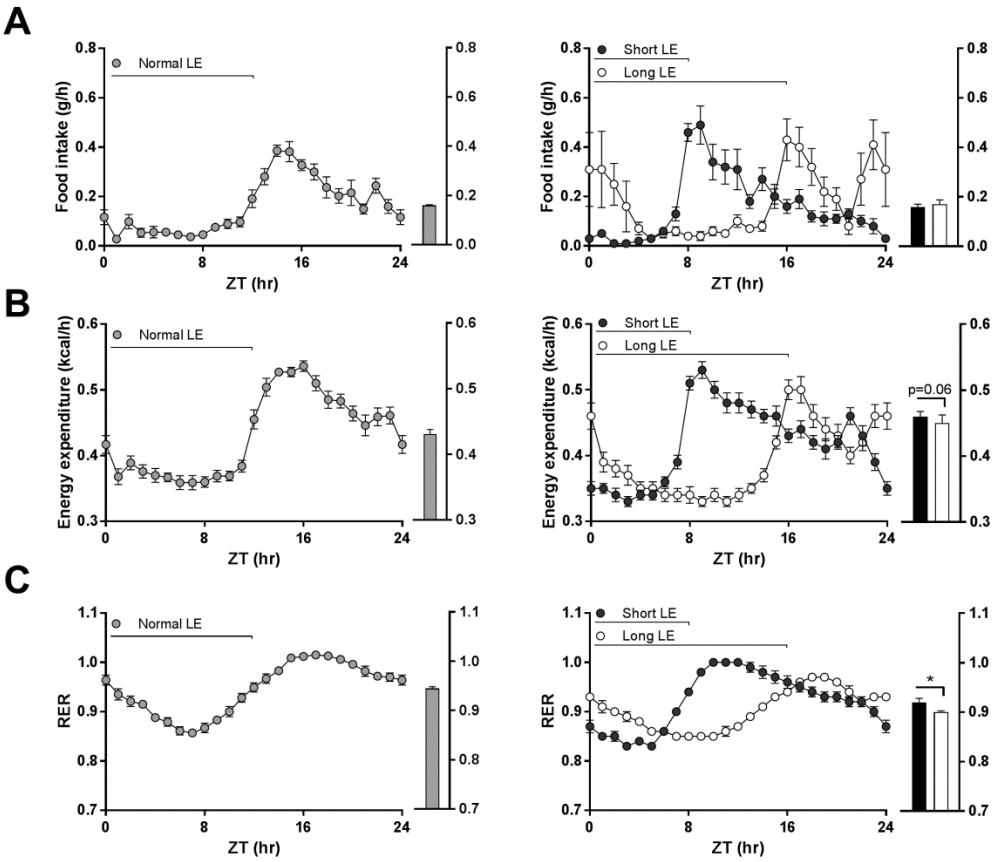

Figure S2. Indirect calormetry under short LE and long LE. Related to Figure 1. Wild-type mice were first housed at normal LE and then subjected to either short LE of long LE for 5 weeks. Food intake (A) was measured and energy expenditure (B) and respiratory exchange ratio (RER) (C) were calculated from the $V{\cdot}O_2$ and $V{\cdot}CO_2$. Data points in the curves represent 1h binned values over a total of three consecutive days. Daily averages are indicated in the bar graphs and used for comparisons between groups. Data are presented as means ±SEM of 15-16 mice per group. *$p<0.05$ (independent samples t-test).

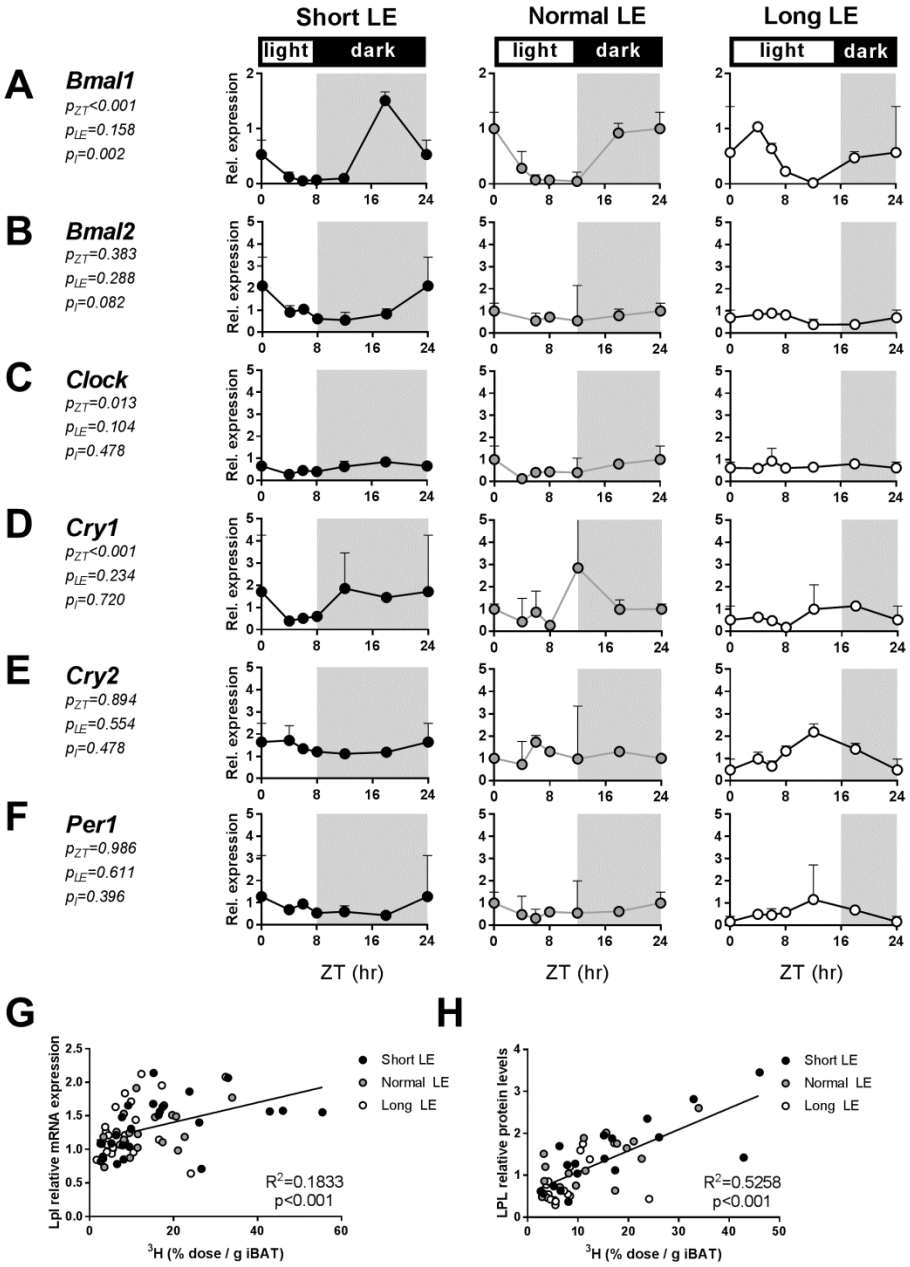

Figure S3. Additional clock gene expression data and correlations between LPL and FA uptake by BAT. Related to Figure 2. Wild-type mice were subjected to short LE, normal LE or long LE. Mice were injected with glycerol tri[^{3}H]oleate-labeled particles at 6 time points (n=4/group) and killed 15 min after the injection for the collection of interscapular brown adipose tissue (iBAT). Gene expression of *Bmal1* (A), *Bmal2* (B), *Clock* (C), *Cry1* (D), *Cry2* (E) and *Per1* (F) was determined by qPCR, calculated relative to *36b4* and *Hprt* expression, and normalized to mean expression of ZT0 of normal LE group. Uptake of [^{3}H]oleate was plotted against *Lpl* gene expression (qPCR; G) and LPL protein levels (Western blot; H). Data are presented as means ± SEM and ZT0/ZT24 was double plotted for visualization purposes. p_{ZT}, p_{LE} and p_I represent p-values for the factors Zeitgeber Time (ZT), LE and interaction respectively (two-way ANOVA). Correlation analysis was performed by linear regression analysis.

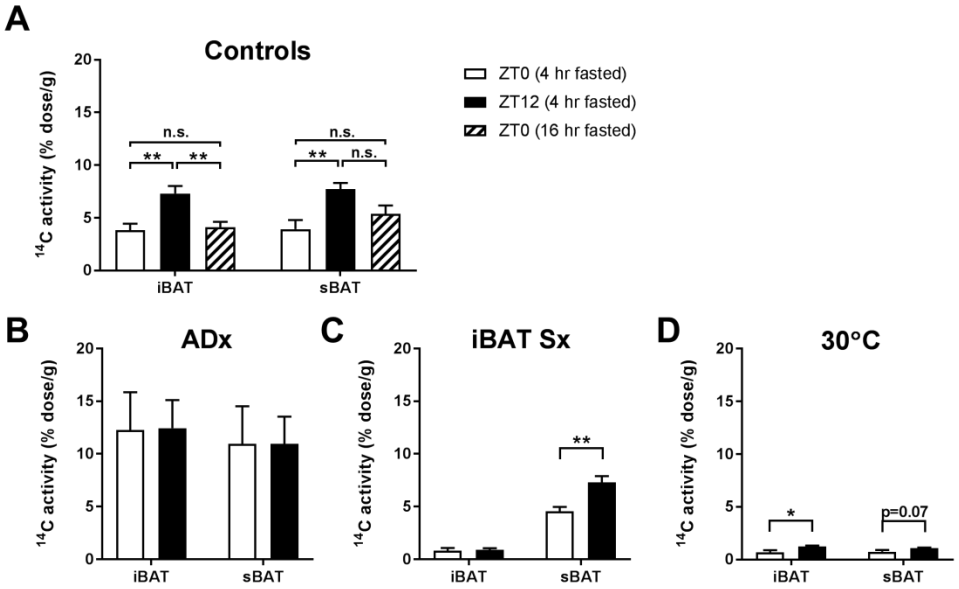

Figure S4. 24h rhythm of glucose uptake by BAT after prolonged fasting, adrenalectomy, sympathetic denervation and at thermoneutrality. Related to Figure 3. Wild-type mice were subjected to normal LE and injected with [^{14}C]deoxyglucose at lights on (ZT0) or lights off (ZT12). The uptake of [^{14}C]deoxyglucose was determined for interscapular BAT (iBAT) and subscapular brown adipose tissue (sBAT) after short and prolonged fasting (n=8/group; A), after adrenalectomy (n=4-5/group; B), after sympathetic denervation of iBAT (n=8/group; C), or after housing at thermoneutrality (n=7/group; D). Data are presented as means ± SEM. *p<0.05, **p<0.01, ***p<0.001 (ANOVA with Tukey's post hoc test (A); independent samples t-test (B-D)).

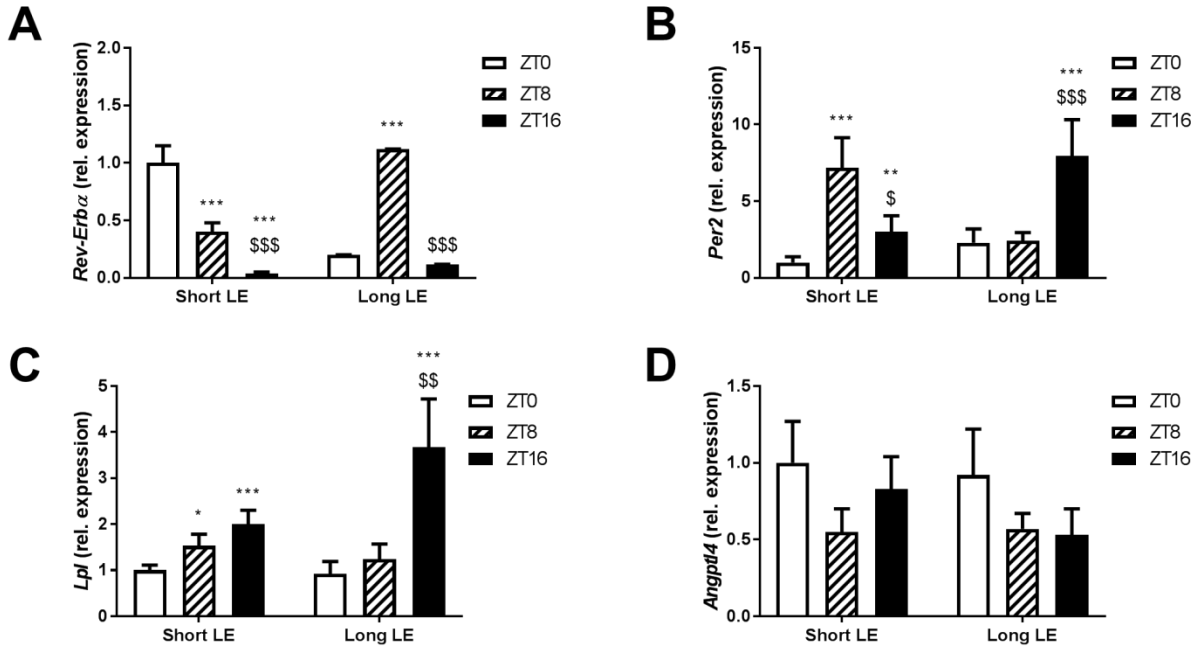

Figure S5. Gene expression in BAT of APOE*3-Leiden.CETP mice. Related to Figure 5. Interscapular brown adipose tissue (iBAT) of APOE*3-Leiden.CETP mice, fed a western type diet and subjected to short LE or long LE, was collected at three time points (n=7-8/group). Gene expression of *Rev-Erbα* (A), *Per2* (B), *Lpl* (C) and *Angptl4* (D) was determined by qPCR, calculated relative to *36B4* and *Hprt* expression, and normalized to mean expression of ZT0 of the short LE group. Data are presented as means ± SEM. *p<0.05, **p<0.01, ***p<0.001 (lights on compared to lights off), $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001 (ZT8 compared to ZT16) (ANOVA, Tukey's post hoc test).

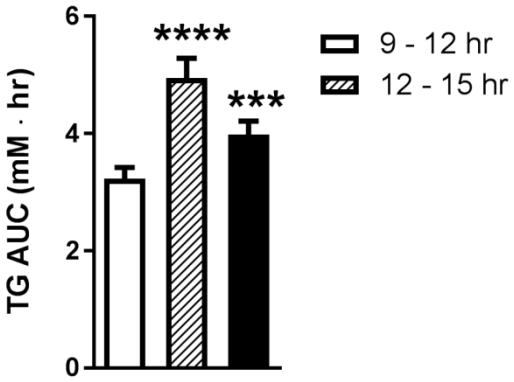

Figure S6. Postprandial triglyceride levels in humans. Related to Figure 6. 37 healthy individuals were fasted overnight and diurnal venous blood sampling was started at clock time 9.00h. At 9 hr, 12 hr and 18 hr a standard liquid meal was consumed. TG levels were determined every 30 minutes and postprandial AUC was calculated 3h after ingestion of an isocaloric meal (a). Data presented as means ±SEM. ***$p<0.001$ (compared to 9–12 hr), based on one-way ANOVA (Tukey's post-hoc test). Abbreviations: AUC= area under the curve, TG = triglycerides.

	Minimum FA uptake (% of dose/g)			Maximum FA uptake (% of dose/g)			Range of FA uptake (% of dose/g)			Fold range (max/min)			Peak time (ZT)			Trough time (ZT)		
	Short LE	Normal LE	Long LE	Short LE	Normal LE	Long LE	Short LE	Normal LE	Long LE	Short LE	Nor-mal LE	Long LE	Short LE	Nor-mal LE	Long LE	Short LE	Nor-mal LE	Long LE
Liver	8.0±0.8	8.7±0.1	8.4±0.7	11.2±0.3	14.7±1.8	12.5±1.4	3.2	6.0	4.2	1.4	1.7	1.5	18	18	18	0	4	6
sWAT	0.9±0.1	1.1±0.1	1.1±0.1	1.8±0.3	2.2±0.5	1.9±0.4	0.9	1.1	0.8	1.9	2.0	1.8	12	18	12	4	8	6
gWAT	0.5±0.1	0.6±0.1	0.6±0.1	1.2±0.2	1.8±0.6	1.3±0.4	0.7	1.3	0.7	2.5	3.3	2.2	12	18	18	4	8	4
Heart	3.9±0.3	4.2±0.7	4.0±0.4	8.6±2.1	7.9±0.6	6.5±1.1	4.7	3.8	2.5	2.2	1.9	1.6	0	4	8	8	12	12
Muscle	1.6±0.2	1.4±0.1	1.0±0.2	3.5±0.3	3.2±0.3	2.9±0.2	1.9	1.8	1.9	2.1	2.3	2.8	4	4	4	6	18	6
pVAT	2.0±0.5	1.7±0.2	1.5±0.2	24.5±7.8	25.2±2.3	16.4±4.6	22.5	23.6	14.9	12.4	15.3	11.2	18	18	18	0	4	0
sBAT	4.9±3.1	2.4±0.5	5.5±1.6	64.5±10.5	28.6±10.8	28.9±6.6	59.5	26.3	23.4	13.0	12.0	5.2	8	12	12	0	4	4
iBAT	3.4±0.6	4.1±0.8	4.4±0.9	39.6±7.0	17.7±5.8	17.6±5.1	36.1	13.7	13.2	11.6	4.3	4.0	8	12	18	0	4	0

Table S1. Parameters of [^{3}H]oleate uptake by metabolic organs in mice adapted to short, normal and long daily light exposure. Related to Figure 1. Wild-type mice were subjected to short (8h), normal (12h) or long (16h) daily light exposure (LE) at an ambient temperature of 22°C for five weeks. Mice were injected with glycerol tri[^{3}H]oleate-containing lipoprotein-like particles at 6 time points (n=4/ group), sacrificed and uptake of [^{3}H]oleate derived activity was determined for liver, subcutaneous white adipose tissue (sWAT), gonadal white adipose tissue (gWAT, heart, muscle, perivascular white adipose tissue (pVAT), subscapular brown adipose tissue (sBAT) and interscapular brown adipose tissue (iBAT). Minimum and maximum fatty acid (FA) uptake (% of injected dose per gram) were taken from observations at two of these 6 time points, the difference being defined as range of FA uptake. Peak and trough times are given as Zeitgeber times (ZT) in hours (ZT0 represents lights on). Data presented as means ± SEM.

	All (n=37)	Men (n=19)	Women (n=18)
Age (y)	65.2 ± 0.9	65.7 ± 1.2	64.6 ± 1.2
BMI (kg/m^2)[#]	25.1 ± 0.6	25.5 ± 0.7	24.6 ± 1.1
Fat mass (%)[#]	31.5 ± 1.4	24.8 ± 1.0	38.2± 1.2 [$$$]
Current smokers (n)	1	1	0

Table S2. Characteristics of the study population. Related to Figure 5. Data are presented as means ± SEM or number (current smokers). [$$$] $p<0.001$ men *vs.* women. [#]data of 1 person are missing.

Gene	Forward primer	Reverse primer
Angptl4	GGAAAGAGGCTTCCCAAGAT	TCCCAGGACTGGTTGAAGTC
Bmal1	ATGCCAAGACTGGACTTCCG	TGCAGAAGCTTTTTCGATCTGC
Bmal2	CAGGAGTATCAGCGCCCAGT	CCTTCTGCTGCCTTGAGGATTA
Clock	AGTTAGGGCTGAAAGACGGC	GGTGTGGAGGAAGGGTCTGA
Cry1	AGAGGGCTAGGTCTTCTCGC	GTGAGTCTGCTGACTGTCCC
Cry2	CCAGAGCACTATCCAGTGGC	GCGATGGCTCTCCAGTCTC
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Lpl	CCCTAAGGACCCCTGAAGAC	GGCCCGATACAACCAGTCTA
Per1	ACGGCCAGGTGTCGTGATTA	CCCTTCTAGGGGACCACTCA
Per2	TGTGTGCTTACACGGGTGTCCTA	ACGTTTGGTTTGCGCATGAA
Rev-Erbα	GTGCTTGTCTCTGCAGACCG	TTGGTGAAGCGGGAAGTCTC
Rplp0 (36b4)	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Ucp1	TCAGGATTGGCCTCTACGAC	TGCATTCTGACCTTCACGAC

Table S3. Primer sequences.

Supplemental Experimental Procedures

Animal Husbandry
For all experiments, mice were single housed in clear plastic standard type II cages with stainless steel wire lids and a hinged divider. The cage was enriched with a 1-cm layer of sawdust bedding (Woody Clean S 8/15, Rettenmaier, Germany) and paper tissues. Cages were placed within light-tight cabinets at a constant room temperature of 22°C with *ad libitum* access to food and water, unless indicated otherwise. The cages were illuminated with white fluorescent light with an intensity of approximately 85 $\mu W/cm^2$. Upon arrival at our facility, mice were kept for at least two weeks on a standard laboratory 12h light: 12h dark cycle. Experiments that took place during dark period were performed under red light illumination.

Wild type mice experiments
12 week old male C57Bl/6J mice (Charles River) were matched on body weight. Mice were housed on 12h light: 12h dark cycle (normal daily light exposure (LE), 8h light: 16h dark cycle (short daily LE) or a 16h light: 8h dark cycle (long daily LE) (n=24 per group). Mice were fed a standard laboratory chow (Macronutrient distribution: 7% fat, 18% protein, 75% carbohydrates; Special Diets Services, UK) and after five weeks, the uptake of triglyceride (TG)-derived fatty acid (FA) by metabolic organs was assessed (see below) at time points ZT (Zeitgeber Time, time (h) after lights on) 0, 4, 6, 8, 12 and 18. At the end of this experiment, mice were killed via cervical dislocation, perfused for 5 minutes with ice cold PBS and organs were collected for radiolabel uptake, gene and protein expression analysis (see below).
In an addition group of male C57Bl/6J mice, 24 h rhythms in food intake, energy expenditure and substrate utilization was determined by means of indirect calorimetry in metabolic cages (PhenoMaster, TSE systems, Bad Homburg, Germany) containing automatized scales placed inside the calorimeters. All mice were first measured at 12h LE and subsequently housed in light tight cabinets and split into two groups which were acclimated to either short or long LE for five weeks. During the last week, mice were again housed in metabolic chambers to assess the effects of LE on energy metabolism.
Bilateral sympathetic denervation of iBAT was performed under isofluorane inhalation. A midline incision of the skin was made and the anterior side of both iBAT pads was exposed. Sympathetic branches were visualized and cut on both sides. Wounds were closed and mice received postoperative analgesia (0.03 mg/kg buprenorphine; Temgesic, Merck). After one week of recovery, the uptake of TG-derived FA by BAT was assessed (see below) at time points ZT0 and ZT12.
Bilateral adrenalectomy was performed under isoflurane inhalation through a dorsal midline skin incision and lateral retroperitoneal incisions. Wounds were closed using a Michel suture clip and to compensate for the loss in adrenal function all mice were given an subcutaneous injection with 0.5 mL of 0.9% NaCl. After one week of recovery, the uptake of TG-derived FA by BAT was assessed (see below) at time points ZT0 and ZT12.

Indirect calorimetry and food intake
For the measurements of food intake, energy expenditure and respiratory exchange ratio, male C57Bl/6J mice fed chow diet were previously acclimated to the indirect calorimeters for a 2-day period. All experiments took place at 22°C, while food and water were provided *ad libitum*. Data points were collected at 20-min intervals.

Preparation of radiolabeled lipoprotein-like particles
Radiolabeled lipoprotein-like emulsion particles were prepared from 100 mg of total lipid including triolein (70 mg), egg yolk phosphatidylcholine (22.7 mg), lysophosphatidylcholine (2.3 mg), cholesteryl oleate (3.0 mg) and cholesterol (2.0 mg), with addition of glycerol tri[^{3}H]oleate (100 μCi). Sonication was performed using a Soniprep 150 (MSE Scientific Instruments, UK) equipped with a water bath for temperature (54°C) maintenance, at 10 μm output. The emulsion was fractionated by consecutive density gradient ultracentrifugation steps. After centrifugation for 27 min at 20,000 rpm at 20°C, an emulsion fraction containing chylomicron-like particles (average size 150 nm) was removed from the top of the tube by aspiration. Following a subsequent centrifugation step for 27 min at 40,000 rpm large VLDL-like particles (average size 80 nm) were obtained in a similar manner and used for in vivo kinetic experiments. Emulsions were stored at 4°C under argon and used within 5 days following preparation.
We previously showed that these particles rapidly acquire an array of exchangeable apolipoproteins from serum, including apoE, apoCs, apoAIV, apoAI, apoAII and apoD (Rensen et al., 1997; Rensen et al., 1995), and that the hepatic uptake of their core remnants is mediated by apoE (Rensen et al., 1997). In fact, the *in vivo* kinetics of

[^{3}H]cholesteryl oleate-labeled TRL-mimicking particles (Rensen et al., 1997) are very similar to those of [^{3}H]vitamin A-labeled native chylomicrons (van Dijk et al., 1992), with similar clearance rate from plasma (t½ ~2 min) and uptake by the liver (~65%-75% after 30 min). Lactoferrin reduced the uptake by the liver of both TRL-mimicking particles and chylomicrons by ~75% (van Dijk et al., 1992) and liver cell distribution studies confirmed that hepatocytes accounted for ~75% of the total uptake by the liver (van Dijk et al., 1992). The use of glycerol tri[3H]oleate-labeled TG-rich lipoprotein-like particles has been extensively described by our group, often to study changes in BAT activity as reviewed in (Hoeke et al., 2016). LPL is required for the liberation of [^{3}H]oleate as has been shown *in vitro* and *in vivo* (Rensen and van Berkel, 1996). Importantly, we have shown that BAT takes up TG preferentially by means of LPL-mediated uptake of TG-derived FA (Khedoe et al., 2015) and this processing is strongly reduced by the endogenous LPL inhibitor ANGPTL4 (Dijk et al., 2015)

Dyslipidemic mice experiments
Homozygous human cholesteryl ester transfer protein (CETP) transgenic mice were crossbred with hemizygous APOE*3-Leiden mice at our Institutional animal facility to obtain APOE*3-Leiden.CETP mice (C57Bl/6J background). Female mice were used as these specifically develop high levels of plasma triglycerides and cholesterol. Mice of 9-12 weeks old were fed Western-type diet (WTD; caloric energy: 35% fat, 17% protein, 48% carbohydrates; addition of 0.1% cholesterol; AB Diets, Woerden, the Netherlands). After 3 weeks, mice were matched for body weight and plasma TG and adapted to either short or long daily LE (n=27/group). In a subset of mice (n=5/group) a telemetric transmitter was implanted under isoflurane anesthesia. A small incision was made between the shoulder blades via which a TA-F10 miniature transmitter (Data Sciences International, MN, USA) was inserted subcutaneously in the flank. Data acquisition was performed using Dataquest A.R.T. TM Software (Data Sciences International). In the same subset of mice, after four weeks, seven consecutive blood samples via venous tail bleeding were obtained at time points ZT 0, 4, 8, 12, 16, 20, 24 and one week later, stress-free blood samples were obtained at ZT0, 8 and 16 by drawing blood within one minute of handling the mouse. Fasting and postprandial lipids were determined either at ZT 0, 8 or 16 (see below). After five weeks, the uptake of TG-derived FA by metabolic organs and clearance from the circulation was determined (see below) at the same time points.

Biochemical analyses
Mouse blood was collected into chilled paraoxon-coated capillaries to prevent *ex vivo* lipolysis. Capillaries were centrifuged at RT and plasma was isolated and stored at -80°C. Human blood samples were collected and kept at RT for 1 h. Serum was isolated and aliquoted into 500 µL tubes and stored at -80°C. Plasma was assayed using a commercially available enzymatic kit for TG (Roche Diagnostics, Mannheim, Germany) and free FA (NEFA-C kit, Wako Diagnostics, Instruchemie, Delfzijl, the Netherlands).

Gene expression
A part of iBAT was snap frozen and stored at -80°C for gene expression analysis. Total RNA was isolated using TriPure (Roche) according to the manufacturer's instructions (for Rev-erb$^{-/-}$ experiments, RNA was isolated by TRI reagent (Sigma) using a Tissuelyzer (Qiagen)). 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was carried out on a CFX96 PCR machine using IQ SYBR-Green Supermix (Bio-Rad). Primer sequences are indicated in Table S3. Melt curve analysis was included to assure a single PCR product was formed. Expression levels were normalized to *36b4* and *Hprt* housekeeping gene expression. Data was plotted as relative expression to expression in normal 12h daily LE group at ZT0. Primer sequences are available on request.

Western blots
A part of iBAT was snap frozen and stored at -80°C for gene expression analysis. To extract proteins, BAT was lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 1% SDS; Thermo Fisher-Scientific, Landsmeer, the Netherlands) supplemented with Complete Protease and phosSTOP phosphatase inhibitors (Roche Diagnostics, Almere, the Netherlands) with the Qiagen Tissuelyser II (Qiagen, Venlo, the Netherlands). Upon homogenization, lysates were placed on ice for 30 minutes and centrifuged 4 times at 13,000 rpm for 10 min at 4˚C to remove fat and cell debris. Protein concentrations were determined by using a bicinchoninic acid (BCA) assay (Thermo Fisher-Scientific). Equal amounts of protein (11.25 µg (LPL/HSP90) or 40 µg (ANGPTL4) protein per lane) were combined with 2x Laemmli sample buffer, boiled at 95˚C for 5 minutes, and loaded onto 8-16% (LPL/HSP90) or 10% (ANGPTL4) Criterion gradient gels (Bio-Rad, Veenendaal, The

Netherlands). Next, proteins were transferred onto a PVDF membrane using the Transblot Turbo System (Bio-Rad). Membranes were probed with a goat anti-mouse LPL antibody (kind gift from André Bensadoun); a rabbit anti-mouse HSP90 antibody (Cell Signaling Technology, #4874); or a rat anti-mouse ANGPTL4 antibody (Adipogen, Kairos 142-2) at 1:5000 (LPL), 1:2000 (HSP90) or 1:1000 (ANGPTL4) dilution. Tris-buffered saline, pH 7.5, with 0.1% Tween-20 (TBS-T) and 5% w/v skimmed milk was used to block membranes and for primary and secondary antibody incubations. In between, membranes were washed in TBS-T only. Imaging of western blots was performed with the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad). Western blot quantifications were done with Image Lab software (Bio-Rad).

Mature glycosylated LPL (LPL in the Golgi and on the cell surface) was determined by digesting 40 µg of protein with EndoH (New England BioLabs) according to the manufacturer's instructions. Briefly, BAT protein lysates were combined with glycoprotein denaturing buffer and boiled for 10 minutes at 95˚C. Following the addition of EndoH, the samples were incubated at 37 ˚C for 1h and, subsequently, combined with 5x Laemmli sample buffer, boiled at 95˚C for 5 minutes and loaded onto 10% Criterion gradient gels (Bio-Rad) for analysis by western blotting as described above. EndoH-resistant LPL represents mature glycosylated LPL located in the Golgi or on the cell surface, whereas EndoH-sensitive LPL represents LPL located in the endoplasmic reticulum with oligosaccharide side chains high in mannose residues.

Human study

38 healthy participants were recruited within the Switchbox study for in-depth endocrine and metabolic phenotyping, which included diurnal venous blood. Participants were included over a total period of 1.5 years without preference for inclusion during a particular season. To be included in the Switchbox study, participants had to have a fasting glucose level <7 mM, hemoglobin level <7.1 mM, a body mass index (BMI) between 19 kg/m^2 and 33 kg/m^2 and had to be free of any significant chronic disease. Of the 38 participants, one participant was excluded because of a newly diagnosed hypertriglyceridemia, leaving 37 participants for the present study. After an overnight fast of 10-14 h, a catheter was inserted before start of the study, and blood sampling started at 09:00h. Every 10 min 1.2 mL of blood was collected in a K3-EDTA tube and 2 mL of blood in a serum separator (SST)-tube. Participants received three standardized liquid meals at 09:00h, 12:00h and 18:00h during a day. All meals consisted of 600 kcal Nutridrink, containing a standard percentage of energy derived from fat (35%), carbohydrates (49%) and protein (16%) (Nutricia Advanced Medical Nutrition, Zoetermeer, the Netherlands). All participants were sampled in the same room with standardized ambient conditions, 630 ± 10 lux of light intensity and 22 ± 2 °C. Participants were not allowed to sleep during the day, and except for lavatory use no physical activity was allowed during the study period. Lights were turned off between 23:00h to 08:00h to allow the participants to sleep.

Supplemental References

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