Title: Obesity in mice with adipocyte-specific deletion of clock component *Bmal1*

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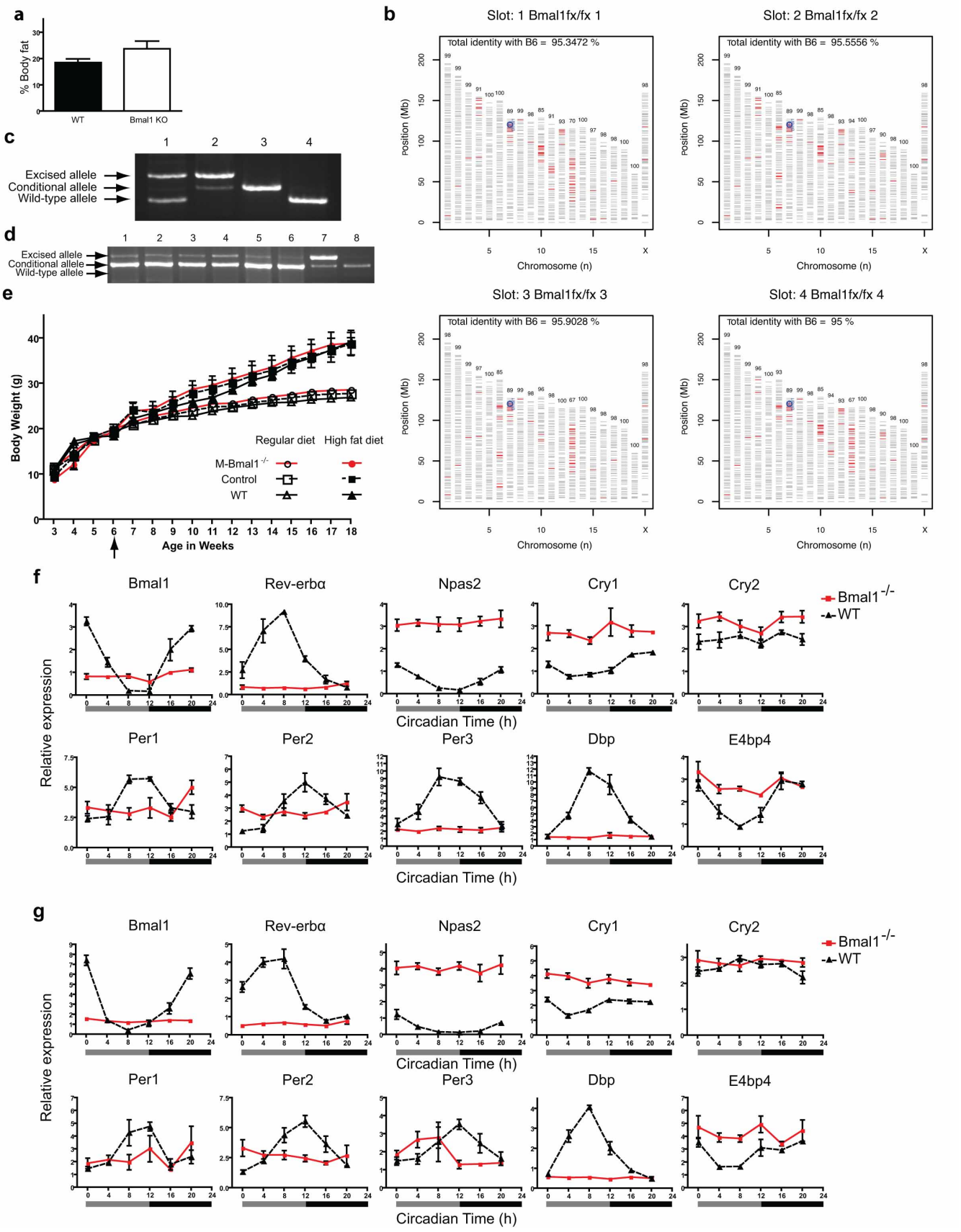
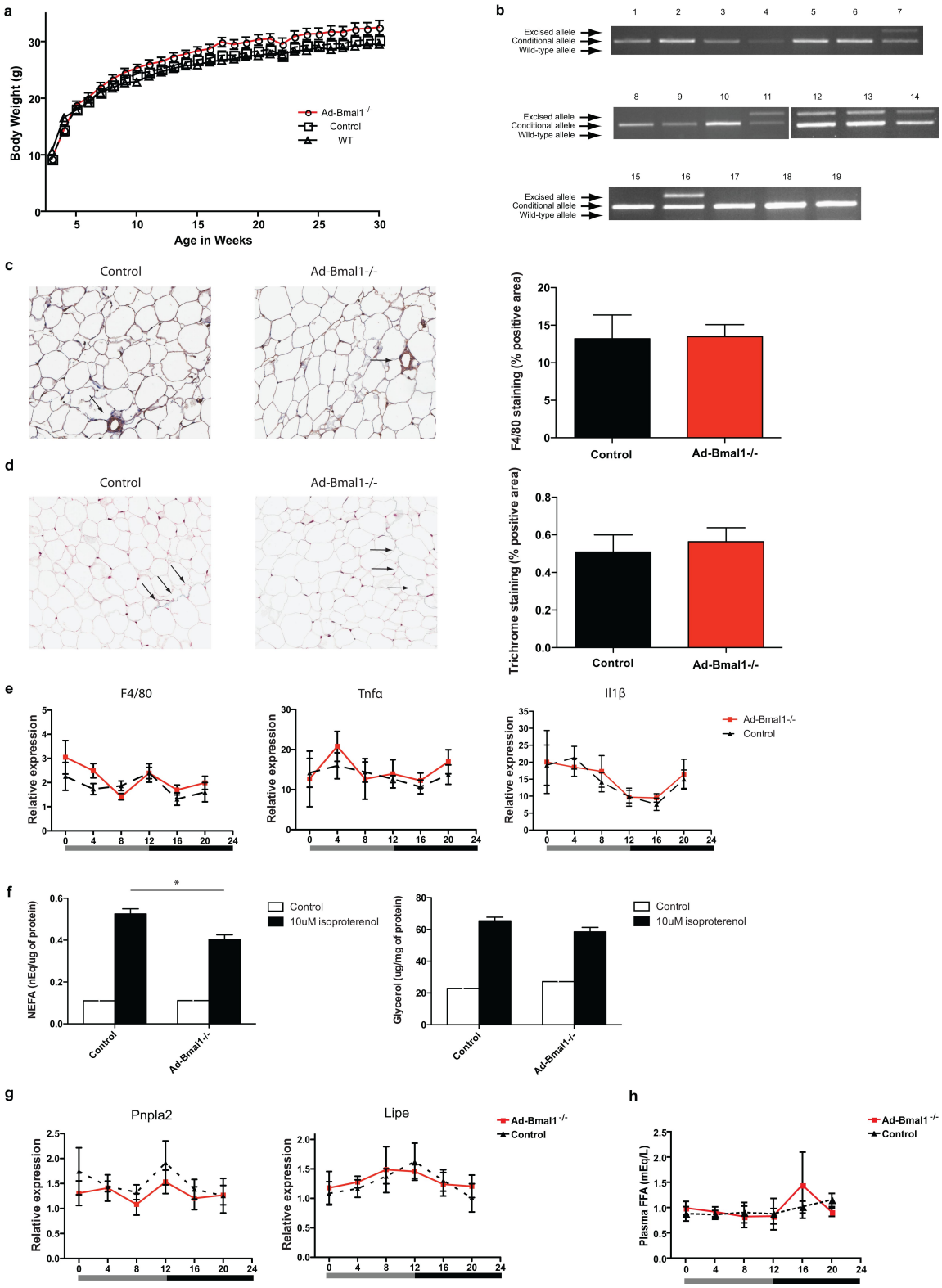


Supplementary Figure 1. Characterization of *Bmal1* knockout mice, adipocyte-specific *Bmal1*$^{-/-}$ mice and myeloid cell specific *Bmal1*$^{-/-}$ mice

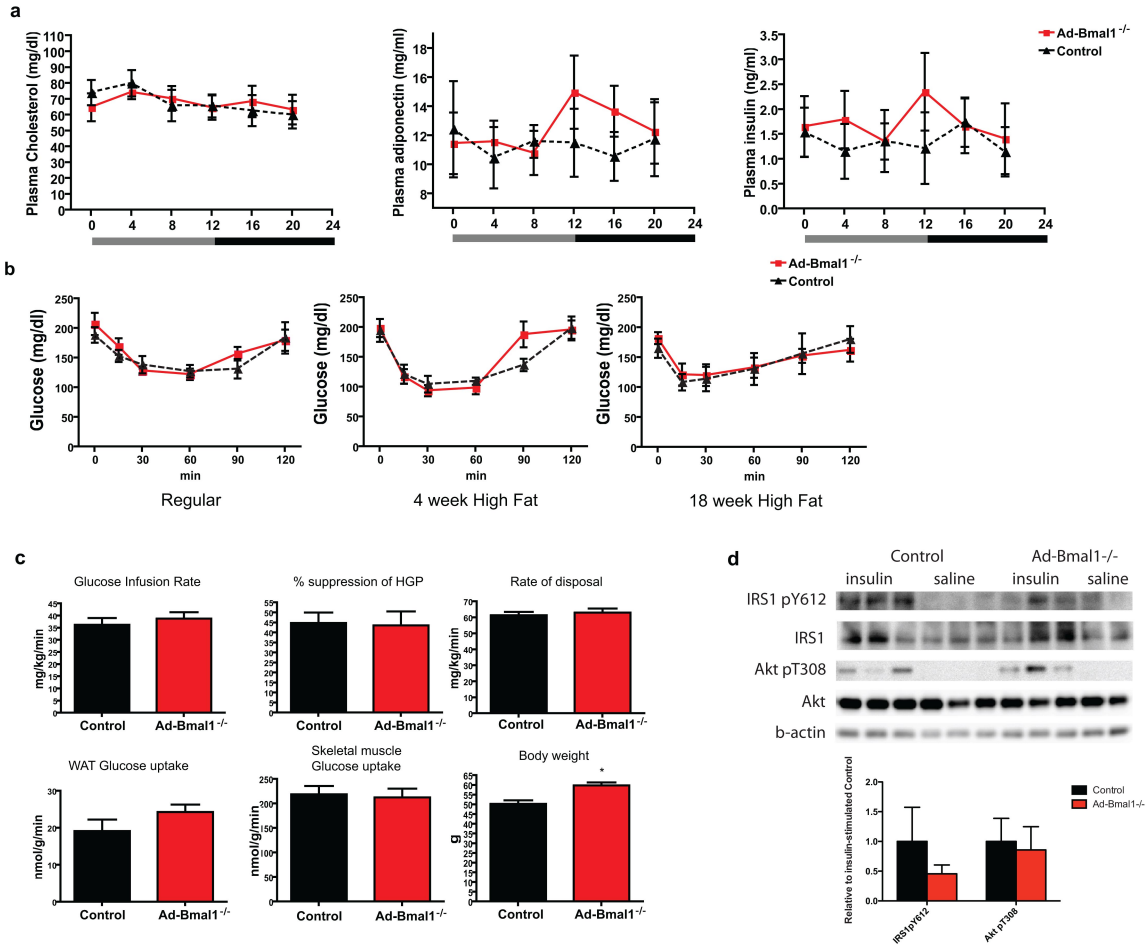
(**a**) Adipose tissue mass in WT and Bmal1 KO mice, presented as a percentage of body weight (n=4, *P*=0.08). Error bars, s.e.m. (**b**) Similarity to the C57BL/6J background for aP2-Cre+/- Bmal1fx/fx mice analyzed with a mouse genome SNP array (illumina Mouse MD Linkage). (**c**) PCR products amplified from genomic DNA isolated from peritoneal macrophages. (1), ubiquitous Ella-Cre+/- Bmal1fx/+; (2), LysM-Cre +/- Bmal1fx/fx; (3), Bmal1fx/fx, no Cre; (4), Bmal1+/+, no Cre. (**d**) PCR products amplified from genomic DNA isolated from different tissues of LysM-Cre +/- Bmal1fx/fx mice. (1), liver; (2), adrenals; (3), skeletal muscle; (4), white adipose tissue; (5), brown adipose tissue; (6), hypothalamus; (7),

peritoneal macrophages; (8), epididymal adipocytes. (**e**) Body weights of mice with myeloid cell-specific deletion of Bmal1 (M-Bmal1$^{-/-}$), Bmal1fx/fx control and wild-type (WT) mice fed either a regular chow (open symbols) or high-fat diet starting at 6 weeks of age (regular chow diet, n=10; high fat diet, n=8). (**f,g**) White adipose tissue (**f**) and brown adipose tissue (**g**) from Bmal1 knockout mice (Bmal1$^{-/-}$) having ad libitum access to food. Tissues were collected from 14-week-old mice (n=3 for each time-point and group). Relative expression levels are normalized to Gapdh and plotted in arbitrary linear units (mean±s.e.m.).



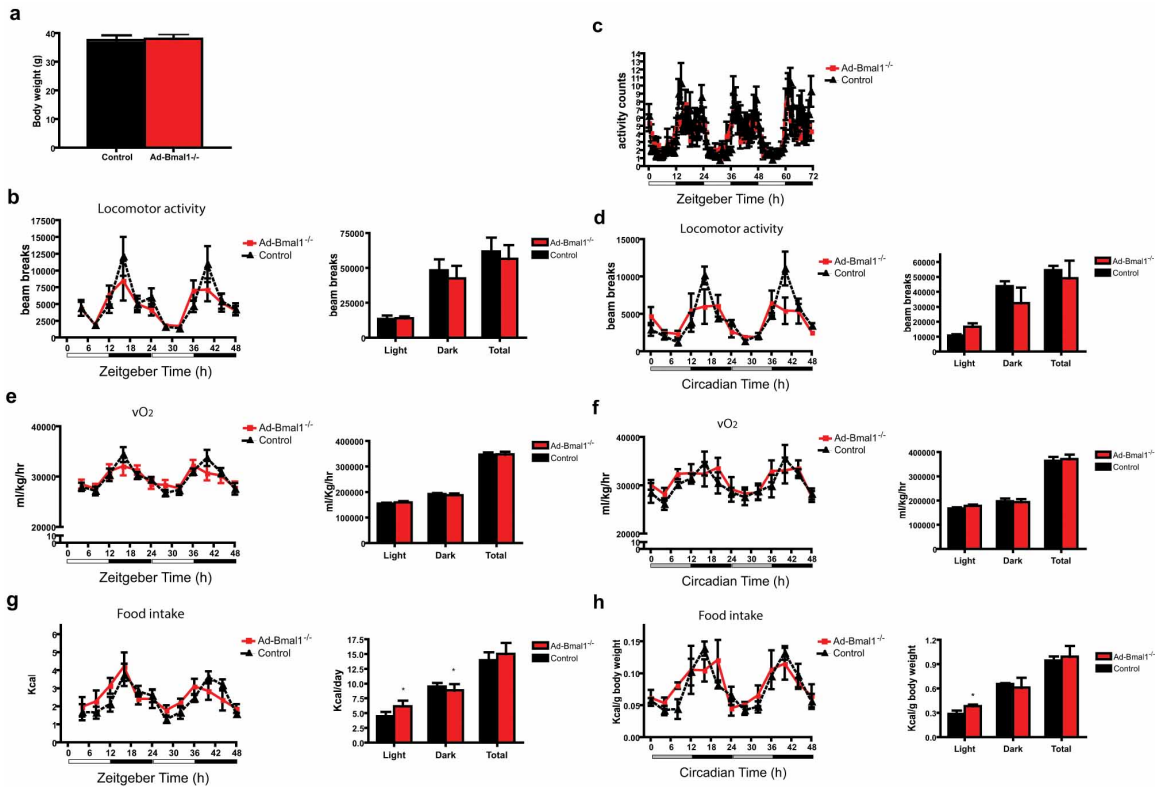
Supplementary Figure 2. Body weight and metabolic parameters of Ad-Bmal1$^{-/-}$ mice
(**a**) Body weights of Ad-Bmal1$^{-/-}$, Bmal1fx/fx control and wild-type (WT) mice fed a regular chow diet followed for up to 30 weeks of age (n=12). (**b**) PCR products amplified from genomic DNA isolated from different tissues of adiponectin-Cre+/- Bmal1fx/fx mice. (1), heart; (2), lung; (3), liver; (4), kidney; (5), spleen; (6), adrenals; (7), epididymal white adipose tissue; (8), hypothalamus; (9), forebrain; (10), cerebellum; (11), interscapular brown adipose tissue; (12), perirenal white adipose tissue; (13), epididymal

white adipose tissue; (14), subcutaneous white adipose tissue; (15), skeletal muscle; (16), interscapular brown adipose tissue; (17), midbrain; (18), aorta; (19), posterior brain cortex. (**c**) Indicative images of immunohistochemistry with antibody against F4/80 on epididymal adipose tissue from 20-week-old mice. Arrows show crown like structures. Bar graph shows quantification of F4/80 staining signal in 10 selected areas from each of four mice per group. (**d**) Indicative images of Trichrome staining on epididymal adipose tissue from 20-week-old mice. Arrows show areas of staining. Bar graph shows quantification of Trichrome staining signal in 10 selected areas from each of four mice per group. (**e**) Gene expression in epididymal adipose tissue from Ad-Bmal1$^{-/-}$ and control mice. (**f**) Ex vivo lipolysis in epididymal adipose tissue collected at ZT4 from Ad-Bmal1$^{-/-}$ and control mice. Adipose tissue explants were incubated for 4 hours in culture media containing or not 10uM isoproterenol. Non-esterified fatty acid and glycerol concentrations were determined in the media as an indication of lipolysis. (**g**) Adipose triglyceride lipase (*Pnpla2*) and hormone sensitive lipase (*Lipe*) expression in epididymal adipose tissue from Ad-Bmal1$^{-/-}$ and control mice. Tissues were collected from 20-week-old mice kept in constant darkness and fed ad libitum a regular diet (n=4 for each time-point and group). Relative expression levels are normalized to Gapdh and plotted in arbitrary linear units (mean±s.e.m.). (**h**) Plasma levels of free fatty acids (FFA) at different time-points of circadian time. 20-week-old mice were kept under constant darkness and had ad-libitum access to regular chow diet (n=4 for each time-point and group). Error bars, s.e.m.



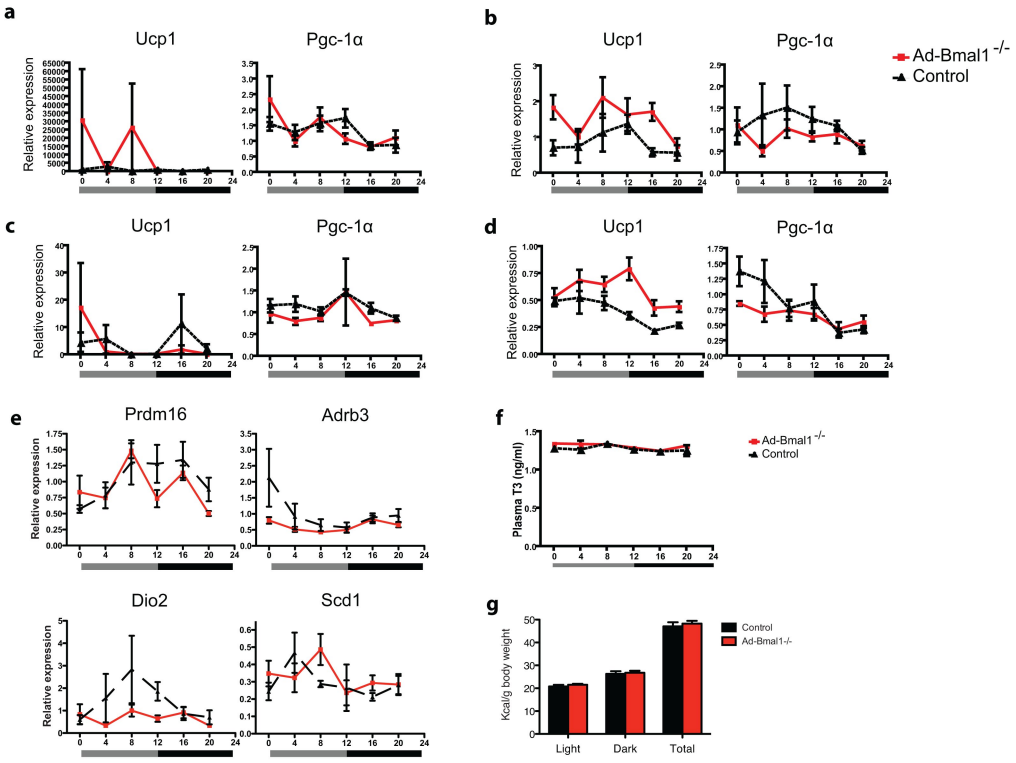
Supplementary Figure 3. Sensitivity to insulin in Ad-Bmal1$^{-/-}$ mice

(**a**) Plasma levels of total cholesterol, adiponectin and insulin at different time-points of circadian time. 20-week-old mice were kept under constant darkness and had ad-libitum access to regular chow diet (n=4 for each time-point and group, *$P < 0.05$). (**b**) Insulin tolerance tests in Ad-Bmal1$^{-/-}$ and control mice on different diets (mice 20 weeks, 20 weeks and 24 weeks old respectively, n=4). (**c**) Euglycemic hyperinsulinemic clamp in 1 year old Ad-Bmal1$^{-/-}$ and control mice on high fat diet for 40 weeks (n=8, *$P < 0.05$). (**d**) 20-week old mice were fasted for 16 hours, injected intraperitoneally with saline or insulin at 10 units/kg of body weight, and sacrificed 30 min thereafter. Insulin stimulated site-specific phosphorylation of IRS1 and Akt as assessed by immunoblotting analysis. For quantification, phosphorylated proteins were normalized to the corresponding total proteins. Beta-actin served as loading control. Error bars, s.e.m.



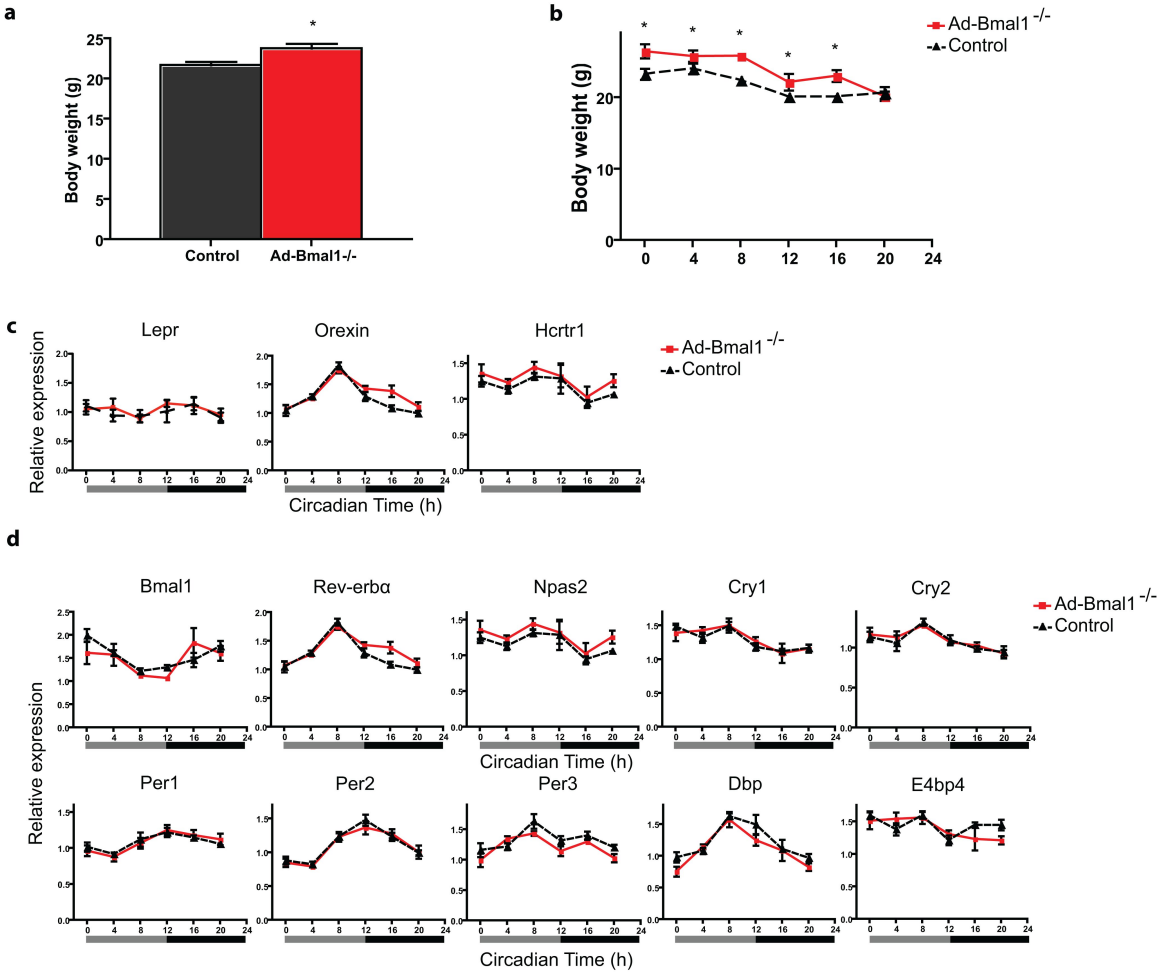
Supplementary Figure 4. Behavioral analysis of regular diet fed Ad-Bmal1$^{-/-}$ mice under light-dark and constant darkness conditions

(**a**) Body weight of 20-week-old mice undergoing behavioral and calorimetric analysis. Mice were fed HFD for one week. Locomotor activity (**b-d**), O_2 consumption rate (vO_2) (**e,f**), and food intake (**g,h**) were monitored over two consecutive 24 hour cycles for mice kept in 12h light: 12h dark conditions (**b,c,e,g**) and constant darkness (**d,f,h**) (n=8 per group, *$P < 0.05$, ** $P < 0.01$). vO2 values are corrected for body weight. Correction for lean body mass produced similar results. Body weight and lean mass was not different between mice in the two groups.

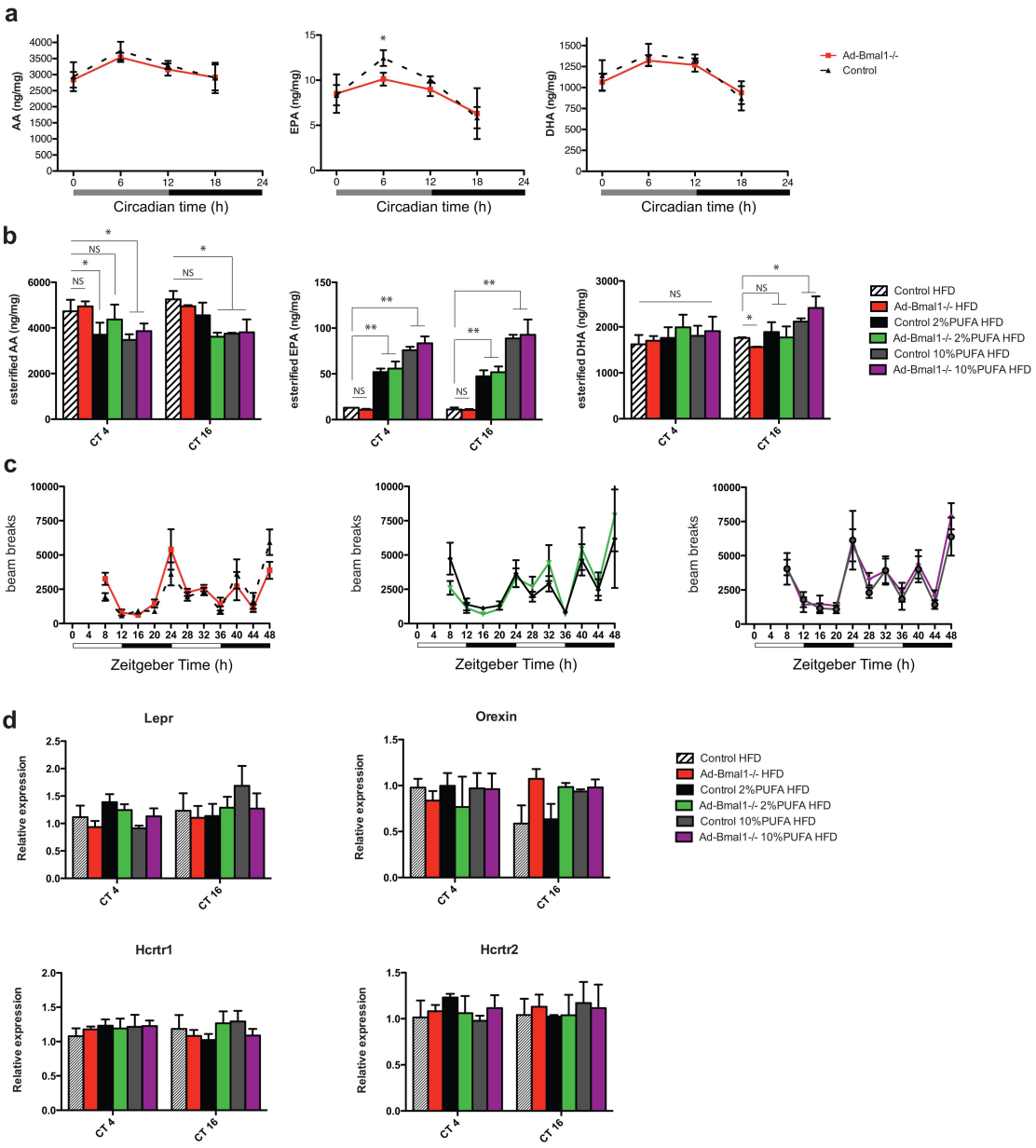


Supplementary Figure 5. Expression of genes encoding regulators of thermogenesis in white and brown adipose tissue, plasma triiodothyronine (T3) and heat production in vivo

(**a-d**) Ucp-1 and Pgc-1a gene expression in white (**a,c**) and brown (**b,d**) adipose tissue from adipocyte-specific Bmal1 knockout mice (Ad-Bmal1$^{-/-}$) fed ad libitum (**a,b**) or fasted for 24 hours prior to tissue collection (**c,d**). Tissues were collected from 20-week-old mice fed a regular diet (n=4 for each time-point and group). Relative expression levels are normalized to Gapdh and plotted in arbitrary linear units (mean±s.e.m.). (**e**) Expression of genes encoding key regulators of thermogenesis in brown adipose tissue from Ad-Bmal1$^{-/-}$ mice fed ad libitum. (**f**) Plasma levels of T3 at different time-points of circadian time. 20-week-old mice were kept under constant darkness and had ad-libitum access to regular chow diet (n=4 for each time-point and group). Error bars, s.e.m. (**g**) Heat production was monitored over two consecutive 24 hour cycles for 20-week-old mice undergoing behavioral and calorimetric analysis. Mice were kept in 12h light: 12h dark conditions. Results are represented as totals for light and dark periods (n=8 per group), corrected for lean body mass.



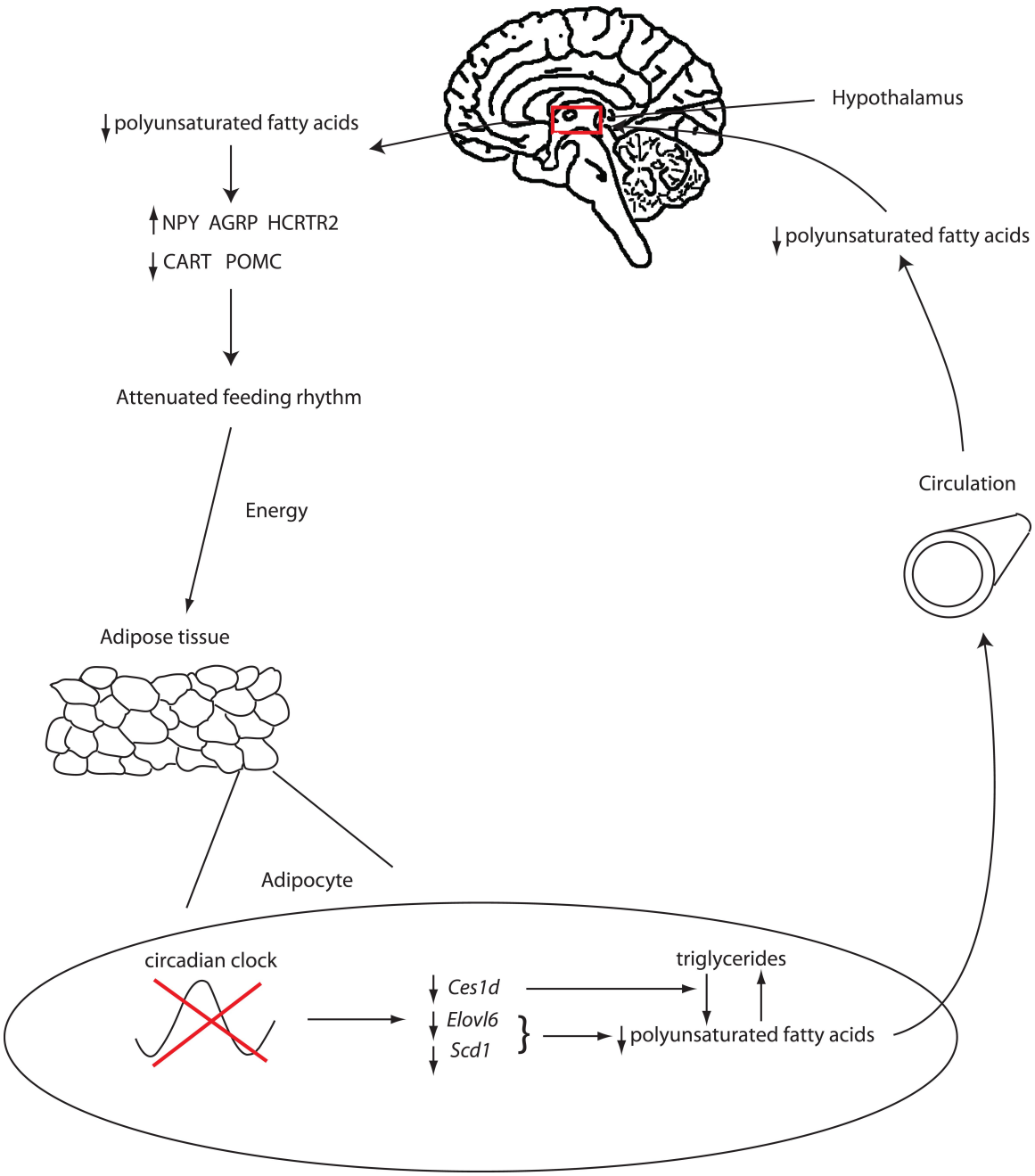
Supplementary Figure 6. Hypothalamic expression of neuropeptide and clock genes
(**a**) Body weight of 20-week-old mice. Ad-Bmal1$^{-/-}$ mice have increased weight compared to controls. (**b**) Body weight of mice participating at different time points of the experiment. (**c**) Expression of leptin receptor (Lepr), orexin, orexin receptor 1 (Hcrtr1) in hypothalamic sections from adipocyte-specific Bmal1 knockout mice (Ad-Bmal1$^{-/-}$) being fasted for 24 hours prior to tissue collection. Hypothalamic sections were collected from 20-week-old mice fed a regular diet (n=4 for each time-point and group). Relative expression levels are normalized to Gapdh and plotted in arbitrary linear units (mean±s.e.m.). Results are representative of three independent experiments. (**d**) Clock and clock output gene expression in hypothalamic sections from adipocyte-specific Bmal1 knockout mice (Ad-Bmal1$^{-/-}$) being fasted for 24 hours prior to tissue collection. Hypothalamic sections were collected from 20-week-old mice fed a regular diet (n=4 for each time-point and group). Relative expression levels are normalized to Gapdh and plotted in arbitrary linear units (mean±s.e.m.). Results are representative of three independent experiments.



Supplementary Figure 7. Hypothalamic esterified fatty acids and neuropeptide expression in Ad-Bmal1$^{-/-}$ mice fed high polyunsaturated fatty acid diets

(**a**) Hypothalamic levels of esterified forms of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in regular diet-fed 20-week-old mice kept in constant darkness and fasted for 24 hours prior to tissue collection (n=6 for each time-point and group; values are ng/mg of tissue). (**b-d**) 30-week old Ad-Bmal1$^{-/-}$ and control mice were fed three diets: a high fat diet providing 43% of total energy from fat (HFD), a diet providing 2% of energy from eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) (1.2% EPA, 0.8% DHA), 43% of total energy from fat (2% PUFA HFD), a diet providing 10% of energy from EPA, DHA (6% EPA, 4% DHA), 43% of total energy from fat (10% PUFA HFD) (n=6 for each group and diet, *$P < 0.05$, ** $P < 0.01$, NS: non significant). (**b**) Hypothalamic levels of esterified arachidonic acid (AA), EPA, DHA after 8 weeks in the diet at circadian time (CT) 4 and 16. Ad-Bmal1$^{-/-}$ and control mice were kept in constant darkness and fasted for 24 hours prior to tissue collection. (**c**) Locomotor activity monitored over two consecutive 24 hour cycles. Mice in the three diets were kept in 12h light: 12h dark conditions. (**d**) Hypothalamic expression of appetite regulating neuropeptides after 8

weeks in the diet at CT4, CT16. mRNA levels are quantified in hypothalamic sections isolated from mice kept in constant darkness and fasted for 24 hours prior to tissue collection. Relative expression levels are normalized to *Gapdh* and plotted in arbitrary linear units. In all experiments, data are expressed as mean±s.e.m.



Supplementary Figure 8. Graphical representation of the suggested mechanistic explanation of the obesity in Ad-Bmal1$^{-/-}$ mice

Disruption of the adipocyte circadian clock results in a reduced capacity of the cell to synthesize long-chain polyunsaturated fatty acids and a time-specific reduction in triglyceride hydrolysis. This leads to reduced plasma long-chain polyunsaturated fatty acids during the light (rest) period of the day. The reduction is reflected in hypothalamic neurons regulating food intake, leading to changes in orexigenic, anorexigenic neuropeptides that promote increased food intake during the light period. Energy from food consumption during the light period (an "inappropriate" time) is preferably stored in the adipose tissue compared to energy from food intake during the night period.

Supplementary Methods

Mouse genotyping

Mouse genotypes were assessed by PCR on genomic DNA from tail tips using the following primers: Cre recombinase, 5'-CGA TGC AAC GAG TGA TGA GG-3' (forward) and 3'-GCA TTG CTG TCA CTT GGT CGT-5' (reverse); *Bmal1* WT or fx, 5'-AAT CAC CTT TTG GGG AGG AC-3' (forward), 5'-TCA TCA GAG GAA CCA GGG TAA-3' (forward), and 5'-CCC TGA ACA TGG GAA AGA GA-5' (reverse).

Real-time PCR primers

Bmal1 5'-AGGCCCACAGTCAGATTGAAA-3' 3'-CCAAAGAAGCCAATTCATCAATG-5'

Rev-erbα 5'-CCCAACGACAACAACCTTTTG-3' 3'-CCCTGGCGTAGACCATTCAG-5'

Npas2 5'-GCTGATGTTGGAGGCATTAGATG-3' 3'-CATAGATGATGCTGCCGTCTGT-5'

Cry1 5'-TCGCCGGCTCTTCCAA-3' 3'-TCAAGACACTGAAGCAAAAATCG-5'

Cry2 5'-CCCACGGCCCATCGT-3' 3'-TGCTTCATTCGTTCAATGTTGAG-5'

Per1 5'-TCGAAACCAGGACACCTTCTCT-3' 3'-GGGCACCCCGAAACACA-5'

Per2 5'-GCTCGCCATCCACAAGAAG-3' 3'-GCGGAATCGAATGGGAGAATA-5'

Per3 5'-GGCTGCTTTGATCCTGAATTCT-3' 3'-GAACGCCCTCACGTCTTGAG-5'

Dbp 5’-CACCGTGGAGGTGCTAATGA-3’ 3’-GCTTGACAGGGCGAGATCA-5’

E4bp4 5’-GCTCAAGAGATTCATAGCCACACA-3’ 3’-GCTCGGTCAGCAGCCATTT-5’

Npy 5’-TCCGCTCTGCGACACTACAT-3’ 3’-GGGCTGGATCTCTTGCCATA-5’

Agrp 5’-AGCTTTGGCGGAGGTGCTA-3’ 3’-GCGACGCGGAGAACGA-5’

Cart 5’-CCGAGCCCTGGACATCTACTC-3’ 3’-CGCTTCGATCAGCTCCTTCT-5’

Pomc 5’-GACTAGGCCTGACACGTGGAA-3’ 3’-GGCCCCTGAGCGACTGTA-5’

Ucp-1 5’-CAGTACCCAAGCGTACCAAGCT-3’ 3’-GGCCGTCGGTCCTTCCT-5’

Pgc-1α 5’-GGACAGTCTCCCCGTGGAT-3’ 3’-TCCATCTGTCAGTGCATCAAATG-5’

Elovl6 5’-TGCTCTTCTGCCATTTCTTCTTT-3’ 3’-TCAGCCTTCGTGGCTTTCTT-5’

Scd1 5’-CAACACCATGGCGTTCCA-3’ 3’-TGGGCGCGGTGATCTC-5’

Ces1d 5’-CATAGTGGGCATCAACAAGCA-3’ 3’-GCGAGTGGATAGCCCATAAGC-5’

Cnr1 5’- GGCCCAAGGCCGTAGTG-3’ 3’- GGAGAGGCAACACAGCAATTACT-5’

Gapdh 5’-CATGGCCTTCCGTGTTCCT-3’ 3’-GCGGCACGTCAGATCCA-5’

Hyperinsulinemic-euglycemic clamp Intravenous infusion of [3-^{3}H] glucose (5 μCi bolus, 0.05 μCi/min) was used as described previously[1,2]. Human insulin (16 mU/kg; Eli Lilly, Indianapolis, IN) was injected as a bolus, followed by continuous infusion at 2.5

mU/kg/min. Tail blood glucose was measured by glucometer at 10 min intervals, and 20% glucose was infused to maintain blood glucose at euglycemic levels (120 and 140 mg/dl). After steady state had been maintained for 1 h, the glucose uptake in various tissues was determined by injecting 2-deoxy-D-[1-^{14}C] glucose (2-[^{14}C]DG) (10 mCi) 45 min before the end of clamps. During the final 50 min of basal and clamp infusions, 20 µl blood samples were collected at 10 min intervals for measurement of ^{3}[H] glucose, $^{3}H_20$ and 2-[^{14}C]DG.

Statistical analysis of the dampening of the rhythmic expression of clock genes

The dampening of the rhythmic expression of clock genes was tested using a permutation test[3]. The data consists of N=6 time points and M=3 to 4 replicates per time point for each genotype, where all measurements were obtained from distinct and independent animals. Define the "average change" statistic as the average over time of $\Delta s / \Delta t$, where Δs is the change in mean signal from time point t to t+1, and Δt is the length of the time interval from t to t+1. The average change statistic is sensitive to non-uniform behavior over time. Define the null hypothesis H_0 = the average change statistic has the same distribution in both genotypes. We test the null hypothesis with a one-sided permutation test of the average change statistic. Permutated data is obtained by resampling M replicates per time point from the pool of all measurements from both genotypes, for that time point. Before resampling, the data are mean normalized independently in each condition, so that the average time course in each condition is balanced around zero. This normalization assures that any observed differences are due only to a change in shape and not in overall level of intensity. The null hypothesis implies that the average change of the unpermuted data should not be extreme with respect to the permutation distribution.

The *P*-value for this test is the tail probability of the observed value in the permutation distribution. A small *P*-value indicates a difference in the process generating the measurements between WT and mutant, with respect to the average change statistic, implying a dampening of the non-uniform behavior over time in the mutant as compared to the WT. Software available at cbil.upenn.edu/UPSIDE.

1. Qi, Y.*, et al.* Adiponectin acts in the brain to decrease body weight. *Nat Med* **10**, 524-529 (2004).
2. Qi, Y.*, et al.* Loss of resistin improves glucose homeostasis in leptin deficiency. *Diabetes* **55**, 3083-3090 (2006).
3. Ewens, W.S. & Grant, G.R. *Statistical methods in bioinformatics: an introduction*, (Springer Verlag, 2005).