Supplemental Data

Lack of Food Anticipation in *Per2* Mutant Mice

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Supplemental Experimental Procedures

Animals and Housing

F2 homozygous *Per1* and *Per2* mutant mice and their WT C57BL/6 × 129SvEvBrd littermates were used. The loss-of-function Per1 mutation (*Per1*$^{-/-}$) and the Per2 mutation (*Per2*^{Brdm1}) are described in [S1] and [S2], respectively. All mice were placed in individual cages with running wheels in light- and soundproof-ventilated chambers (12 cages per box) under a 12 hr light/12 hr dark cycle. Under these lighting conditions, times of day were converted to Zeitgeber times (ZT) in which ZT0 and ZT12 were the onsets of light and darkness, respectively. Food and water were provided ad libitum unless otherwise stated.

Procedure of Behavioral Experiments

In a first series, male and female WT, *Per1* mutant, and *Per2* mutant (3- to 7-month-old) mice were kept in individual cages with a wheel and exposed to a light-dark cycle. Wheel-running activity was recorded every 5 min by a PC computer with ClockLab software (actimetrics, Evanston, IL). Daily food intake was determined during 2 weeks of baseline with food ad libitum. Food access was progressively limited to 8 hr a day, from ZT4 to ZT12 (Temporal restriction: TR group) in half of the animals. The mice could eat as much as they wanted during that period. Mice and food were weighed every week. The other half of mice was submitted to a hypocaloric diet (HF group), i.e., they were provided at ZT4 75% of their normal daily food intake during the first week and 66% of their initial intake during the following 2 weeks. Under HF conditions, mice eat their diet in the first 3 hr after food is provided [S3]. Mice were weighed every week. If an animal lost more than 20% of its initial body mass, it was refed accordingly.

After 3 weeks of food restriction, TR and HF mice were provided food ad libitum and released from ZT12 (i.e., lights off) in constant darkness (DD) so that phase shifts in the locomotor activity rhythm in response to food restriction could be assessed. One week later, animals were fasted for one day and a half, starting at midday.

In a second series, female WT and *Per2* mutant (5- to 6-month-old) mice were kept in individual cages with no wheel and exposed to a light-dark cycle 12/12, as above. Under gaseous anesthesia (mixture of oxygen, nitrogen protoxide, and isoflurane), each animal was implanted intraperitoneally with an E-Mitter transmitter (Ref: PDF-4000, MiniMitter Co., Sunriver, OR) to record general activity and body temperature every 5 min with VitalView data acquisition system (MiniMitter). Baseline food intake was determined as above. Mice were released in constant light and submitted to a hypocaloric diet for 3 weeks, as above except that food was provided at projected ZT4 (instead of ZT4). Mice were then transferred back to a light-dark cycle and fed ad libitum for 2 weeks. Thereafter, mice were released in constant darkness and submitted to a hypocaloric diet for 3 weeks with food given at projected ZT4, as before. After the last day of hypocaloric feeding, food was provided ad libitum with a 6 hr delay compared to the previous mealtime.

Activity and Temperature Analysis

Mean daily activity was quantified for each genotype during the last 8 days of restricted feeding period and compared to the last 8 days of the first ad libitum period with Clocklab software. Running-wheel revolutions were quantified every 2 hr so that mean activity profiles for each genotype during ad libitum and restricted feeding periods could be assessed. Onset of activity was determined in light-dark conditions with food ad libitum and in DD conditions with food ad libitum. The first day of DD was not considered so that a possible transient cycle could be avoided. Projected lines from these onsets were used to determine phase shifts measured on the first day of DD (Clocklab). The endogenous period in DD was determined by χ^2 periodogram (Clocklab). For *Per2* mutant mice of the first behavioral series, data analysis was performed only on animals showing a significant circadian rhythmicity in DD. For the second behavioral series, daily profiles of general cage activity and body temperature in both WT and *Per2* mutant mice were determined on the 18th and tenth day of HF in constant light and constant darkness, respectively. These dates were chosen because at that time, the active phase of the free-running circadian rhythm did not overlap with the prior daily meal time and thus confounds between FAA and spontaneous activity that defined the subjective night were avoided. Mean general cage activity and body temperature were averaged during 2 hr before mealtime. Diet-induced thermogenesis was defined as the increase of body temperature between the 2 hr prior to mealtime and the 2 hr period starting 1 hr after food was provided.

Procedure of Molecular Experiments

WT, *Per1* mutant, and *Per2* mutant (3- to 7-month-old) mice were placed in individual cages with a wheel. After 2 weeks of baseline, animals of each genotype chosen randomly were fed ad libitum throughout the experiment (AL group). The other mice were submitted to a hypocaloric regimen (HF group) as described above. Mice of each genotype under AL or HF conditions were sacrificed at ZT2, ZT8, ZT14, and ZT20. Brains were dissected out and frozen in 2-methylbutane; livers were removed and stored at −70°C.

RNA Extraction and Real-Time qPCR Analysis

Pieces of frozen livers were homogenized in RNA zol B and extracted according to the manufacturer's instructions. The RNA samples were further purified by consecutive precipitations with 4 M LiCl and ethanol. The synthesis of single-stranded complementary DNA (cDNA) from the RNA was performed as described with the probes for *Bmal1*, *Rev-erbα*, *Per1*, *Per2*, and *Gapdh* [S4]. The probe for *Dbp* was forward 5′-CGTGGAGGTGCTTAATGACCTTT-3′, reverse 5′-CATGGCCTGGAATGCTTGA-3′, and 5′-FAM-AACCTGATCCCGC TGATCTCGCC-TAMRA-3′.

In Situ Hybridization

Antisense and sense RNA probes were generated with an in vitro transcription kit (Maxiscript, Ambion, TX). Here, we used riboprobes of *mBmal1b*, *mCry1*, *mDbp*, and *Vasopressin* (*Avp*). 14 μm brain sections were postfixed in 4% phosphate buffered paraformaldehyde and rinsed twice with phosphate buffered saline (PBS) and then acetylated twice in 0.1 M triethanol-amine, washed again with PBS, and dehydrated in a graded ethanol series. Sections were hybridized overnight with either denatured antisense or sense riboprobe in a humid chamber at 54°C. Sections were then rinsed with SSC, treated with ribonuclease A (Sigma, St. Louis, MO), rinsed with stringency washes of SSC, and dehydrated in a graded ethanol series. Slices and radioactive standards were exposed for 1 week to an autoradiographic film (Biomax MS-1 Kodak, Sigma). Standards were included in each cassette to verify that the measured values of optical densities were in the linear response range of the film. Densitometric analysis of hybridization signals was performed with ImageJ (National Institutes of Health, Bethesda, MD). The optical density of specific signals was calculated by subtraction of the intensity of staining background area (defined as a circle of 100 μm diameter) measured in the anterior hypothalamic area above the SCN from that of a circle of 100 μm diameter measured in the right and left SCN. Measures were made on three consecutive slices in the rostro-caudal middle of the SCN and averaged for a given brain. Data were expressed as relative optical-density values.

Body-Composition Analysis

After sacrifice, the skull, thorax, and abdominal cavity of the animals were incised and the gut was cleaned of undigested food. The whole carcasses were dried to a constant weight in an oven maintained at 70°C, and after weighing and homogenization, the dried carcasses were analyzed for fat content by the Soxhlet extraction method [S5]. Fat-free dry mass was calculated as the difference between dry weight and body fat.

Diets and Energy Intake

High-Fat Diet

This is a pelleted high-fat and high-sucrose diet (# D12331 from Research Diet, New Brunswick, NJ) consisting of, by energy, 18% protein, 24% carbohydrates, and 58% saturated fat (hydrogenated coconut oil); it contains a metabolizable energy value of 5.25 kcal/g (i.e., 21.9 kJ/g).

Chow Diet

This is a pelleted chow diet (Kliba, Cossonay, Switzerland) consisting of, by energy, 24% protein, 66% carbohydrates, and 10% fat; it contains a metabolizable energy value of 3.15 kcal/g (i.e., 13.2 kJ/g).

Glycogen

50 mg of liver was lysed in KOH 1M at 95°C and neutralized with HCl 1 M. 100 μl of the obtained solution was digested in sodium acetate 0.3 M containing 10 mg/ml Amyloglucosidase (Roche Molecular Biochemicals, Basel, Switzerland) for 2 hr at 30°C. Glucose was measured on 15 μl of supernatant with a glucose hexokinase assay kit (Sigma) according to the manufacturer's protocol. Glucose was expressed in μmol/g wet liver.

Free Glucose

After lysis and neutralization, 100 μl of liver homogenate was centrifuged and free glucose was measured on 15 μl of the undigested supernatant with a glucose hexokinase assay kit (Sigma) according to the manufacturer's protocol. Its concentration was expressed in μmol/ g wet liver.

Statistical Analysis

Data are presented as mean ± SEM. Unpaired Student's t test was used to compare two groups. ANOVAs, with or without repeated measures, followed by post-hoc comparisons with the Student-Newman-Keuls test were used to compare more than two groups. mRNA levels in the SCN were fitted by a nonlinear least-squares regression with the following cosine-wave equation (cosinor): $y = [A + B \times \cos (2\pi \times (t - C)/24]$ where y is the level of mRNA, A is the mean level of mRNA, B is the amplitude of mRNA oscillation, C is the acrophase of mRNA oscillation, and *t* is the time (hr).

Supplemental References

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S3. Mendoza, J., Graff, C., Dardente, H., Pevet, P., and Challet, E. (2005). Feeding cues alter clock gene oscillations and photic responses in the suprachiasmatic nuclei of mice exposed to a light/dark cycle. J. Neurosci. *25*, 1514–1522.

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S5. Entenman, C. (1957). General procedures for separating components of tissue. In Methods in Enzymology, *Vol. 3*, S.P. Colowick and O. Kaplan, eds. (New York: Academic), pp. 299–317.

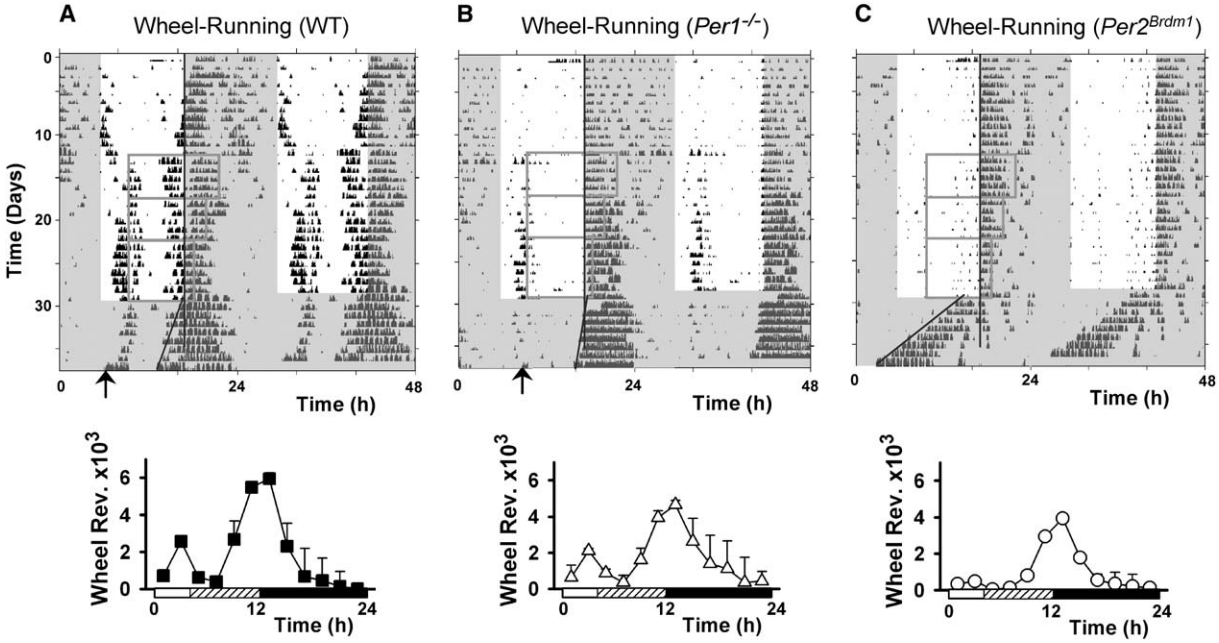

Figure S1. Daily Wheel-Running Activity under Temporally Restricted Food Access

Wild-type (A), *Per1* (B), and *Per2* (C) mutant mice exposed to a light-dark cycle. Activity is plotted as an actogram with each horizontal line corresponding to two consecutive days, with the second day being double plotted on the next line. Gray shading indicates lights off. Animals were fed ad libitum, provided with temporally restricted food access indicated by the rectangles, and subsequently released into constant darkness with food ad libitum, with the exception of the last day when no food was accessible. The arrow shows expression of food-anticipatory activity at the expected time in both wild-type (A) and *Per1* (B) mutant mice fed ad libitum in constant darkness. The bottom graph represents the mean daily-activity profile during the last 8 days of temporally restricted food access (n = 6 in WT and $Per1^{-/-}$ mice and n = 5 in $Per2^{Brdm1}$ mice; mean ± SEM). The hatched area on the X axis indicates food access. Note the lack of food-anticipatory activity in *Per2* mutant mice (C).

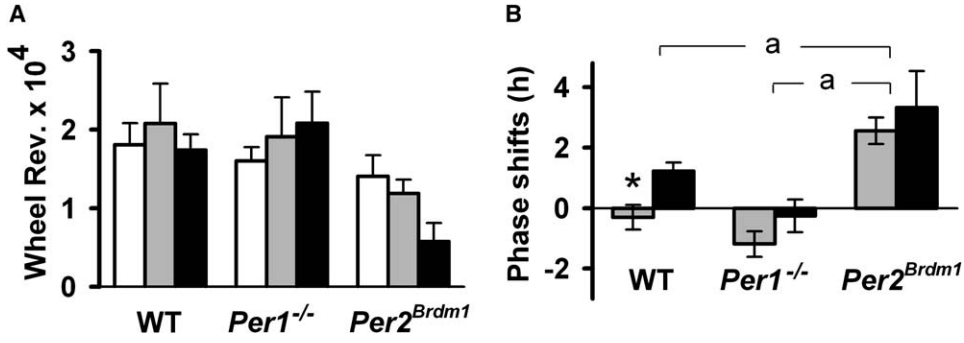

Figure S2. Locomotor Activity and Phase Shifts

Levels of daily locomotor activity (A) and phase shifts (B) with food restriction under light-dark conditions.

(A) Mean of wheel revolutions per day in wild-type, *Per1*, and *Per2* mutant mice during the last 8 days of the initial ad libitum period (white bars) and during the last 8 days of temporally restricted (gray bars) and hypocaloric (black bars) feeding conditions under a light-dark cycle.

(B) Phase shifts in locomotor activity of wild-type, *Per1*, and *Per2* mutant mice after temporally restricted (gray bars) or hypocaloric (black bars) feeding conditions. The phase shifts were calculated after transfer to constant darkness and ad libitum refeeding. *, $p < 0.05$ between feeding conditions for a given genotype; a, $p < 0.05$ between genotypes regardless of the feeding conditions (for temporally restricted feeding, n = 6 in WT and $Per1^{-/-}$ mice and n = 5 in $Per2^{Brdm1}$ mice; for hypocaloric feeding conditions, n = 6 in WT and $Per1^{-/-}$ mice and n = 4 in $Per2^{Brdm1}$ mice; mean ± SEM).

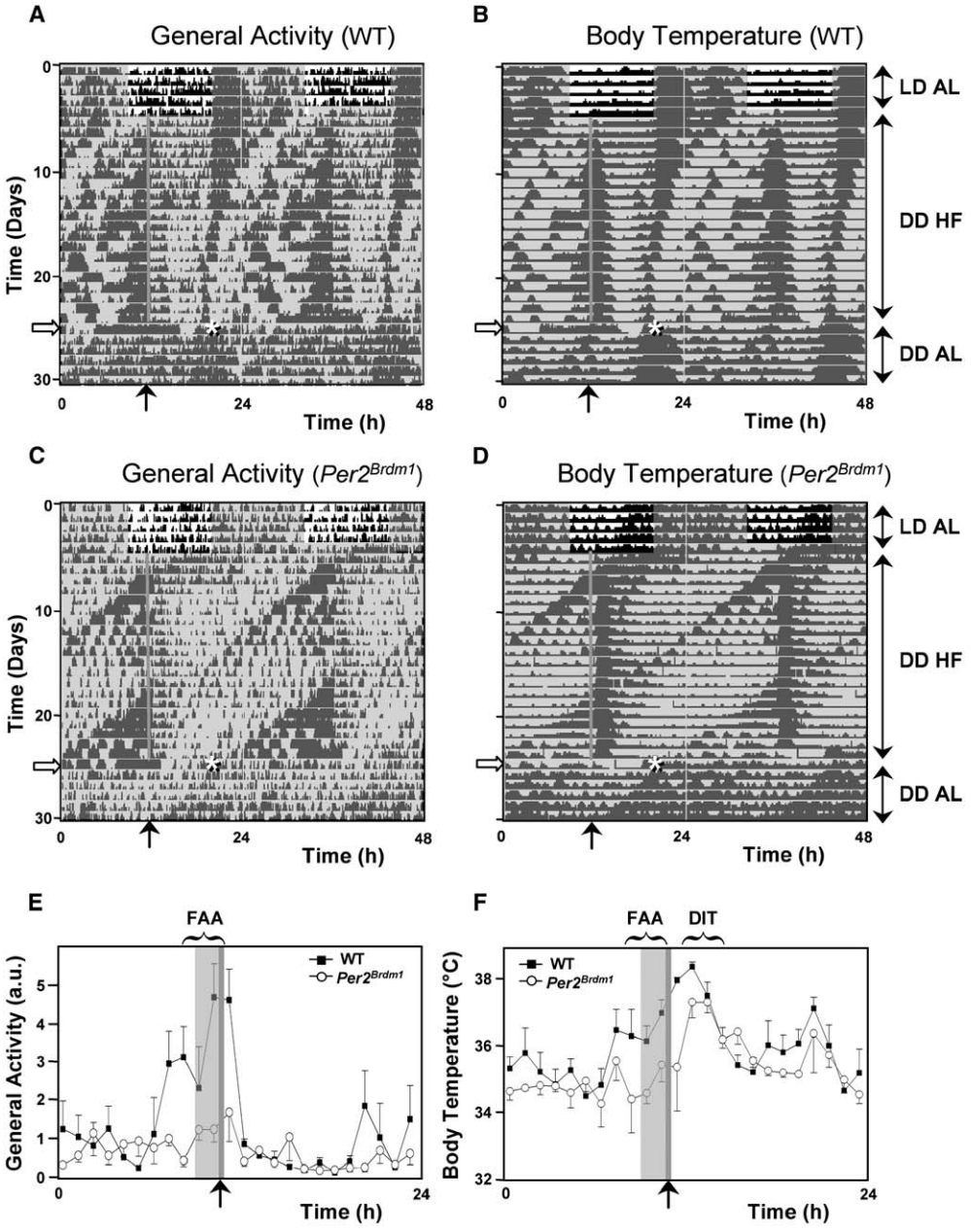

Figure S3. Daily General Cage Activity and Body Temperature

Wild-type (A and B) and *Per2* mutant (C and D) mice under hypocaloric feeding conditions in constant darkness. General activity (A and C) and temperature (B and D) are double plotted. Gray shading indicates lights off. Animals were fed ad libitum under a light-dark cycle (days 1–5), submitted to hypocaloric feeding, released into constant darkness (days 6–24), and fed ad libitum for a period at the end (days 25–30). The gray line and vertical black arrow indicate the time when hypocaloric food was provided. Mealtime was associated with a large increase in body temperature, corresponding to the so-called diet-induced thermogenesis (DIT) in both wild-type (B) and *Per2* mutant (D) mice. The bottom graphs represent daily profiles of general activity (E) and temperature (F) in wild-type (black squares) and *Per2* mutant (open circles) mice during the hypocaloric-feeding period in constant darkness (n = 5 for both genotypes; mean ± SEM). The arrow below the X axis indicates the time of feeding. Brackets with FAA and DIT show the periods during FAA and DIT are expected, respectively. The shaded area indicates the 2 hr period prior to mealtime. Note the lack of food-anticipatory general activity (C and E) and temperature (D and F) in *Per2* mutant mice. After the period of hypocaloric feeding, food was given 6 hr later than before on day 25 (see the horizontal white arrow). Note that the increase in body temperature in the wild-type mouse (probably due to food-anticipatory activity) is concomitantly absent in the *Per2* mutant mouse. In both mice, there was a specific increase of thermogenesis (white asterisk) once food was given ad libitum.

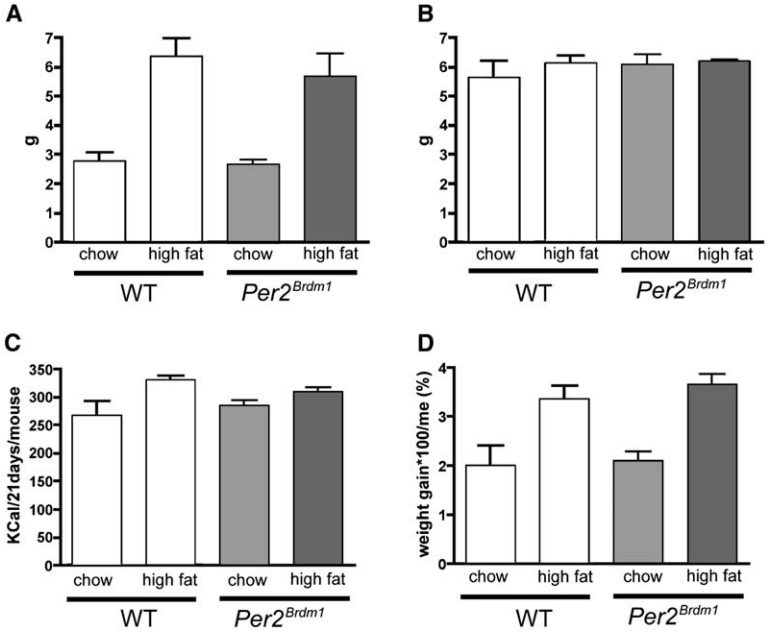

Figure S4. Physiological Parameters Related to Feeding

Body-fat contents (A), free-fat mass (B), mean energy intake (C), and feeding efficiency (D) under regular diet (chow) and high-fat diet in wild-type (white bars) and *Per2* mutant (black bars) mice fed ad libitum. g stands for grams, and me stands for mean energy intake. (n = 6 for all groups; mean ± SEM)

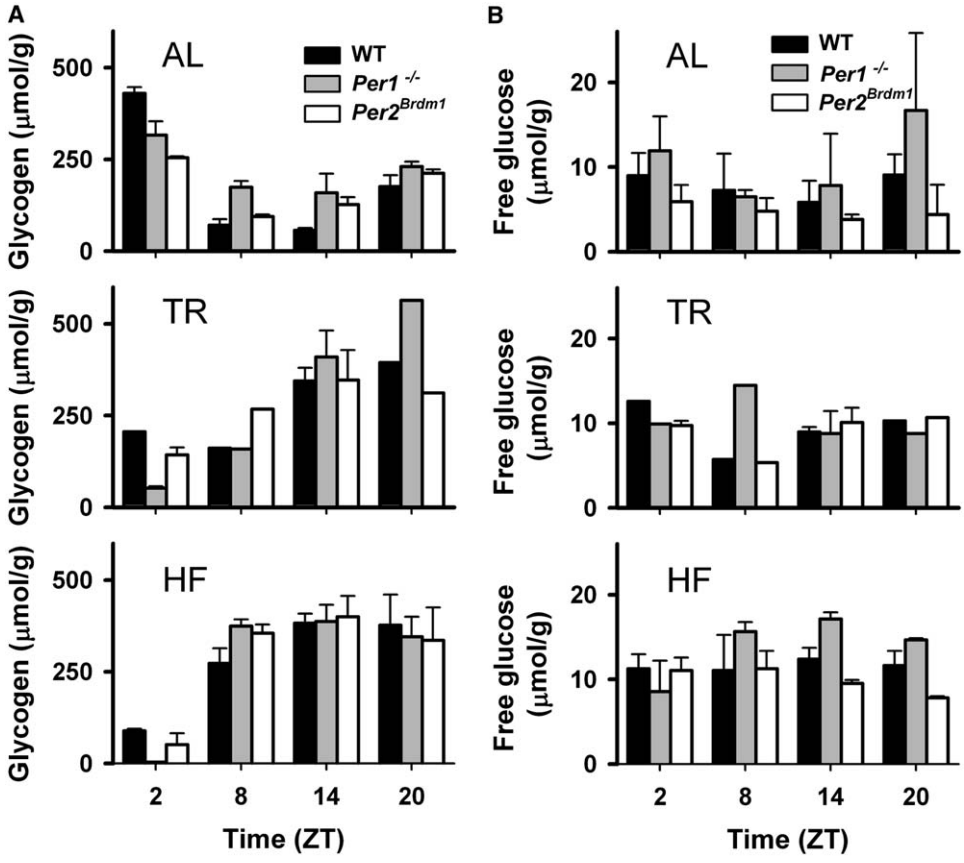

Figure S5. Glycogen and Free Glucose

Determined of mice held under ad libitum (AL), temporally restricted (TR), and hypocaloric feeding (HF) conditions. Liver glycogen (A) and free-liver glucose (B) in wild-type (black bars), *Per1* mutant (gray bars), and *Per2* mutant (white bars) mice (n = 1–4; mean ± SEM). ZT stands for Zeitgeber time.

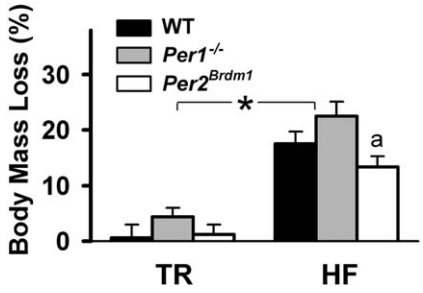

Figure S6. Body Mass Loss in Wild-Type and *Per1* Mutant Mice

Wild-type (black bars) and *Per1* mutant (white bars) mice under temporally restricted (TR) and hypocaloric feeding (HF) conditions (n = 6 in WT and $Per1^{-/-}$ mice and n = 4 in $Per2^{Brdm1}$ mice; mean ± SEM). *, $p < 0.05$ between feeding conditions regardless of the genotype; a, $p < 0.05$ between *Per* mutants during HF conditions.

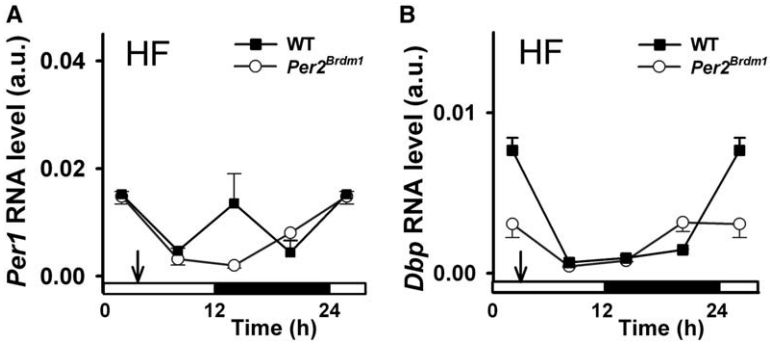

Figure S7. Expression of Clock and Clock-Controlled Genes in the Kidney of Mice under Hypocaloric Feeding Conditions

Expression of *Per1* (A) and *Dbp* (B) in wild-type (black squares) and *Per2* mutant (open circles) mice (n = 2; mean ± SEM). Data for ZT2 were double plotted. Nighttime is indicated by a black bar on the X axis.