Supplemental Information for Shi et al.:

"Circadian disruption leads to insulin resistance and obesity"

Inventory of Supplemental Information

Figure S1 is the protocol for entrainment and fasting prior to the hyperglycemic-euglycemic clamps shown in Figs. 1 & 2.

Figure S2 is the immunoblot analysis from muscle extracts for phospho-AKT (p-AKT S473 and T308) and total AKT of mice after hyperinsulinemic-euglycemic clamps. The densitometric analysis of this figure is shown in Fig. 3C.

Figure S3 are data showing locomotor activity and circadian period in high fat-fed mice. This figure is related to Fig. 4

Figure S4 are data showing Per2 expression measured as luminescence from mouse embryonic fibroblasts (MEFs) and isolated tissues of the P_{mPer2}::mPER2-Luc reporter mouse monitored *in vitro*. Related to Fig. 6.

Table S1 is an analysis of body composition, food consumption, and activity levels in high-fat diet fed B1koB2Tg mice with or without a clearly rhythmic behavior in constant darkness. Related to Fig. 4.

Supplemental Experimental Procedures give further details of materials and methods used in this study.

Supplemental References provides additional references for Supplemental Information.

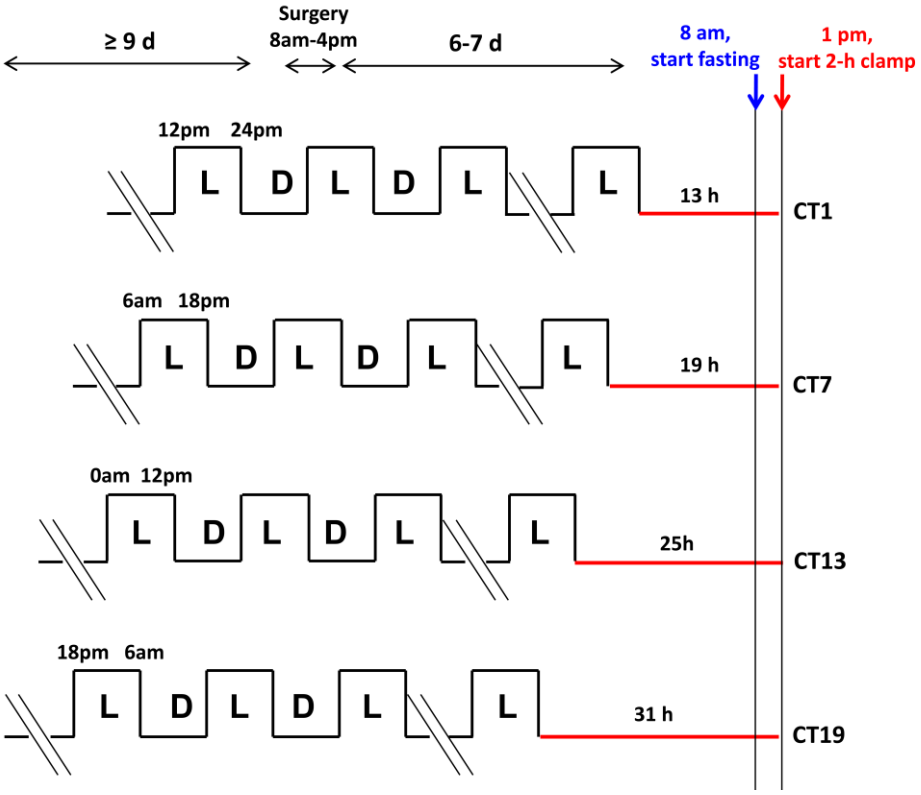


Fig S1: Protocol for entrainment and fasting prior to the hyperglycemic-euglycemic clamps shown in Figs. 1 & 2. Mice were entrained to different circadian phases under LD 12:12 (12 h light: 12 h dark) cycles in a light-tight box for at least 9 days before surgery. In the figure, the 12 h light intervals are indicated as "L" and the 12 h dark intervals are indicated as "D" for the uppermost treatment schedule (13 h in red light; CT1). The catheterization surgery was performed under room light. Then the mice were re-entrained under the same LD conditions as those prior to the surgery for an additional 6-7 days. After the final 12 h light exposure, all subsequent treatments were performed under very dim red light (red light intensity was ~1.8 μmol/m^2s). At 8:00 am on the day of the clamp, fasting was begun, and then at 1:00 pm (after a 5 h fast), two-hour hyperglycemic-euglycemic clamps were performed under dim red light.

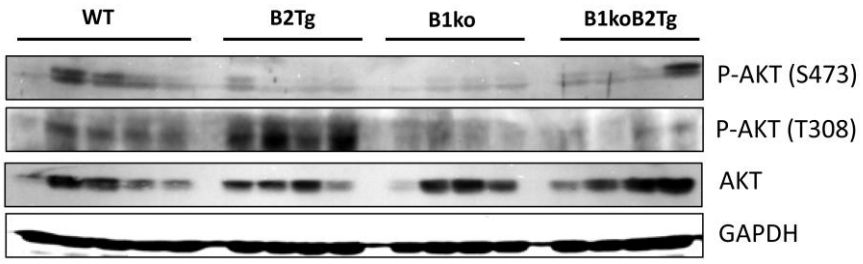


Fig S2: Related to Fig. 3. Immunoblots from muscle extracts for phospho-AKT (p-AKT S473 and T308) and total AKT of mice after hyperinsulinemic-euglycemic clamps. GAPDH was used as a loading control. Each lane comes from a separate mouse (n = 5 for WT, n = 4 for the other groups). The densitometric analysis is shown in Fig. 3C.

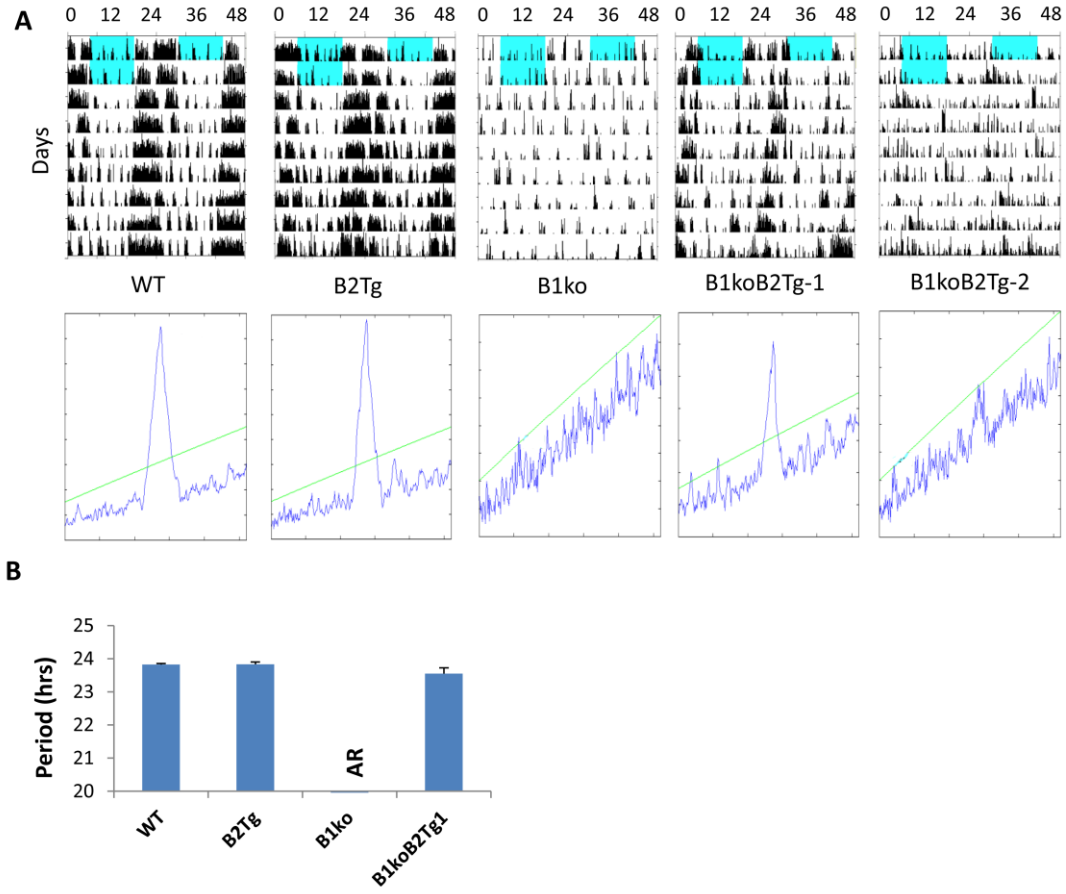
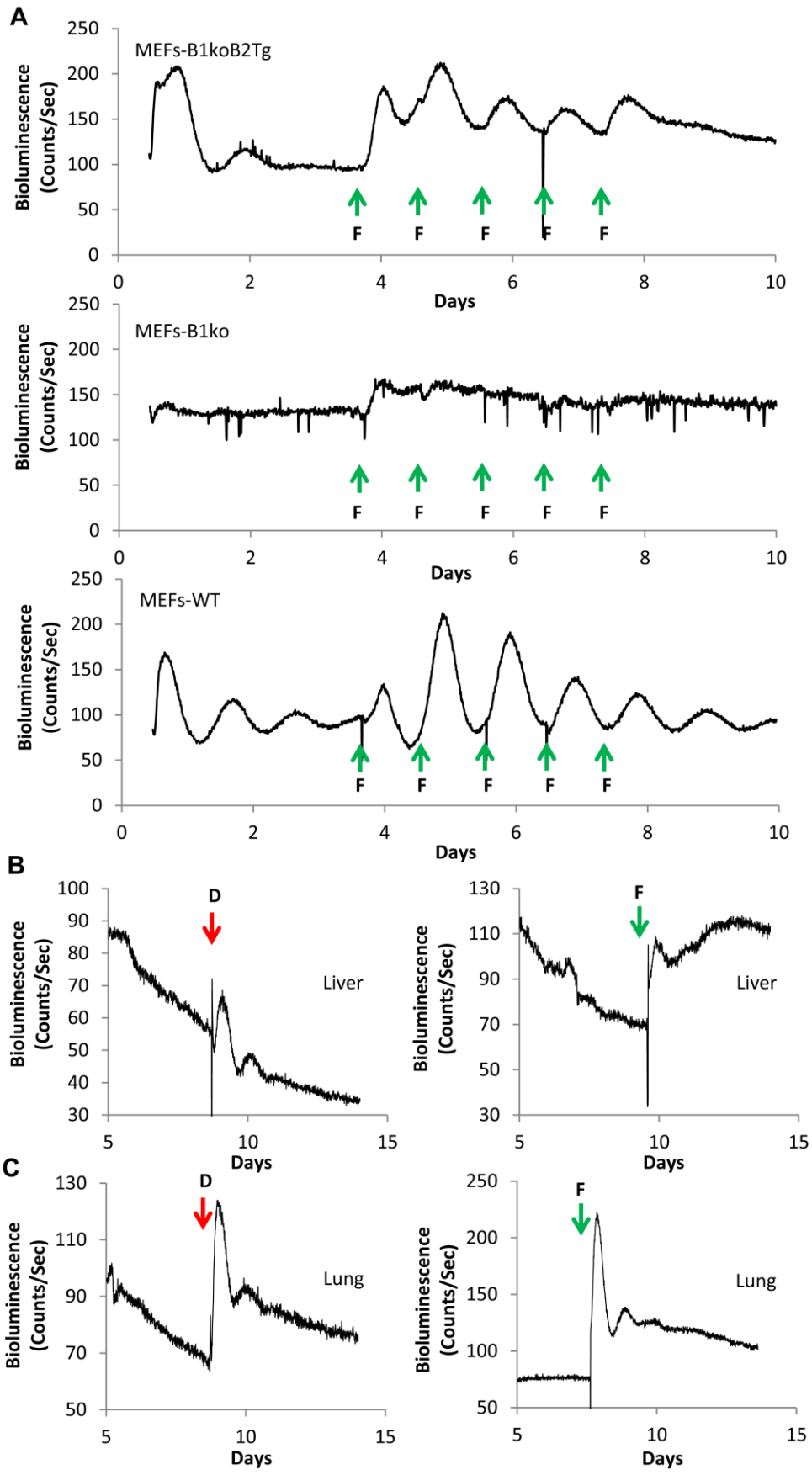


Fig S3: Related to Fig. 4. Locomotor activity and circadian period in high fat-fed mice.
(A) Representative locomotor activity records as monitored by infrared sensors of mice with four different genotypes in light-dark cycles (LD 12:12) and constant darkness (DD). Blue color denotes the lights-on interval. Beneath each panel is the chi-square periodogram analysis of the locomotor activity rhythm in DD (for these particular examples, the period estimates were 23.7 h for WT, 23.8 h for B2Tg, and 23.7 h for B1koB2Tg-1 {B1ko and B1koB2Tg-2 were arhythmic}). The plots represent analyses of at least one week of data in DD from a representative mouse. These analyses were used as the criterion for separating the individual B1koB2Tg data into two groups on the basis of whether the rhythmicity at ~24 h exceeds the threshold (green diagonal line) for rhythmicity. B1koB2Tg-1 represents a *Bmal1* knockout mouse with a *Bmal2* transgene that shows rescue of rhythmicity, while B1koB2Tg-2 represents a B1koB2Tg mouse that expresses minimal rescue. Each error bar is mean ± SEM.

(B) Period analyses of locomotor activity recorded by infrared sensors in mice at 3 months of age in DD. All WT and B2Tg mice tested were behaviorally rhythmic in both LD and DD conditions. All B1ko mice measured were clearly arrhythmic in DD. Seven out of ten B1koB2Tg mice showed clearly rhythmic behavior in DD and were labeled as B1koB2Tg-1, while another 3 mice appeared not to be clearly rhythmic in DD for first 7-10 days and were labeled as B1koB2Tg-2 (the B1koB2Tg-2 mouse data were not analyzed for period in this panel). Each bar represents mean ± SEM, n = 7-10/genotype for WT, Bmal2 Tg, and Bmal1 ko mice.

Fig S4: Related to Fig. 6. Per2 expression measured as luminescence emission from mouse embryonic fibroblasts (MEFs) and isolated tissues of the P_{mPer2}::mPER2-Luc reporter mouse monitored *in vitro*.
(A) Bioluminescence monitored from MEFs. Three days after initiation of the culture, cells were synchronized by cyclic addition of forskolin (5 μM) at the times indicated by green arrows ("F") for 5 22.5-h cycles.
(B) Bioluminescence monitored *in vitro* from liver slices of B1ko/B2Tg mice.
(C) Bioluminescence monitored *in vitro* from lung slices of B1ko/B2Tg mice.
The tissue slices were given dexamethasone (100nM, "D") or forskolin (5 μM, "F") at the times indicated by red or green arrows. The drug application was performed in a temperature-controlled room (29°C) and the bioluminescence measurement was performed at 36°C.



	Rhythmic (B1koB2Tg-1)	**Arrhythmic** (B1koB2Tg-2)	**p-Value** (Two-tail T test)
N	7	3	
Body Weight (g)	27.96±1.54	35.30±5.63	0.16
Fat mass (g)	4.58±0.71	12.72±4.98	0.03
Lean mass (g)	20.12±0.92	20.27±0.97	0.93
Activity (IR, LD)	0.76±0.12	0.28±0.06	0.04
Food Intake (g/day)	3.00±0.21	3.15±0.24	0.72

Table S1. Related to Fig. 4. Analysis of body composition, food consumption, and activity levels in high-fat diet fed B1koB2Tg mice with or without a clearly rhythmic behavior in DD for first 7-10 days (see representative examples in Fig. S3A, where B1ko/B2Tg-1 samples are rhythmic animals and B1ko/B2tg-2 are arhythmic animals). Data are represented as mean ± SEM.

Experimental Procedures

Animal care

Mice harboring the *mPer2*-luc knock-in allele [1] and *Bmal1*+/- mice [2] have been back-crossed with the C57BL/6J strain (JAX, Bar Harbor, Maine) for more than 10 generations. The *Bmal2* transgenic mouse was made in a C57BL/6 background [30]. Mice were housed in temperature-controlled environments on a 12:12-h light-dark cycle (lights on 6 am to 6 pm, light intensity is 300 Lux of cool-white fluorescence light) unless otherwise stated and fed chow (5001; Purina Mills, St. Louis, MO) containing 13.5% calories from fat. For high-fat diet (HFD, F3282; BioServ, Frenchtown, NJ) experiments, mice were fed HFD containing 60% calories as fat, beginning at one month of age. All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted according to that committee's guidelines.

Hyperinsulinemic-euglycemic clamp

As shown in Figure S1, mice were entrained to different circadian phases under LD 12:12 (12 h light: 12 h dark) cycles in a light-tight box for at least 9 days before surgery. In each experiment, two groups of mice were entrained to reverse phase LD cycles (i.e., CT1 and CT13 in one experiment, CT7 and CT19 in another experiment). The catheterization surgery was performed under room light. Briefly, catheters were chronically implanted in the jugular vein (for infusions) and carotid artery (for sampling). Then the mice were re-entrained under the same LD conditions as those prior to the surgery for an additional 6-7 days. After the final 12 h light exposure, mice were placed in free-running conditions under very dim red light (red light intensity was ~1.8 $\mu mol/m^2s$ from red fluorescent lamps with an extra blocking filter to allow only light emission of >630 nm). In the interval of 13-31 h of free running in dim red light, mice were fasted for 5 h, beginning at 8:00 am and ending at 1:00 pm. The clamp procedure was begun at 1:00 pm. Because the mice had been entrained to various LD cycles prior to the day of the clamp procedure, this protocol means that the clamp was performed at four different circadian phases (CT1, CT7, CT13, and CT19 for WT mice). After the final 12 h light exposure of LD, mice were exposed to only dim red light for the free-run, fasting, and clamp procedure.

Beginning at 1:00 pm, two-hour hyperinsulinemic-euglycemic clamps were performed on conscious 5-h fasted mice under the dim red light conditions (~1.8 $\mu mol/m^2s$). Insulin was continuously administered at 2 mU kg^{-1} min^{-1} (t = 0 to 120 min). Blood was continuously infused from a donor animal to replace blood removed during the clamp. Arterial glucose levels were measured at baseline (t = -10 and 0 min) and every 10 min during the clamp period. The glucose infusion rates were adjusted to maintain euglycemia. Baseline (at -10 or 0 min of the clamp procedure) and clamp (at time 120 min of the clamp procedure) arterial blood was taken and stored at -80°C for insulin and corticosterone measurements. After the clamp procedure was completed, the mice were sacrificed and tissues were rapidly excised and flash-frozen in liquid nitrogen and stored at -80°C for future analyses. Plasma insulin and corticosterone were determined by radioimmunoassay using a double antibody procedure.

Determination of Akt phosphorylation by immunoblotting

Samples of liver and of vastus muscle harvested from mice of the CT13 group at the end of the hyperinsulinemic-euglycemic clamp procedure were sonicated in RIPA buffer containing a cocktail of protease inhibitors (Sigma) and a cocktail of phosphatase Inhibitors (Sigma) and clarified by centrifugation. Protein from the supernatant was quantified by the Bradford assay (Bio-Rad). For cMyc-Bmal2 blots (100 μg of total protein from liver, WAT, brain, or muscle) or for Akt blots (40 μg of total muscle protein or 100 μg of total liver protein), proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes for immunoblotting. Phosphorylated and total Akt was probed using antibodies directed against Akt and Ser473-Akt (from Cell Signaling), and against Thr308-Akt (from BD Pharmingen). Anti-cmyc monoclonal IgG (a gift of Dr. Heping Yan), anti-Bmal1 antibody (a gift of Dr. Charles Weitz), and anti-β-actin monoclonal antibody (Sigma) were used to probe myc-Bmal2, Bmal1, and β-actin, respectively. Densitometric analyses were performed using Image J software (NIH).

Locomotor behavior, body weight/fat composition, and food intake assays

Age and gender matched mice with or without the *Per2*-luc reporter construct were fed HFD beginning at one month of age. After two months on HFD, some mice were singly housed in cages equipped with infrared sensors on a LD 12:12 cycle (lights on 6 am to 6 pm) for several days before being released into constant

darkness (DD). After 6-10 days in DD, mice were returned to LD 12:12. ClockLab software (Actimetrics, Evanston, IL) was used to collect data and produce double-plotted actograms as well as periodogram and activity analyses. Chi-Square periodogram analyses were performed to obtain the period using the data in DD. The amount of food that was consumed in 24 h was measured when mice were in individual cages under LD cycles. We used young B1ko mice that do not suffer from accelerated aging and ankle/joint disease of older B1ko mice [3, 4, 5]. For mice in constant light conditions (LL), six male mice were fed HFD beginning at one month of age, meanwhile, the light-dark conditions were changed from LD 12:12 to LL (light intensity 300 lux). Locomotor activity and food intake of the mice was measured at 2 months on HFD. Body fat composition was determined with an mq10 nuclear magnetic resonance analyzer (Bruker Optics).

Tissue culture and *in vitro* luminescence recording

The mice for tissue culture were fed HFD for 3 to 4.5 months or they were fed chow. One to two hours before lights-off of LD 12:12, the mice were sacrificed by cervical dislocation under dim red light, and cultures of SCN and peripheral tissues were prepared as previously described [1, 6] and cultured in recording medium containing fetal bovine serum and luciferin. After 7-8 days in culture, the SCN explants from HFD-fed mice were given a half hour pulse of 10 μM foskolin (Tocris) in fresh medium at 36.5°C, and then the medium was replaced with fresh recording medium and recording was continued for several days. The *in vitro* rhythms were analyzed for period and amplitude using LumiCycle analysis software (Actimetrics, version 2.31).

Statistics

Data are expressed as means ± SEM. Statistical analyses were performed by two-tail unpaired T test for comparison between two groups or as indicated. When appropriate, two-way and one-way ANOVA statistical calculations were performed with the program JMP (JMP version 10, SAS Institute, Cary, NC) and SPSS (version 17, Chicago IL).

Supplemental References

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