Supporting Information

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SI Materials and Methods

Animal Husbandry. All procedures were approved by the Animal Care and Use Committee, University of Wisconsin. Mice were housed under normal 12h:12h light:dark cycle with standard laboratory chow (Mouse diet 9F 5020; PMI Nutrition International) and water ad libitum.

Bmal1$^{fx/fx}$ mice were bred to C57BL/6J mice for 10 generations. The *Cre*^{sf1} mouse line (from Keith L. Parker, University of Texas Southwestern, Dallas) was bred to C57BL/6J background for seven generations. *Cre*^{sf1} mice are outwardly healthy and breed well. *Cre*^{sf1} conveys recombination in the somatic cells of the gonads (ovarian theca and granulosa cells), the adrenal cortex, the anterior pituitary (gonadotropes), the spleen, and the ventromedial hypothalamic nucleus as early as embryonic day 10.5 (1).

Cre^{sf1} mice were crossed with the conditional *Bmal1*$^{fx/fx}$ mice. Although both sexes were used to carry the *Cre* transgene, breedings were set up so that only one copy of *Cre* existed in the offspring.

Genotyping. For genotyping, genomic DNA was isolated by using "PUREGENE DNA Isolation kit" (Gentra Systems). Genotyping for the *Cre* transgene was performed by PCR using the forward primer, OL2642 (all sequences are listed in Table S5) and reverse primer, OL2643, in a reaction consisting of 2.5 units Taq polymerase (Promega) according to the manufacturer's protocol. The PCR was carried out by using the following conditions: (95°/5 min→[95 °C/30 s→ 60 °C /30 s→ 72 °C /30 s] × 30 cycles→72°/5 min → 4°/∞). A 450-bp band confirmed the presence of the *Cre* transgene. Samples negative for *Cre* did not amplify a product.

Genotyping for *Bmal1* was detected by using a combination of the primers (positions of the primers on the alleles are shown in Fig. S1 and sequences in Table S5), OL5436, OL6013, and OL6014, in a PCR program with the conditions (95°/5 min → [95°/30 s → 61°/30 s → 72°/5 s] × 31 cycles → 72°/5 min → 4°/∞). The wild-type allele (wt or +) generates a ∼310-bp band, whereas the floxed allele generates an ∼360-bp band. The excised allele is amplified with the OL6013/OL6014 primer pair and generates an ∼450-bp band due to the loss of the floxed region. Tissue-specific *Bmal1*$^{fx/fx}$ excision in SF1-*Bmal1*$^{-/-}$ mice is shown in Fig. S1*B*. The *Bmal1*^{fx} excised allele is detected in the brain, pituitary, adrenal gland, and ovaries and is absent in liver, muscle, uterus, and oviduct in SF1-*Bmal1*$^{-/-}$ females, consistent with previous reports.

Wheel Running Activity Analysis. Mice were singly housed in cages equipped with running wheels on an light-dark (LD) 12:12 cycle for 3 wk before transfer to constant darkness (DD) for 3 wk. All wheel running activity analyses were performed by using ClockLab software (Actimetrics) as described (2). The free running period was extracted from the constant darkness phase by linear regression analysis of activity onset. The amplitude of circadian rhythm was analyzed by using fast Fourier transform (FFT), which estimates the relative power of an ∼24-h period rhythm in comparison with all other periodicities in time series for 20 d in DD. For activity level study, the total number of wheel revolutions was counted and averaged over days 4–13 in LD and days 1–10 in DD, respectively.

Assessment of Feeding Rhythms. Each mouse was singly housed for a week before the assessment of feeding rhythms. A known amount of food was provided on day 1, and food weight was measured at Zeitgeber Time 0 (ZT0, lights on) and ZT12 (lights off) for 10 consecutive days. For each 12-h period, the difference in food weight was taken as a measure of food consumption for that period. Measurements were carried out in the same room over the same time period to control for changes in food weight due to humidity.

Assessment of Estrous Cycles. Estrous cycles were examined in females beginning at 10 wk of age. Vaginal smears were taken at ZT4 for at least 22 consecutive days. Vaginal secretions were collected with a plastic pipette filled with 10 μL of PBS by inserting the tip just into the vaginal opening. Cell morphology was analyzed under a microscope to determine cycle stage (3). The characterization of each phase is based on the proportion of the three types of cells observed in thevaginal smear: nucleated round cells (proestrus), cornified cells (estrus), and leukocytes (diestrus).

Assessment of Early Pregnancy Parameters. After pairing, females were examined for presence of copulation plugs early in the morning (defined as 0.5 dpc). This procedure was repeated for 14 continuous days for *Bmal1*$^{+/+}$, *Bmal1*$^{fx/fx}$, *Bmal1*$^{fx/+}$*Cre*^{sf1}, and SF1-*Bmal1*$^{-/-}$ females. To check for ovulation, uteri were flushed at ZT12 (lights off) on 3.5 dpc and the presence of embryos was scored as ovulation. Implantation was evaluated on 6.5 and 10.5 dpc. At 6.5 dpc, implantation sites were visualized with Chicago sky blue dye (4). Decidual swellings were observed by eye at 10.5 dpc.

Histological Analysis. For histological characterization, tissues were fixed in 10% (vol/vol) buffered formalin overnight, dehydrated in graded ethanol, and embedded in paraffin. Sections (7 μm) were stained with hematoxylin and eosin (H&E) and analyzed under a light microscopy as described (5). For some experiments, 100 μL of 1% (wt/vol) Chicago sky blue (Sigma) was injected through the tail vein 5 min before euthanasia. The female reproductive tracts were then dissected and sectioned for histological study as described (4). To count corpora lutea, 10 serial sections were cut from 3.5 dpc SF1-*Bmal1*$^{-/-}$ or control ovaries. The highest observed number of corpora lutea on a single section was used as the representative number for that ovary.

RNA Isolation. RNA was isolated by using the QIAGEN RNeasy kit (Qiagen). RNA quality and concentration were assessed by using Agilent bioanalyzer2100 (Agilent Technologies) and a NanoDrop spectrophotometer (Thermo Scientific).

Ovarian Gene Expression Assays. In microarray studies, ovary RNA samples were labeled with Cy3 (Low Input Quick Amp Labeling Kit, one-color 5190-2305; Agilent Technologies) and hybridized to mouse whole genome microarray (G4852A 8 × 60k; Agilent Technologies). Microarray images were quantified and data were extracted by Agilent feature extraction software 10.3 (Agilent Technologies). The hybridization characteristics were verified by the internal control probe sets (spike-in kit5188-5282; Agilent Technologies) by using feature extraction quality control metrics. Genespring 12.0 (Agilent Technologies) is used to analyze the extracted data. Briefly, expression values were percentile normalized and filtered by quality control (expressed in 100% of samples in at least one of four conditions) before analysis. Microarray data has been deposited in National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus (accession no. GSE48758). Cosine wave-optimization algorithm program (6) was used to test rhythmic gene expression.

A two-way ANOVA was used to identify transcripts differentially expressed due to genotype (*Bmal1*$^{fx/fx}$ and SF1-*Bmal1*$^{-/-}$) and time (ZT0 and ZT12) ($n = 4$ per genotype·time point^{-1}) in female mice on 3.5 dpc. Genes with >1.5-fold change and relaxed FDR cutoff (Benjamini–Hochberg Corrected $P < 0.15$ on genotype) were identified. The list of differentially expressed transcripts (Table S2) was imported into the database of DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov; version 6.7) for functional annotation (Table S2).

Quantitative PCR was carried out by using TaqMan expression assays (ABI Applied Biosystems; Table S5) according to the manufacturer's protocol. Assays were selected to span exon junctions and *Gapdh* was used as a loading control.

Pituitary Gene Expression Studies. On 3.5 dpc, *Bmal1*$^{fx/fx}$ at ZT0 ($n = 3$) or ZT12 ($n = 4$) and SF1-*Bmal1*$^{-/-}$ at ZT0 ($n = 3$) or ZT 12 ($n = 3$) were killed and pituitaries were collected. Gene expression was analyzed by mouse whole genome microarray (G4852A 8 × 60k; Agilent Technologies) as described above. Microarray data has been deposited in NCBI's Gene Expression Omnibus (accession no. GSE48758). We identified differentially expressed transcripts between *Bmal1*$^{fx/fx}$ and SF1-*Bmal1*$^{-/-}$ at ZT0 and ZT12 on 3.5 dpc by using a similar method to the ovarian gene expression study (two-way ANOVA).

Hormone Measurements. Mice were euthanized by CO_2 asphyxiation in their home cages. Whole blood samples were collected by cardiac puncture. Blood was allowed to coagulate at room temperature for 1 h before centrifugation. Serum was separated by centrifugation at 10,000 min^{-1} for 10 min and stored at −30 °C until assayed. Progesterone and prolactin levels were analyzed by RIA in the laboratory of the National Hormone and Peptide Program (Harbor-University of California, Los Angeles Medical Center).

Hormone Supplementation. Progesterone supplementation was begun at 3.5 dpc with daily s.c. injections at ZT4 of 2 mg progesterone (Sigma) dissolved in corn oil or 100 μL of corn oil as the vehicle control (7). PRL supplementation started at 1.5 dpc to SF1-Bmal1$^{-/-}$ females with twice-daily s.c. injections, at ZT0 and ZT12, of 150 μg of PRL (Sigma) dissolved in saline or 100 μL of saline as the vehicle control. This PRL supplementation protocol has been shown to rescue implantation in CDK4$^{-/-}$ females (8).

Statistical Analysis. The Wilcoxon rank sum test (two-sided) was used in all hypotheses testing for the difference in locations in two populations. The χ^2 test was consulted in all hypotheses testing for difference in proportion. In data that includes more than two groups to compare, one-way ANOVA or two-way ANOVA is used as indicated in the text.

1. Bingham NC, Verma-Kurvari S, Parada LF, Parker KL (2006) Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44(9):419–424.
2. Siepka SM, Takahashi JS (2005) Methods to record circadian rhythm wheel running activity in mice. *Methods Enzymol* 393:230–239.
3. Allen E (1922) The estrous cycle in the mouse. *Am J Anat* 30(3):297–371.
4. Hogan B, Beddington R, Costantini F, Lacy E (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*. (Cold Spring Harbor Lab Press, Plainview, NY), 2nd Ed.
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6. Straume M (2004) DNA microarray time series analysis: Automated statistical assessment of circadian rhythms in gene expression patterning. *Methods Enzymol* 383:149–166.
7. Ratajczak CK, Boehle KL, Muglia LJ (2009) Impaired steroidogenesis and implantation failure in Bmal1-/- mice. *Endocrinology* 150(4):1879–1885.
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9. Bunger MK, et al. (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* 41(3):122–132.

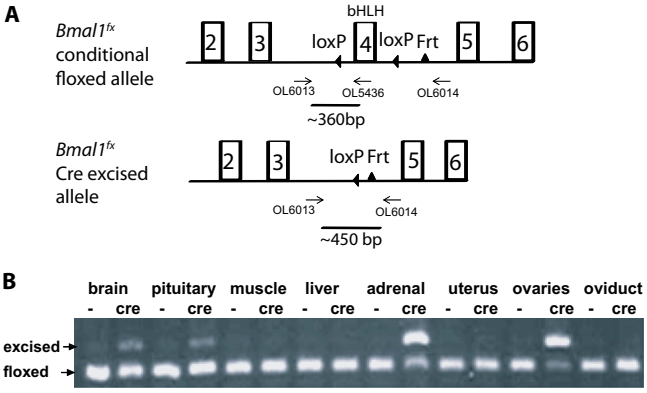

Fig. S1. Generation and tissue-specific excision of the conditional *Bmal1*^{fx} allele. (*A*) Schematic diagram illustrating the region surrounding the basic-helix–loop–helix domain of the murine *Bmal1* locus. The conditional *Bmal1*^{fx} allele and the *Bmal1*^{fx}-excised allele are shown. Exon numbers reflect known coding exons. The *lox-P* sites flank exon 4. Oligonucleotides (OL) used for genotyping are indicated. (*B*) PCR analysis of *Bmal1*$^{fx/fx}$ excision in selected tissues from SF1-*Bmal1*$^{-/-}$ mice. Arrows indicate the positions of the excised and intact conditional alleles. The primer pair OL6013/5436 amplifies the intact conditional alleles (~360 bp). The *Bmal1*^{fx}-excised allele is amplified with the OL6013/6014 primer pair and generates an ~450-bp band.

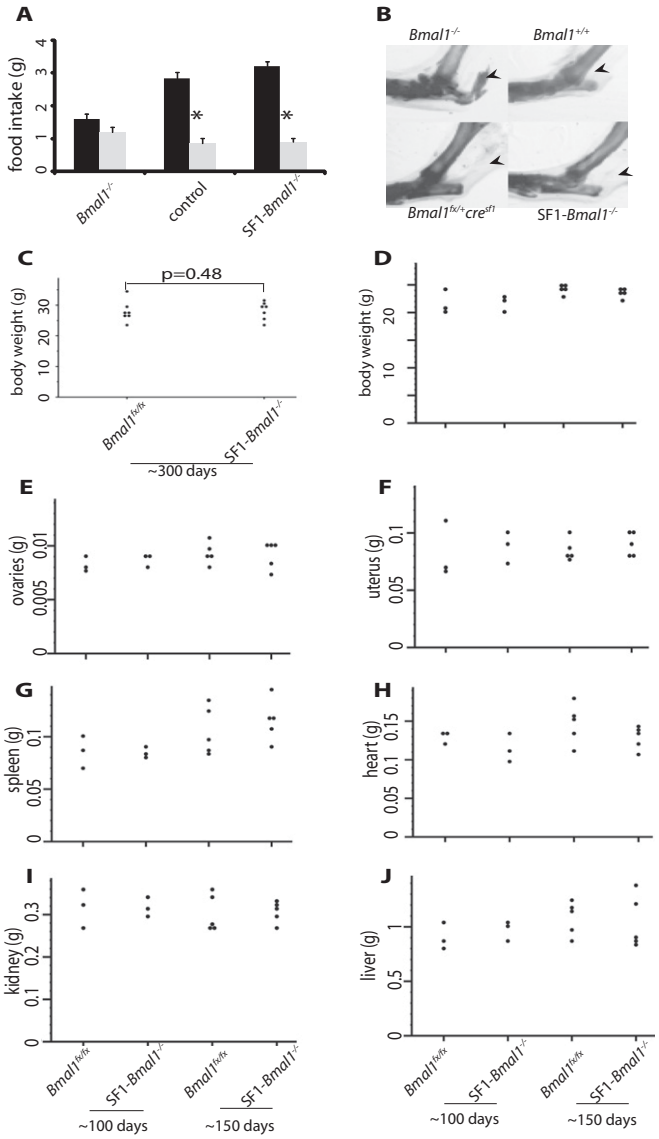

Fig. S2. Effect of *Bmal1* deletion on feeding rhythms, tendon ossification, and body and organ weights. (*A*) Food intake during the day (gray) and night (black) was measured in *Bmal1*$^{-/-}$ ($n = 2$), control ($n = 3$, 1 *Bmal1*$^{fx/fx}$ and 2 *Bmal1*$^{+/+}$), and SF1-*Bmal1*$^{-/-}$ ($n = 3$) females. Values are shown as mean ± SEM over a period of 10 d. *$P < 0.05$, Wilcoxon rank sum test. (*B*) Representative hind limb tendons of 180-d-old *Bmal1*$^{+/+}$, *Bmal1*$^{-/-}$, *Bmal1*$^{fx/+}$*Cre*^{sf1}, and SF1-*Bmal1*$^{-/-}$ females ($n \geq 3$ for each genotype) stained with Alizarin Red (9). The arrow indicates tendon calcification in *Bmal1*$^{-/-}$ mice, which is not present in mice of the other three genotypes. (*C*) Body weights of *Bmal1*$^{fx/fx}$ and SF1-*Bmal1*$^{-/-}$ females at ~300 d of age. Each dot represents a single animal. *P* value, calculated by Wilcoxon rank sum test, is indicated. (*D–J*) Body weights and organ weights of *Bmal1*$^{fx/fx}$ and SF1-*Bmal1*$^{-/-}$ females measured on ~100 or ~150 d with indicated genotypes shown below. Each dot represents a single animal. No significant differences detected in all measurements ($P > 0.1$, Wilcoxon rank sum test).

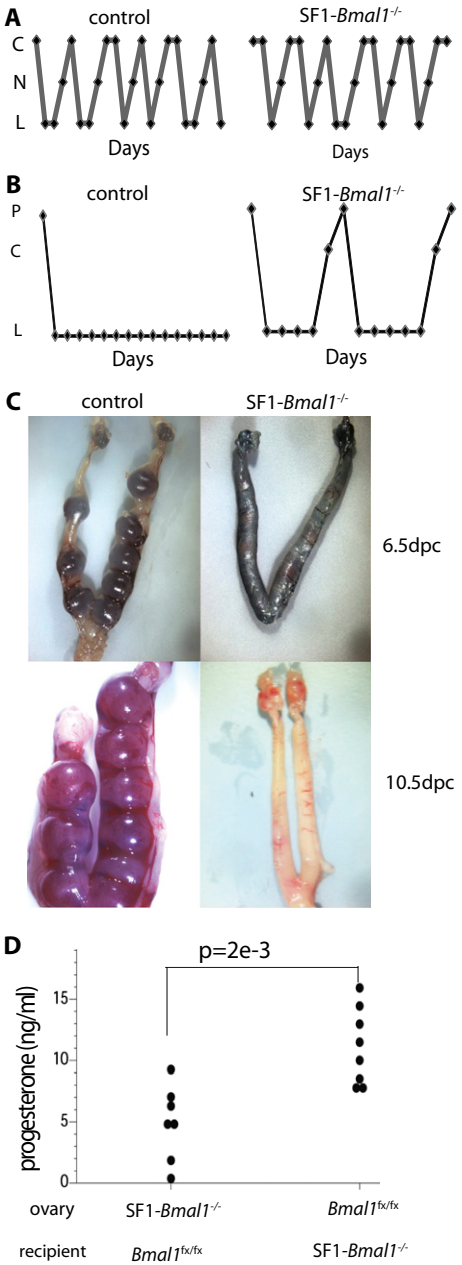

Fig. S3. Effect of *Bmal1* deletion on estrous cycling, presence of copulation plugs, implantation, and progesterone levels. (*A*) Representative estrous cycle patterns of virgin SF1-*Bmal1*$^{-/-}$ ($n = 6$) and control (4 *Bmal1*$^{fx/fx}$ and 1 *Bmal1*$^{fx/+}$Cre^{sf1}) females, as determined by vaginal cytology. Each day is represented by a diamond. C, cornified (estrus); L, leukocytic vaginal smears (diestrus); n, nucleated (proestrus). (*B*) Representative estrous cycle patterns of mated control females ($n = 5$) and SF1-*Bmal1*$^{-/-}$ as determined by vaginal cytology ($n = 5$). Each day is represented by a diamond. C, cornified; L, leukocytic; P, presence of plug. (*C*) Representative pictures of implantation. Uteri were isolated from control and SF1-*Bmal1*$^{-/-}$ females at 6.5 dpc (*Upper*) and 10.5 dpc (*Lower*) and stained with Chicago Sky Blue (6.5 dpc) or photographed directly (10.5 dpc). (*D*) Progesterone levels in *Bmal1*$^{fx/fx}$ recipients receiving SF1-*Bmal1*$^{-/-}$ ovaries and SF1-*Bmal1*$^{-/-}$ recipients receiving *Bmal1*$^{fx/fx}$ ovaries are shown. *P* value, by Wilcoxon rank sum test, is indicated.

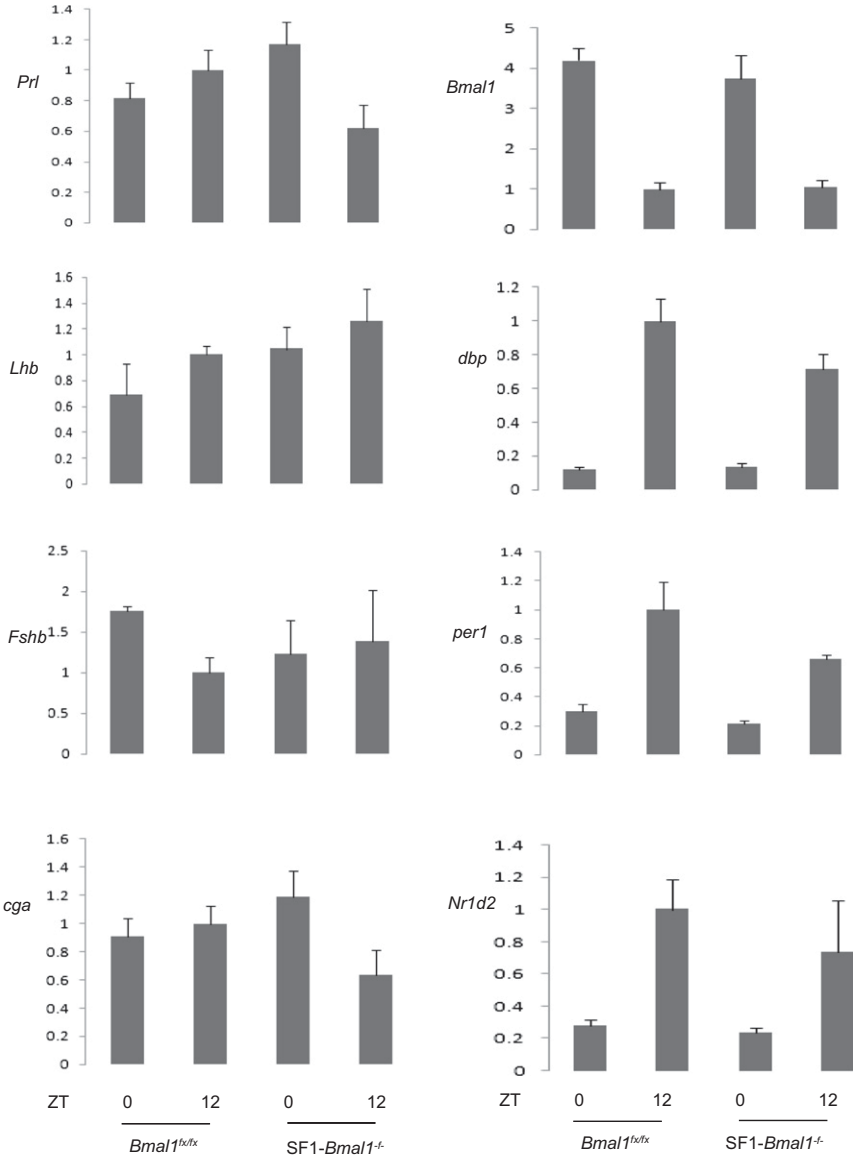

Fig. S4. Pituitary gene expression in *Bmal1*$^{fx/fx}$ and SF1-*Bmal1*$^{-/-}$ females. Microarray-derived gene expression values are represented in mean ± SEM. Expression value from *Bmal1*$^{fx/fx}$ at ZT12 on 3.5 dpc set to 1.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)
Table S5 (DOCX)