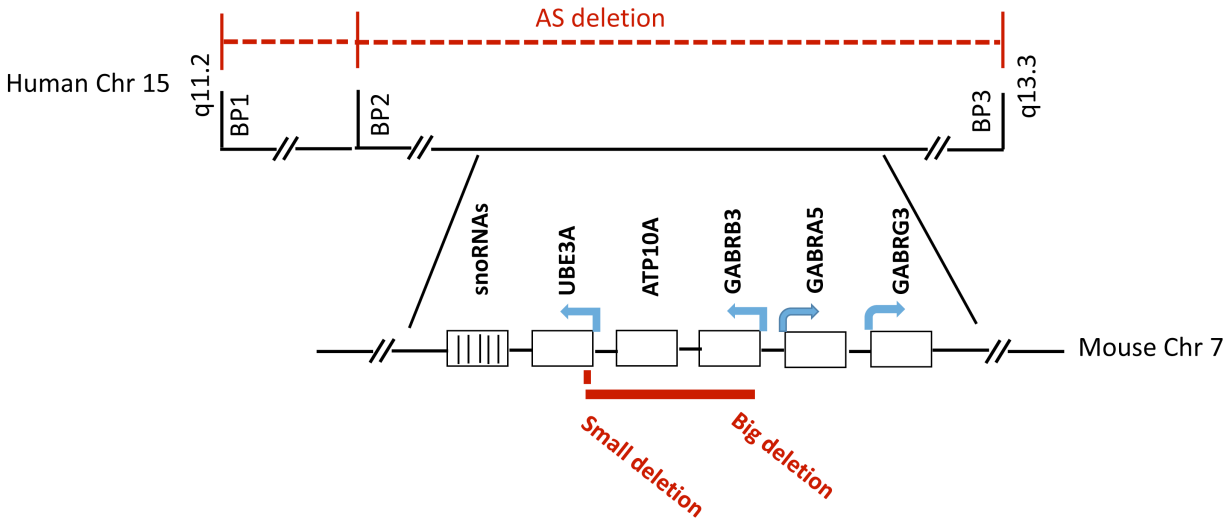
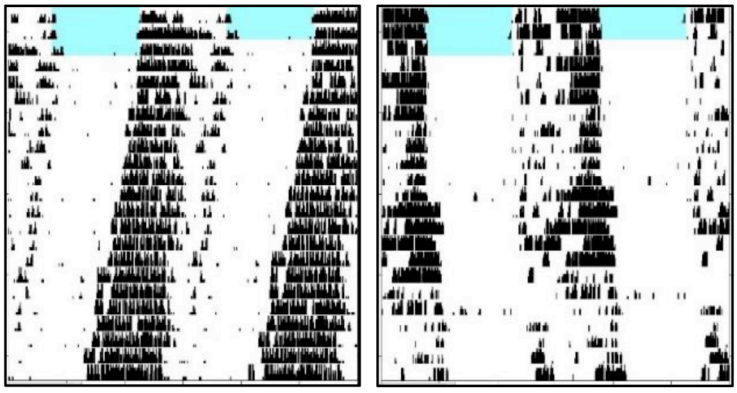
Supplemental Information

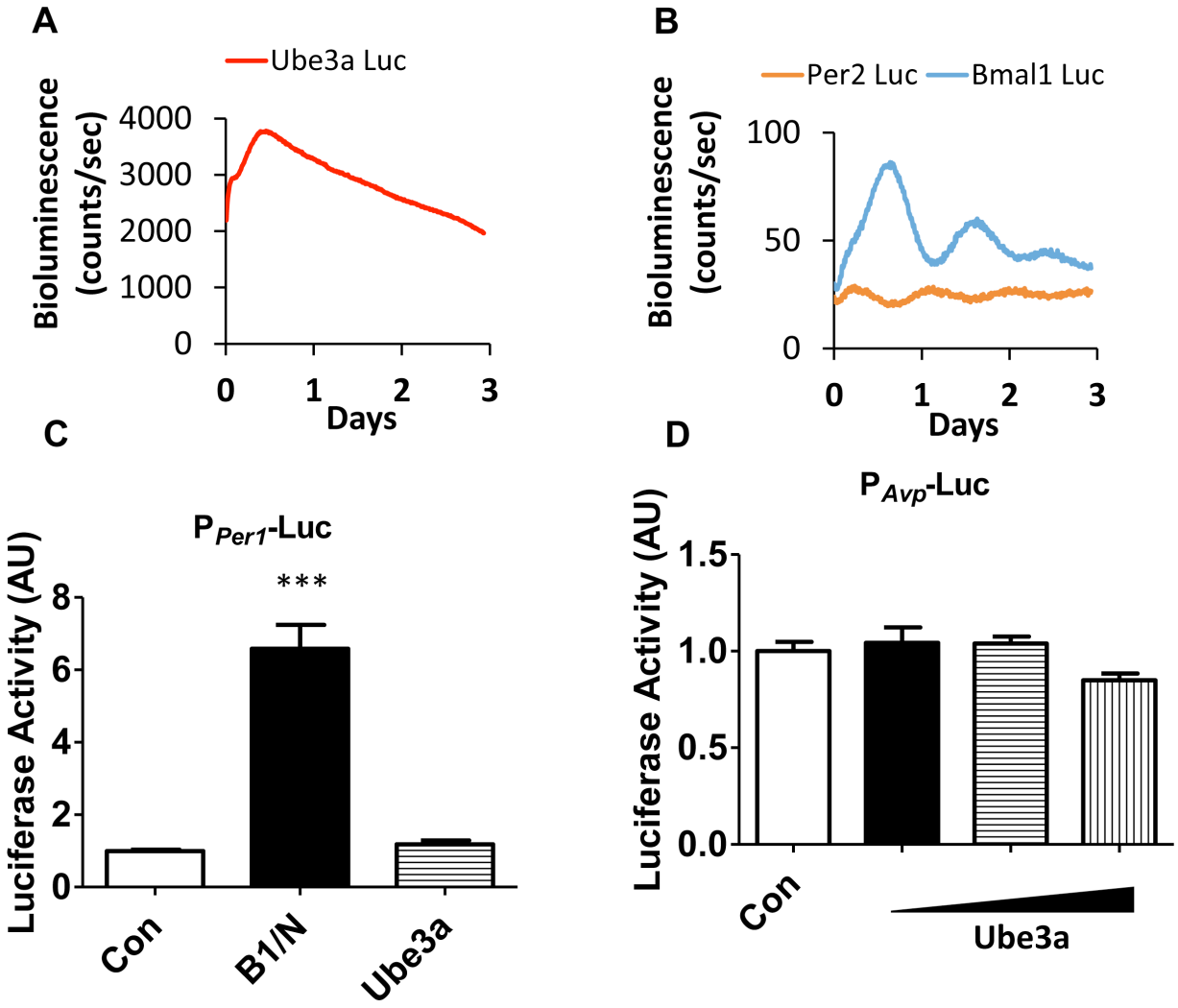
Supplemental Figures



Supplemental Figure S1 (related to Fig.1). Map of the region of human chromosome 15 (H) and mouse chromosome 7 (M) indicating the two Angelman syndrome mouse models used in this study: U-m-/p+ (specific deletion of Ube3a locus [S1] and UG-m-/p+ (inactivation of the *Ube3a* and *Gabrb3* genes and deletion of the *Atp10a* gene [S2].

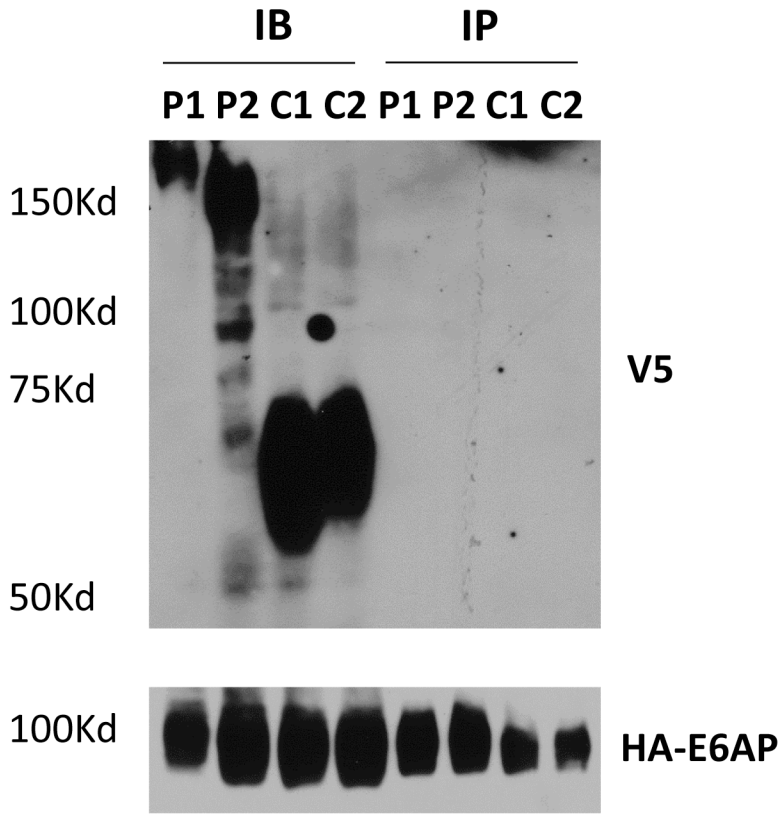


Supplemental Figure S2 (related to Fig.1). Maternal deletion of the *Ube3a-Gabrb3* region (UG-m-/p+) lengthens the period of the wheel-running rhythm. Wheel-running activity records (actograms) of mice are shown in the double-plotted format. Mice (4 month old littermates) were wildtype (WT, left), or *Ube3a* maternal deletion (UG-m-/p+, right) that were exposed to a 12 h light/12 h dark cycle (blue indicates light, white indicates dark) and were subsequently transferred to constant darkness (DD).

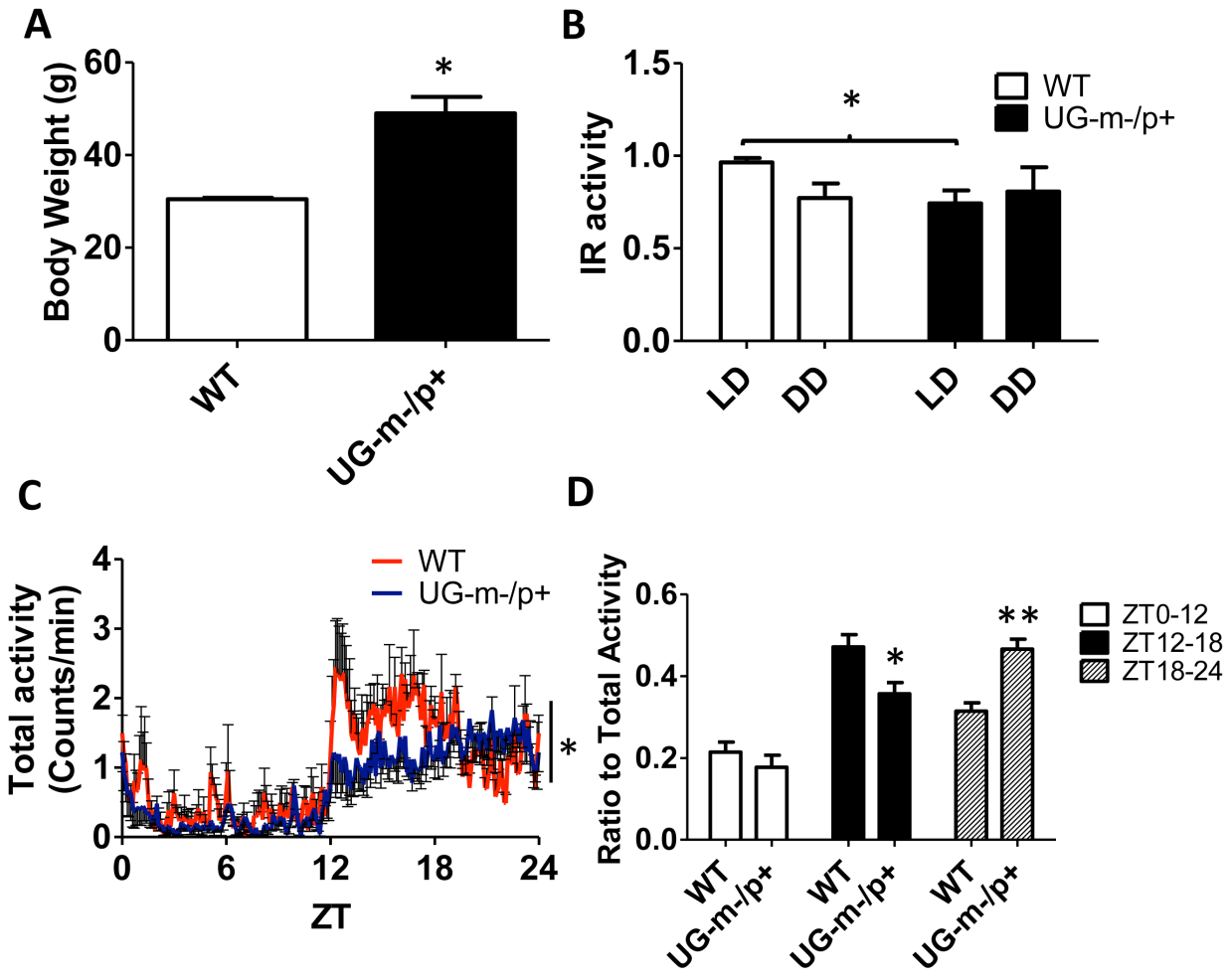


Supplemental Figure S3 (related to Fig. 4). The upstream region of *Ube3a* is not activated rhythmically, and *Ube3a* overexpression does not enhance the activity of the *mPer1* or *mAvp* promoters.

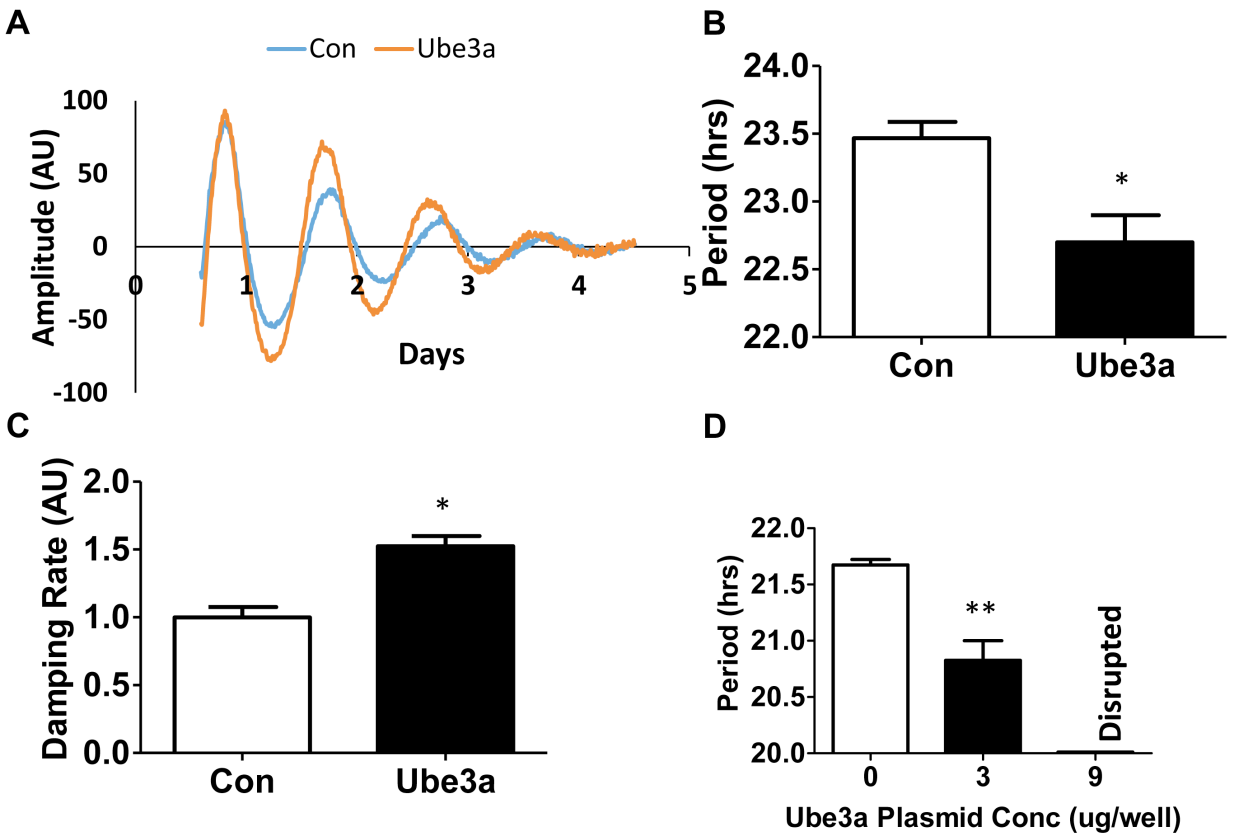
A: Transcription of an upstream region of *Ube3a* is not rhythmic, as indicated by luminescence from a P_{mUbe3a}::Luc reporter that has been transiently transfected into Rat-1 fibroblasts. **B:** Under similar conditions P_{mPer2}::Luc and P_{Bmal1}::Luc reporters exhibit circadian rhythms of luminescence (transient transfection of these luciferase reporters into Rat-1 cells as in panel **a**). **C,D:** HEK 293 cells were transiently transfected with (i) a *Ube3a* cDNA construct that expresses E6-AP and (ii) either P_{mPer1}::Luc (**C**) or P_{AVP}::Luc (**D**) reporter plasmids. In panel **C**, one set of samples was transfected with plasmids expressing BMAL1 and NPAS2 (B1/N). Data are mean ± SEM (n = 4) of firefly luciferase activity, normalized with the *Renilla* luciferase control (P_{CMV}::Rluc). *** $p<0.001$ compared with the empty vector control (Con), which is set as 1.0, one-way ANOVA, post hoc Tukey test.



Supplemental Figure S4 (related to Fig. 5). The Interaction between PER1/2 (P1/P2) or CRY1/2 (C1/C2) and E6-AP expression *in vivo*. Interaction between Per1/2 (P1/P2) or Cry (C1/C2) and E6-AP in HEK293 cells transfected with V5 tagged clock expression plasmids and HA tagged hUbe3a. Immunoprecipitates were prepared using an anti-HA antibody and the immunoprecipitates (IP) and original cell lysates (IB) were separated by electrophoresis and immunoblotted with either anti-HA (for E6-AP) or anti-V5 (for PER1/2 or CRY1/2).



Supplemental Figure S5 (related to Fig. 6). Maternal deletion of the *Ube3a-Gabrb3* region leads to an increased weight gain and altered daily activity patterns.
A: Body weight of WT and the UG-m-/p+ mice fed regular chow and weighed at 10 months of age. Each bar represents mean ± SEM. *p<0.05 by 2-tailed T test (n ⩾ 3).
B: Total locomotor activity of 4-month old mice in LD and DD. Activity was recorded by infrared sensors (mean ± SEM) (n ⩾ 3).
C: Total daily locomotor activity was recorded with infrared sensors from mice in LD of WT vs. UG-m-/p+ mice (Mean ± SEM over 7 d in LD).
D: Total daily locomotor activity was recorded with infrared sensors from mice in different phases of the LD cycle and quantified in bins separately. ZT0 denotes lights-on, and ZT12 denotes lights-off. Mean ± SEM. *p<0.05, ** p<0.01 compared with corresponding WT controls by 2-tailed T test. (n ⩾ 3).



Supplemental Figure S6 (related to Fig. 5). Induction of *Ube3a* expression levels shortens the circadian period in cell cultures.

A: Overexpression of E6-AP in mouse embryonic fibroblasts (MEFs) shortens the circadian period of PER2 expression as reported by P_{mPer2}::mPer2-Luc luminescence. MEFs were transfected with a construct that expresses Ube3a (P_{CMV}::*Ube3a*) or with an empty vector (Con).

B,C: Period (**B**) and damping rate (**C**) analyses of the rhythms in MEFs shown in panel A (mean ± SEM, n = 3 per treatment, *p<0.05, 2-tailed t-test).

D: Rat-1 fibroblasts were co-transfected with a P_{Bmal1}::Luc reporter plasmid and a plasmid expressing E6-AP (*Ube3a* cDNA-expressing plasmid). Lower concentrations of the Ube3a plasmid (3 μg/well) lead to period shortening, while higher concentrations (9 μg/well) cause arhythmia and/or rhythms whose amplitude is too low to allow an accurate estimation of period. Mean ± SEM, n = 4 per treatment. **p<0.01 as compared with the empty vector control, 2-tailed t-test.

Supplemental Experimental Procedures

Mouse strains

Mice harboring knockout of the *Ube3a* gene [S1] or of the *Ube3a-Gabrb3* region [S2] were backcrossed to the C57BL/6 background for at least seven generations (at this stage, the strains were genetically 87% C57BL/6, as assessed by SNP genotyping performed by the Jackson Laboratory, Bar Harbor, ME), followed by at least two more generations of backcrossing to C57BL/6J (JAX, Bar Harbor, Maine) before using any of the strains in this study. Female mice harboring one *Ube3a* or *Ube3a-Gabrb3* knockout allele were crossed with wild-type background (WT) male mice to obtain maternal deletion mice (U-m-/p+ or UG-m-/p+ mice, respectively). Mice harboring the P_{mPer2}::mPER2-LUC knock-in allele [S3] that have been backcrossed with the C57BL/6J strain for more than 10 generations were crossed with the *Ube3a* or *Ube3a-Gabrb3* knockout mice to obtain U-m-/p+ or UG-m-/p+ mice with the luminescence reporter of mPER2 expression. We used two strains of AS model mice for the following reasons: the U-m-/p+ strain [S1] was used to establish the specificity of the effects for the *Ube3a* gene, and the UG-m-/p+ strain was used as a more accurate model of human AS [S2]. Mice were housed in temperature-controlled environments on a 12:12-h light-dark cycle (lights on 6 am to 6 pm, cool-white fluorescence light ~ 300 lux) and fed chow (5001, Lab diet, 5% fat) for breeding and experiments. All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted according to that committee's guidelines.

Locomotor behavior and body weight/fat composition

Age and gender matched male littermates with or without the mPER2-LUC reporter were singly housed in cages equipped with running-wheel or infrared sensors for locomotor activity measurements. The locomotor activity pattern of the UG-m-/p+ mice was most accurately assessed by infrared sensors of total activity, whereas either method of measurement of activity patterns worked well for U-m-/p+ mice. ClockLab software (Actimetrics, Evanston, IL) was used to collect data and perform periodogram and activity analyses. Circadian period in DD was calculated using Chi-Square periodogram analyses. The amount of food that was consumed in 24 h was measured from mice that were housed individually. For the 6-hour LD shift experiments (Fig. 1E-H), 1.5-2 month old mice were used, otherwise, 4 month-old mice were used for locomotor activity assays. Body fat composition was determined with an mq10 nuclear magnetic resonance analyzer (Bruker Optics).

Immunoblotting and co-immunoprecipitation

Samples of hypothalamus harvested from mice in DD for 26 h and 38 h were sonicated in RIPA buffer containing a cocktail of protease inhibitors (Sigma) and clarified by centrifugation (RIPA buffer = 50mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS,1.0% NP-400.5% Sodium Deoxycholate). Protein from the supernatant was quantified by the Bradford assay (Bio-Rad). Proteins were separated by SDS-PAGE and then transferred to PVDF membranes for immunoblotting. Monoclonal IgG directed against E6-AP (Sigma), anti-BMAL1 antibody (a gift of Dr. Charles Weitz), and anti-β-ACTIN monoclonal antibody (Sigma) were used to probe E6-AP, BMAL1, and β-ACTIN, respectively. Densitometric analyses were performed using Image J software (NIH). To test the physical interaction between E6-AP and BMAL1 proteins, HEK 293 cells were transfected with HA-*hUbe3a* and Myc tagged *Bmal1*/2 or V5 tagged *Per1/2* or *Cry1/2* plasmids. The cell extracts were immunoprecipitated by adding anti-HA antibody (Roche). Precipitated proteins and cell extracts from transfected cells (5-20% of input for IP) were examined by SDS-PAGE and immunoblotting. Anti-HA monoclonal antibody (Roche), anti-Myc monoclonal antibody (a gift of Dr. Heping Yan), anti-V5 antibody (Invitrogen) were used to probe HA tagged

E6-AP, Myc tagged BMAL1/2, and V5 tagged PER1/2 or CRY1/2 respectively.

Tissue culture and *in vitro* luminescence recording
Both male and female littermates harboring one copy of the P_{mPer2}::mPER2-LUC reporter were used for tissue culture and luminescence recording. One to two hours before lights-off of LD 12:12, the mice were sacrificed by cervical dislocation, and cultures of SCN and peripheral tissues were prepared as previously described [S3-S6]. The tissues were cultured in recording medium containing 10% fetal bovine serum (Gibco) and 100 nM of luciferin (Promega) at 36.5°C. The *in vitro* rhythms were analyzed using LumiCycle analysis software (Actimetrics, version 2.31). Topotecan (Tocris) was added to the medium at the beginning of culture (final concentration of 300 nM).

Tissue collection and quantitative RT-PCR
Mice were housed in LD 12:12 with food and water *ad libitum* before being transferred to DD. Animals were sacrificed under dim red light by cervical dislocation beginning at 44 h in DD (CT16). The tissues were collected and stored at -80°C until RNA extraction. Total liver RNA were extracted and transcribed by Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Diluted cDNA was amplified by PCR using the Sybr Green Master Mix (Sigma), and raw threshold-cycle time (Ct) values were calculated with iQ5 software (Bio-Rad). Mean values were calculated from samples and normalized to those obtained for *Hprt* transcripts as reported previously [S5].

Transient transfection assay and *Ube3a* reporter plasmid construction
HEK293 cells were plated in 24-well plates and transfected with Lipofectamine™ 2000 plus various expression constructs and reporter gene constructs (transfection conducted as recommended by Invitrogen). The total amount of DNA for each transfection reaction was kept constant by the addition of an appropriate concentration of empty vector DNA. 24 h after transfection, the cells were lysed, and firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) activities were assayed with the Dual-Luciferase Assay Kit (Promega). A P_{CMV}::Rluc reporter served as the transfection efficiency control. To measure the effect of *Ube3a* expression on circadian rhythms in cell cultures, MEFs (from P_{mPer2}::mPER2-LUC knock-in mouse embryos) or Rat-1 fibroblast cells were transfected with a plasmid expressing E6-AP under the control of the CMV promoter (P_{CMV}::*hUbe3a*). The transfection of MEFs was accomplished with the Neon system (Life Technology). In the case of the Rat-1 fibroblasts, the cells were co-transfected with P_{CMV}::*hUbe3a* and with a P_{Bmal1}::FLuc reporter using Fugene 6 reagent (Roche). One day after transfection, cells were synchronized by 100 nM Dexamethasone for 2 h and then luminescence emission was monitored in a Lumicycle apparatus (Actimetrics). To make a luminescence reporter of *mUbe3a* promoter activity, a fragment extending from the mouse *Ube3a* upstream region (-1880 bp from the transcription start site, NM_173010) to the 5' untranslated region (+220 bp from the transcription start site) was amplified by PCR from mouse genomic DNA and inserted into the pGL4.11 luciferase vector (Promega). The plasmid was verified by sequencing and one canonical (CACGTG) and two non-canonical E-boxes (CA-GC/TG-TG) are present in this 2100-bp upstream region of mouse *Ube3a*.

Supplemental References:

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