Neuron, Volume *79*

Supplemental Information

Translational Control of Entrainment and Synchrony of the Suprachiasmatic Circadian Clock by mTOR/4E-BP1 Signaling

Ruifeng Cao, Barry Robinson, Haiyan Xu, Christos Gkogkas, Arkady Khoutorsky, Tommy Alain, Akiko Yanagiya, Tatiana Nevarko, Andrew C. Liu, Shimon Amir, and Nahum Sonenberg

INVENTORY OFSUPPLEMENTAL MATERIALS

Figure S1 (related to Figure 1)

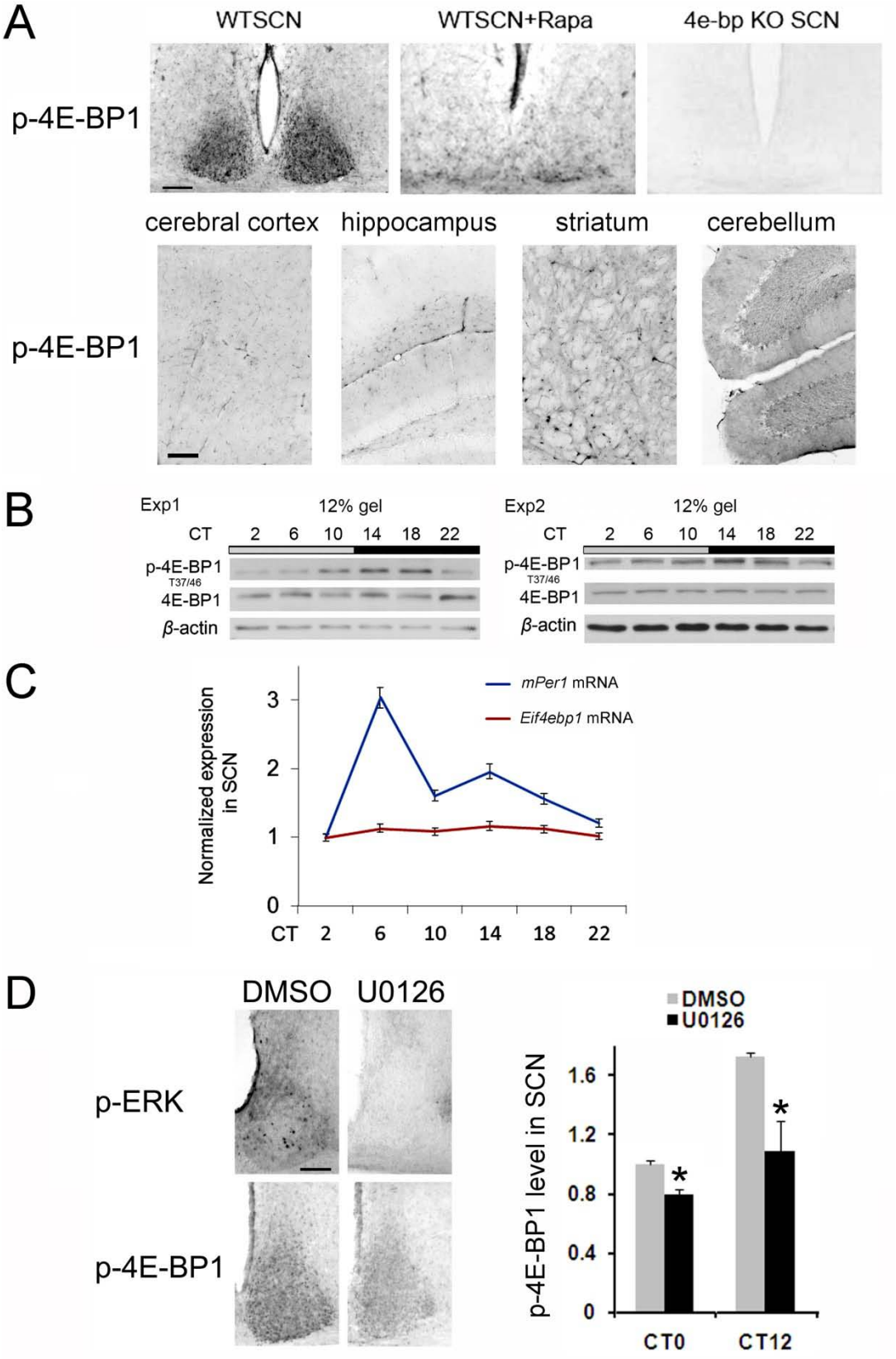

Figure S1 (related to Figure 1). Phospho-4E-BP1 and *Eif4ebp 1* mRNA Expression in the Brain

A. 4E-BP1 phosphorylation (at Thr37/46) in different brain regions. Top panel: phospho-4E-BP1 labeling in WT SCN, WT SCN infused with rapamycin and *Eif4ebp 1/2* KO SCN. Bottom panel: phospho-4E-BP1 labeling in indicated brain regions. For both A and B, the mice were entrained in a 12 h/12 h light/dark (LD) cycle for two weeks and sacrificed at ZT16. For rapamycin infusion, mice were cannulated and rapamycin(100 μM, 2μL) was infused to the lateral ventricle 30 min before sacrifice.

B. Circadian phosphorylation of 4E-BP1 (at Thr37/46) in the SCN. Note that phosphorylation of 4E-BP1 exhibited different levels with circadian time, while 4E-BP1 level did not change. β-actin was used as a protein loading control. In each experiment, SCN tissue from five mice was pooled for each time point. Half of the lysate was used for detecting phosphorylation of 4E-BP1 and the other half was used for 4E-BP1 and β-actin blotting.

C. *Eif4ebp 1* mRNA expression in the SCN. Mice were entrained in a 12 h/12 h LD cycle for two weeks and left in DD for 48 h. SCN tissue was dissected during the next 24 h period and total RNAs were extracted. qRT-PCR was performed to determine *mPer1* and *Eif4ebp 1* mRNA levels in the tissue. Note that *mPer1* mRNA exhibited different levels with circadian time, while *Eif4ebp 1* mRNA level did not significantly change. Five mice were used for each time point.

D. U0126 inhibits 4E-BP1 phosphorylation (at Thr37/46). Left panel: representative micrographs of phospho-ERK (at Thr22/Tyr24) and phospho-4E-BP1 (at Thr37/46) labeling in the SCN at CT12. For these experiments, the mice were cannulated and entrained in a 12 h/12 h light/dark (LD) cycle for two weeks and left in constant dark(DD) for 48 h. Mice were infused with either DMSO or U0126 (10mM, 2μL) at CT23.5 or CT11.5 and sacrificed 30 min after the infusion. Right panel: quantitation of phospho-4E-BP1 (at Thr37/46) expression in the SCN. Five mice were used for each group. Note that U0126 decreased 4E-BP1 phosphorylation at both CT0 and CT12.

Figure S2 (related to Figure 2)

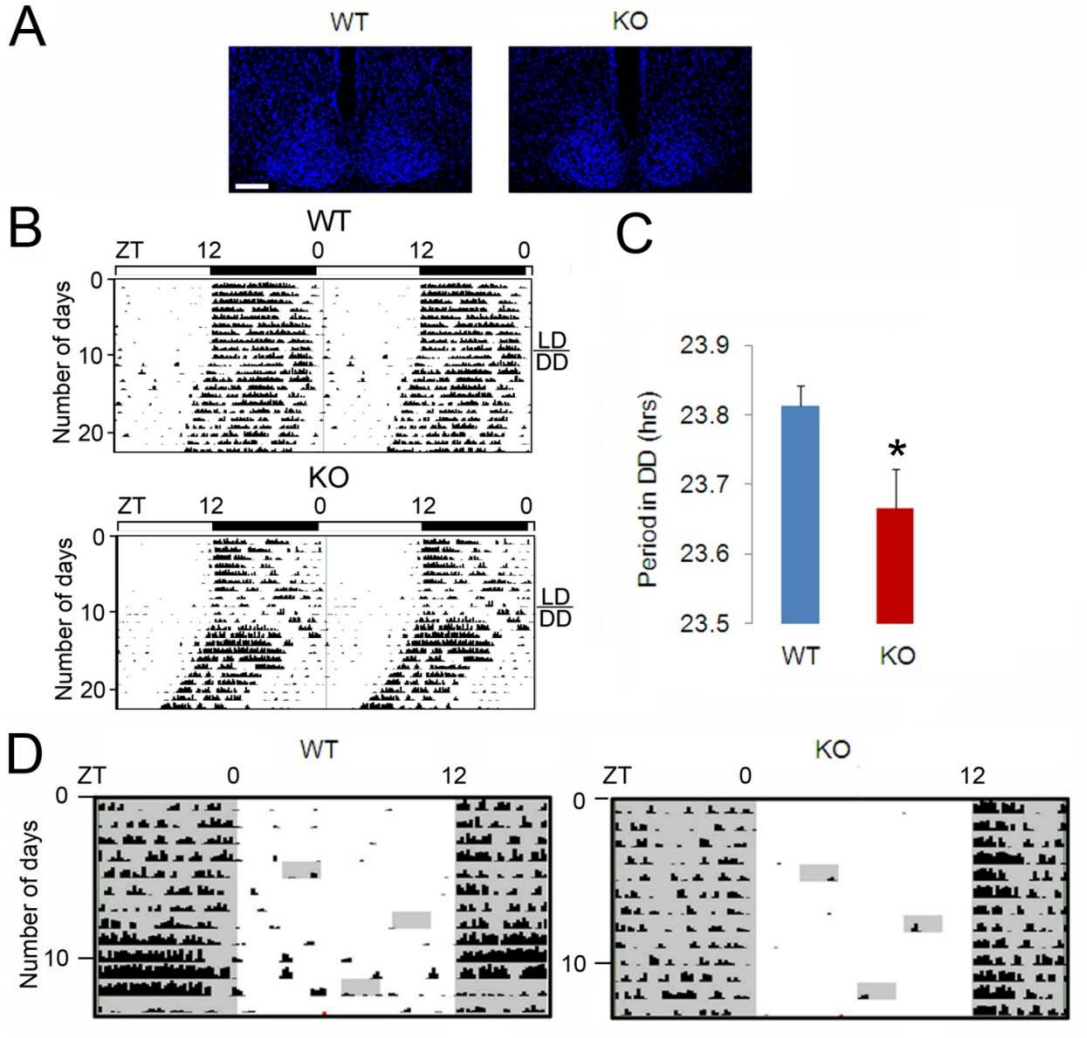

Figure S2 (related to Figure 2). DRAQ5 Labeling and Animal Circadian Behavior in *Eif4ebp1* KO Mice

A. Confocal microscopic imaging shows DRAQ5 (a dye for cell nucleus) staining in the SCN. DRAQ5 staining indicates that the histological architecture of KO SCN is not distinct from the WT SCN. Scale bar: 100 μm.

B. Representative double-plotted actograms of wheel-running activities from WT (top) and *Eif4ebp1* KO mice (bottom). For these experiments, the animals were entrained to a 12 h/12 h light/dark (LD) cycle for 10 days and released into constant dark (DD) for 12 days. The *x*-axis (top) indicates the

Zeitgeber (ZT) time of the day. The *y*-axis (left) indicates the number of days during the experiment.

C. Histograms showing behavioral circadian period in DD. Values are presented as the mean ± SEM. Each data point is averaged from twelve mice. The period was slightly decreased in KO mice compared to WT mice ($p<0.05$, KO vs. WT by Student's t-test).

D. Representative single-plotted actograms of wheel-running activities from WT (left) and *Eif4ebp1* KO mice (right). For these experiments, animals were entrained in a 12 h/12 h LD cycle for 4 days, and on the 5th, 8th and 12th day, three two-hour dark pulses were applied starting from random time points during the light phase (indicated by the grey areas). Note that dark pulses did not induce significant wheel-running activities in WT and KO mice.

Figure S3 (related to Figure 3)

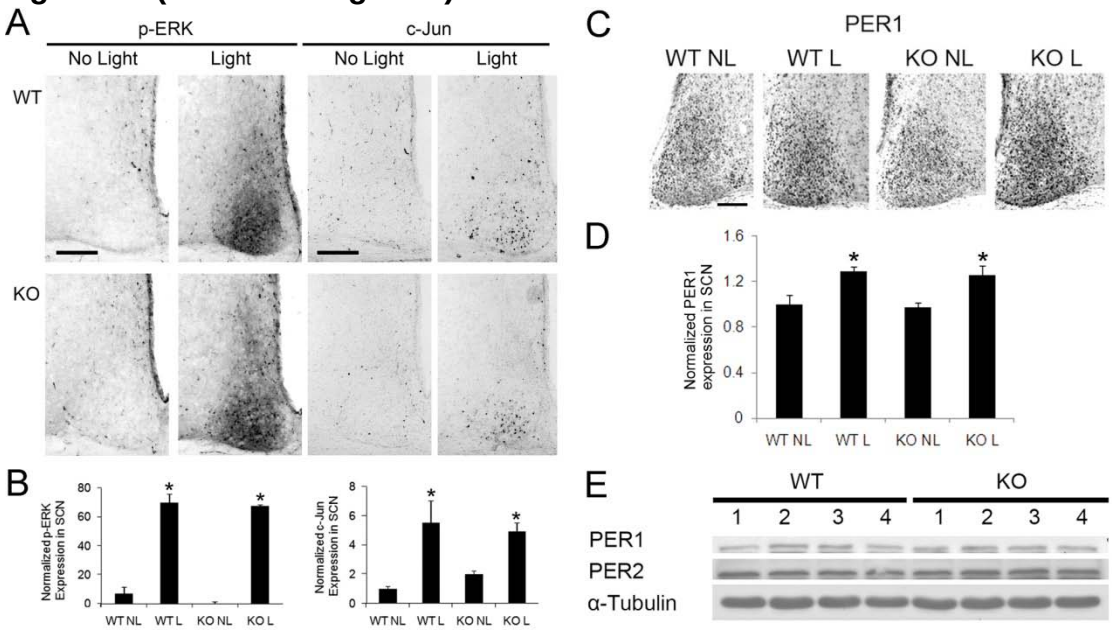

Figure S3 (related to Figure 3). Light-induced p-ERK, c-Jun and PER1 expression in the *Eif4ebp1* KO mice

A. Representative micrographs of SCN tissue labeled for the phosphorylated (at Thr 202 and Tyr 204) form of ERK (p-ERK, left) and c-Jun (right). Relative to the control condition (No Light), photic stimulation (100 lux, 15 min, starting at CT15) triggered a robust increase in ERK phosphorylation and c-Jun expression in the SCN of WT mice. Compared to the WT mice, light induced similar level of p-ERK and c-Jun expression in KO mice. For these experiments, mice were entrained to a 12 h/12 h LD cycle for 10 d and put in DD for 2 d. A light pulse (100 lux, 15 min) was applied at CT15. Animals were killed immediately after cessation of the light pulse. Scale bars: 100 μm.

B. Quantitation of light-induced ERK phosphorylation (left) and c-Jun expression (right). Light evoked significant p-ERK and c-Jun expression in the SCN of both WT and KO mice (*p<0.05, WT L vs. WT NL, KO L vs. KO NL

by ANOVA). The p-ERK and c-Jun induction in the KO mice was not significantly different with that in the WT mice (p>0.05, KO L vs. WT L, by ANOVA). L: light; NL: no light

C. Representative micrographs of SCN tissue labeled for anti-PER1 antibody. Relative to the control condition (No Light), photic stimulation (100 lux, 15 min, starting at CT15) triggered a robust increase in PER1 expression in the SCN of WT mice at CT19. Compared to the WT mice, light induced similar level of PER1 expression in KO mice. For these experiments, mice were entrained to a 12 h/12 h LD cycle for 10 d and put in DD for 2 d. A light pulse (100 lux, 15 min) was applied at CT15. Animals were killed 4h after cessation of the light pulse. Scale bars: 100 μm. L: light; NL: no light

D. Quantitation of light-induced PER1 in the SCN. Light evoked significant PER1 expression in the SCN of both WT and KO mice (*p<0.05, WT L vs. WT NL, KO L vs. KO NL by ANOVA). The PER1 induction in the KO mice was not significantly different with that in the WT mice (p>0.05, KO L vs. WT L, by ANOVA). L: light; NL: no light

E. Western blots of brain lysates. For these experiments, WT and *Eif4ebp1* KO animals were sacrificed at ZT0 and whole brain was lysed for Western blotting analysis. The numbers 1-4 indicate four different animals for each group. Note that PER1 and PER2 levels were not changed in the brain of *Eif4ebp1* KO mice compared to WT mice. α-Tubulin was used as a loading control.

Figure S4 (related to Figure 5)

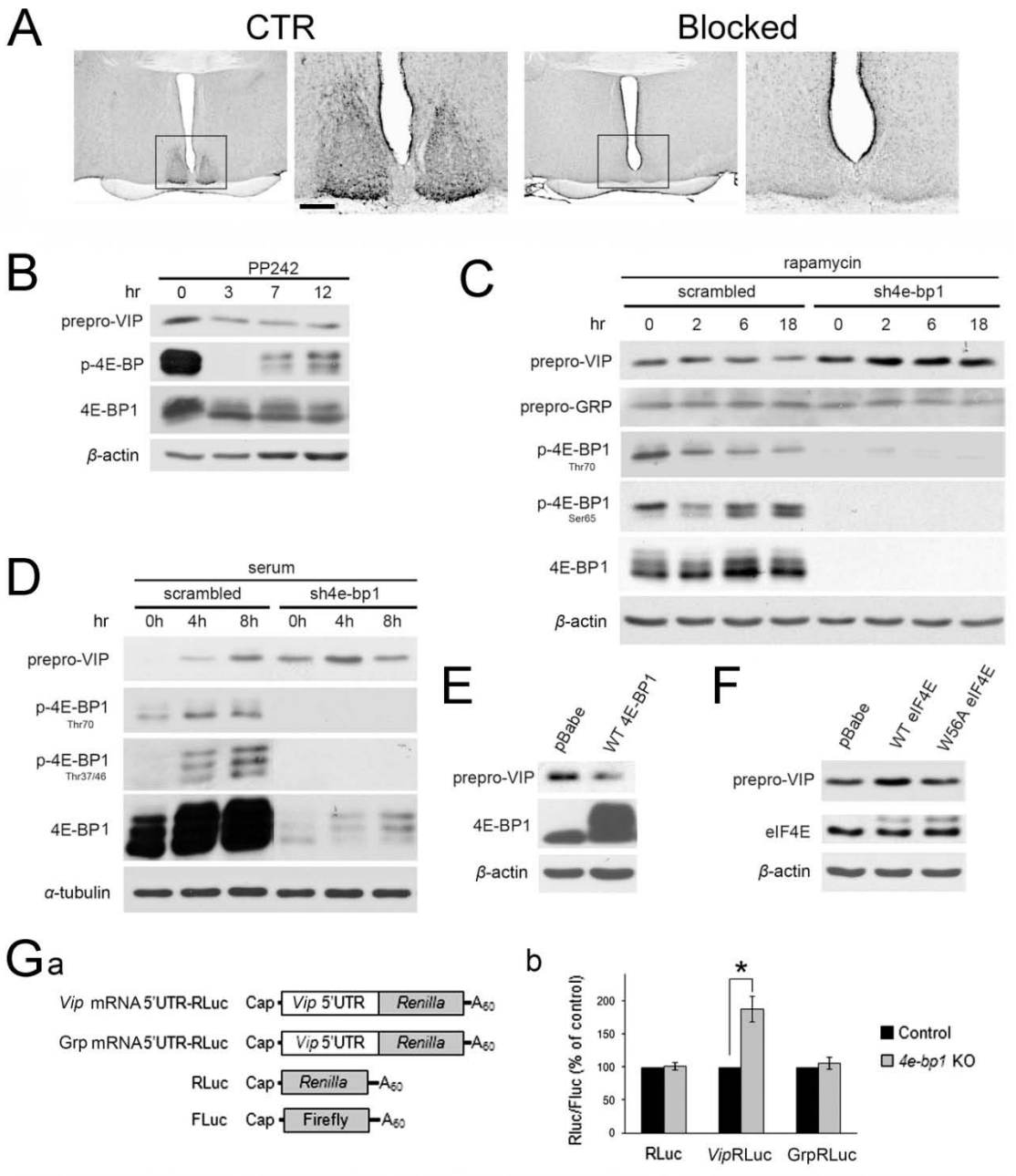

Figure S4 (related to Figure 5). VIP Expression in SCN and Neuroblastoma Cells

A. Representative microscopic images of SCN tissue labeled with control anti-VIP antibody (CTR, left panel) or anti-VIP antibody pre-incubated with the antigen peptide (Blocked, right panel). In each panel, framed area is magnified to the right. Note that VIP labeling signal was completely blocked by the

antigen peptide.

B. Prepro-VIP expression in Neuro2a cells in the presence of PP242 (2.5 μM). Cells were cultured in DMEM supplemented with 10% FBS. Note that PP242 decreased prepro-VIP expression and caused 4E-BP1 dephosphorylation.

C. Prepro-VIP expression in SHEP cells. Cells were cultured in DMEM supplemented with 10% FBS. 4E-BP1 expression was knocked down by lentivirus-mediated shRNAs targeting *Eif4ebp1* mRNA (sh4e-bp1). As a control, cells were infected with lentivirus encoding a scrambled shRNA (scrambled). Cells were treated with rapamycin (100 nM) and harvested at the indicated time points. Note that in sh4e-bp1 cells, prepro-VIP expression was upregulated. Rapamycin induced a persistent decrease of prepro-VIP expression in scrambled cells but not in sh4e-bp1 cells.

D. Prepro-VIP expression in serum-stimulated SHEP cells. Cells were cultured in DMEM supplemented with 10% FBS and serum-starved for 8 h. Then, 10% FBS was added into the medium, and the cells were harvested at the indicated time points. Note that serum induced a persistent increase of prepro-VIP expression in scrambled cells, but the induction was attenuated in sh4e-bp1 cells.

E. Prepro-VIP expression in SHEP cells overexpressing WT 4E-BP1 (with an HA tag).

F. Prepro-VIP expression in SHEP cells overexpressing eIF4E (with a FLAG tag).

G. 5’UTR luciferase assay. (a) Schematic diagram of the *Renilla* (Rluc) and *Firefly* (Fluc) luciferase reporter mRNAs. (b) Translation of the reporter mRNAs in WT (black bars) and *Eif4ebp1* KO (gray bars) MEFs. Fluc-pA mRNA was co-transfected with Rluc mRNAs as a transfection control. The values of Rluc were normalized to those of Fluc. The Rluc/Fluc values in the WT MEFs were set as 100%. Error bars represent the standard deviation of three independent experiments. Note that translation of *Vip* 5’UTR but not *Grp* 5’UTR mRNA reporter was increased in *Eif4ebp1* KO MEFs.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cannulation and Infusion

Mice were cannulated in the lateral ventricles using the techniques described by Cao et al. (2008). The coordinates (posterior, 0.34 mm from bregma; lateral, 0.90 mm from the midline; dorsoventral, −2.15 mm from bregma) were used to place the tip of a 24 gauge guide cannula into the lateral ventricle. To disrupt mTOR signaling, rapamycin (100 μM, 2μL; Cell Signaling Tech., MA) was infused through the cannula at ZT15. Control animals were infused with the same volume of DMSO.

Circadian Behavioral Assay and Light Flash Experiment

Eight- to 10-week-old male mice were individually housed in cages equipped with running wheels. The animals were entrained to a 12 h/12 h light/dark (LD) cycle for 9 days and released into constant dark (DD) for 9 days. In another experiment, the animals were entrained in a 12 h/12 h LD cycle for 4 days, and on the 5th, 8th and 12th day, three two-hour dark pulses were applied starting from random time points during the light phase.

For the light flash experiment, mice were entrained to a 12 h/12 h LD cycle for 10 d and put in DD for 2 d. Animals received a single light exposure (100 lux, 15 min) at CT 15 and were sacrificed immediately or 4h after cessation of the light pulse. Control mice (no light) were sacrificed at CT15.

Immunostaining, DRAQ5 staining and Microscopic Imaging

For immuohistochemical staining, coronal SCN sections (40 µm) were treated with 0.3 % H_2O_2 and 20 % methanol in PBS for 10 min to deactivate endogenous peroxidases and to permeabilize the tissue. The tissue was then blocked for 1 h in 10% goat serum/PBS and incubated in anti-phosphorylated ERK (p-ERK, Thr-202, Tyr-204) antibody, anti-phosphorylated 4E-BP1 (p-4E-BP1, Thr37.46), anti-PER1or anti-c-Jun antibody overnight at 4°C. For the VIP blocking experiment, the diluted VIP antibody was incubated with either scrambled or antigen peptide (1:300, sc-21041) overnight at 4°C, and then used for immunostaining. Next, tissue was incubated for 1.5 h in biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) at room temperature and then placed in an avidin/biotin/HRP complex for 1 h (Vector Laboratories). Sections were washed in PBS (three times, 10 min per wash) between each labeling step. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories) and sections were mounted on slides with Permount media (Fisher Scientific, Houston, TX).

For AVP and VIP double immunofluorescent labeling, tissue was permeabilized with PBST (PBS with 1 % Triton X-100) for 30 min, blocked as described above and then incubated (overnight, 4° C) in 5 % goat serum/PBS with rabbit anti-AVP(1:300) and goat anti-VIP(1:200) antibodies. The

following day, sections were incubated (3 h, room temperature) in Alexa Fluor-conjugated secondary antibodies (1:500; Molecular Probes, Eugene OR) directed against the IgG domains of the primary antibodies. Brain sections were washed in PBS (three times, 10 min per wash) between each labeling step. Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

DRAQ5 was purchased from Biostatus Limited (Leicestershire,United Kingdom). SCN Sections were incubated in PBS with DRAQ5 (1:5000) for 10 min and washed for three times with PBS (10 min each time).

Bright-field microscopy images were captured using a digital camera mounted on an inverted Zeiss microscope (Oberkochen, Germany). Confocal microscopy images were captured using a Zeiss 510 Meta confocal microscope. All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment. The staining intensity in micrographs was quantified as reported (Cao et al., 2008, 2010, 2011). Briefly, all data were quantified using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For the p-4E-BP, p-ERK, and PER1 and PER2 intensity analysis, images of the SCN were acquired (10X), digitally outlined, and the mean pixel values were determined. A digital oval (150×200 pixels) was placed on the adjacent lateral hypothalamus, and the

mean value was subtracted from the value of the SCN signal to generate a normalized SCN intensity value. Notably, the lateral hypothalamic immunolabeling values were not altered with treatment conditions. For the c-Jun and VIP-positive cell counting, an intensity threshold filter was applied to eliminate nonspecific background labeling, and the number of detectable signals above threshold (now defined as positive cells) were counted for each SCN. For all experiments, the data were averaged from three central SCN sections per animal, and these values were pooled to generate a mean value for each group.

Neuroblastoma Cell Culture and Virus Infection

Neuro-2A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). SHEP cell line was a kind gift from Dr. Linda Penn at the University of Toronto. Cells were maintained at 37°C in 5% of CO^2 atmosphere in Dulbecco' modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured and infected by virus as reported (Dowling et al., 2010). The following shRNA vectors and accession numbers were used: human 4E-BP1 (Sigma: TRCN0000040203), Non-Target shRNA Control (Sigma: SHC002 or Addgene: plasmid 1864). The shRNA vectors were cotransduced into HEK293T cells with the lentivirus packaging plasmids PLP1, PLP2, and PLP-VSVG (Invitrogen) using Lipofectamine 2000 (Invitrogen). Supernatants

were collected 48 and 72 h post-transfection, passed through a 0.45 μm nitrocellulose filter, and applied to target cells with polybrene (5 μg/ml). Cells were re-infected the next day and selected with puromycin for 48 h (1 μg/ml, Sigma). pcDNA3-3HA-4E-BP1, pcDNA3-FLAG-eIF4E, and pcDNA3.1-2flag-eIF4EW56A (cap-binding mutant) have been described previously (Poulin et al., 1998; Gingras et al. 1999). The cDNAs were amplified by PCR and inserted into the BamHI/SalI sites of the retroviral vector pBABE. Cell lines stably expressing 4E-BP1 or eIF4E were generated as described previously (Rong et al., 2008).

5'UTR Luciferase Assay

Luciferase reporter mRNAs were generated using the MAXIscript T7 *In Vitro* Transcription kit (Ambion). PCR products encoding the T7 promoter, luciferase, and a poly(A) sequence were used as templates for *in vitro* transcription. WT and *Eif4ebp1* KO MEFs (Dowling et al., 2010) were seeded in 24-well plates and cultured overnight. The cells were co-transfected with either VIP-5'UTR-Rluc-pA , GRP-5'UTR-Rluc-pA or Rluc-pA and Fluc-pA (transfection control) using Lipofectamine (Invitrogen). The cells were lysed 7 h post-transfection, and the luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega).

Table S1. Antibodies used for immunostaining and Western blotting

Antibody	Company	Cat.No	Dilution in immuostaining	Dilution in Western blotting
VIP	Santa Cruz	sc-21041	1-300	
AVP	abcam	ab39363	1-200	
4E-BP1	Cell Signaling	9644	1-250	1-500
p-4E-BP1(Thr37/46)	Cell Signaling	2855	1-500	1-1000
p-4E-BP1(Thr70)	Cell Signaling	9455		1-500
p-4E-BP1(Ser65)	Cell Signaling	9451		1-500
PER1	Millipore	AB2201	1-3000	
PER2	Santa Cruz	sc-7728	1-300	
prepro-VIP	Sigma-Aldrich	V0390		1-500
VPAC2	abcam	ab28624		1-1000
prepro-GRP	ImmunoStar	20073		1-200
prepro-AVP	Calbiochem	PC234L		1-500
Connexin 36	Santa Cruz	sc-14904		1-200
GABAARα1-6(E-8)	Santa Cruz	sc-376282		1-200
eIF4E	BD Transduction Laboratories	610270		1-2000
α-tubulin	Sigma-Aldrich	T9026		1-5000
β-actin	Sigma-Aldrich	A5441		1-5000
p-ERK(Thr202/204)	Cell Signaling	9106	1-2000	
c-Jun	Cell Signaling	9165	1-500	

Table S2. Primers used for qPCR

Primer	Sequence (5' to 3')
VIP Fw mouse	CACAGATAACTACACCCGCC
VIP Re mouse	GAACTCATAATGTAATCACG
AVP Fw	CAACACTACGCTCTCCGCTTGTTT
AVP Re	ACACTGTCTCAGCTCCATGTCAGA
VIPR2 Fw	AGCAGCCAAACGGAGAATCAGAGA
VIPR2 Re	TGACCATCCATCGCTAGTGCAGTT
Actb Fw	TGTGATGGTGGGAATGGGTCAGAA
Actb Re	TGTGGTGCCAGATCTTCTCCATGT
meif4ebp1 Fw1	GCACATACCTCCTTGTGCCT
meif4ebp1 Re1	TCCCAGGTAACCCAGCCTAA
mPer1 Fw	AGCAGGTTCAGGCTAACCAGGAAT
mPer1 Re	AGGTGTCCTGGTTTCGAAGTGTGT

SUPPLEMENTAL REFERENCES

Cao, R., Anderson, F. E., Jung, Y. J., Dziema, H., and Obrietan, K. (2011).Circadian regulation of mammalian target of rapamycin signaling in the mouse suprachiasmatic nucleus. Neuroscience *181*, 79-88.

Cao, R., Lee, B., Cho, H. Y., Saklayen, S., and Obrietan, K. (2008).Photic regulation of the mTOR signaling pathway in the suprachiasmatic circadian clock.Mol Cell Neurosci *38*, 312-324.

Cao, R., Li, A., Cho, H. Y., Lee, B., and Obrietan, K.(2010). Mammalian target of rapamycin signaling modulates photic entrainment of the suprachiasmaticcircadian clock. J Neurosci *30*, 6302-6314.

Dowling, R. J., Topisirovic, I., Alain, T., Bidinosti, M., Fonseca, B. D., Petroulakis, E., Wang, X., Larsson, O., Selvaraj, A., Liu, Y.*, et al.* (2010).mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science *328*, 1172-1176.

Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999). Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev *13*, 1422-1437.

Poulin, F., Gingras, A. C., Olsen, H., Chevalier, S., and Sonenberg, N. (1998). 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J Biol Chem *273*, 14002-14007.

Rong, L., Livingstone, M., Sukarieh, R., Petroulakis, E., Gingras, A. C., Crosby, K., Smith, B., Polakiewicz, R. D., Pelletier, J., Ferraiuolo, M. A., and Sonenberg, N. (2008). Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. Rna *14*, 1318-1327.