Light-regulated translational control of circadian behavior by eIF4E phosphorylation

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The circadian (~24 h) clock is continuously entrained (reset) by ambient light so that endogenous rhythms are synchronized with daily changes in the environment. Light-induced gene expression is thought to be the molecular mechanism underlying clock entrainment. mRNA translation is a key step of gene expression, but the manner in which clock entrainment is controlled at the level of mRNA translation is not well understood. We found that a light- and circadian clock–regulated MAPK/MNK pathway led to phosphorylation of the cap-binding protein eIF4E in the mouse suprachiasmatic nucleus of the hypothalamus, the locus of the master circadian clock in mammals. Phosphorylation of eIF4E specifically promoted translation of Period 1 (Per1) and Period 2 (Per2) mRNAs and increased the abundance of basal and inducible PER proteins, which facilitated circadian clock resetting and precise timekeeping. Together, these results highlight a critical role for light-regulated translational control in the physiology of the circadian clock.

Circadian (~24 h) rhythmicity is a fundamental property of nearly all living organisms1. In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus2. The SCN generates ~24-h rhythms that orchestrate a variety of physiological and behavioral processes3. The circadian rhythms allow animals to predict and prepare for upcoming environmental changes, which is critical for survival. Rhythmic gene expression constitutes the molecular basis of circadian oscillation. The molecular clockwork is composed of transcriptional-translational feedback loops4. The primary negative feedback loop is driven by rhythmic activation of Period (Per) and Cryptochrome (Cry) transcription by the CLOCK (or NPAS2) and BMAL1 heterodimers. The proteins encoded by Per and Cry mRNAs form multimer complexes. As they accumulate in the cytoplasm, the PER-CRY complexes translocate into the nucleus and interact with the CLOCK-BMAL1 heterodimers to repress their own gene transcription.

The circadian clock is entrained (reset) by external cues so that the endogenous rhythms are continuously synchronized with the daily changes in the environment5. Light is the dominant signal for clock entrainment. The phasing of the SCN clock is tightly regulated by ambient light/dark (LD) cycles. At night, transient light exposure causes a rapid resetting of the clock6. Although the precise molecular mechanisms by which photic input drives clock entrainment have not been resolved, it is thought that rapid induction of gene expression drives the resetting process7. Previous studies have revealed multiple signaling mechanisms by which Per transcription is induced by light8–10. For example, light stimulates the mitogen- and stress-activated protein kinase (MSK) 1 (ref. 11), which in turn leads to activation of transcription factors such as the cAMP response element–binding protein (CREB). CREB activates the expression of early response genes that harbor cAMP response elements (CREs) in their promoters, including Fos and Per1 (ref. 12). Little is known, however, about how mRNA translation, a key step in gene expression, is coordinated in the clock entrainment process.

Control of mRNA translation occurs predominantly at the initiation step, which, under most circumstances, is rate-limiting in protein synthesis13. Translation initiation begins with the recognition of the mΓGppN (where N is any nucleotide) structure (mRNA 5′ cap), which is present at the 5′-end of all nuclear transcribed mRNAs, by the eukaryotic translation initiation factor 4E (eIF4E)11. eIF4E binds to the 5′ cap as a subunit of the eIF4F complex together with eIF4A and eIF4G13,14. As a rate-limiting translation factor, eIF4E is a major target for translational control. Its abundance and activities are intricately controlled by several mechanisms. For example, the mTOR-regulated eIF4E binding proteins (4E-BPs) prevent the eIF4E binding to eIF4G, and thus the formation of the eIF4F complex and its binding to the 5′ cap13,14. We have recently reported a key role for 4E-BP1–mediated translational control in the circadian clock15. In addition, the activities of eIF4E can be regulated by phosphorylation. In response to extracellular signals, the p38 and p42/44 mitogen–activated protein kinases (MAPKs) activate the MAPK-interacting serine/threonine-protein kinases (MKNKs or MNKs), including MNK1 and MNK2 (ref. 16), which in turn phosphorylate eIF4E at a single amino acid, Ser209 (ref. 17).
To study the role of eIF4E phosphorylation in the circadian clock, we used the Eif4e<sup>S209A/S209A</sup> mouse (referred to hereafter as the KI mouse), in which Ser209 of eIF4E is mutated to alanine and therefore cannot be phosphorylated<sup>18</sup>. Using a combination of biochemical, molecular and behavioral approaches, we found that phosphorylation of eIF4E is regulated by light and the circadian clock, and that it has an important role in the SCN clock physiology by specifically promoting translation of Per1 and Per2 mRNAs.

**RESULTS**

A light- and clock-regulated MAPK/MNK/eIF4E pathway

We first characterized the expression of phosphorylated eIF4E at Ser209, p-eIF4E by immunostaining. In mice kept under constant dark (DD) conditions, eIF4E was modestly phosphorylated in the SCN at circadian time (CT) 6, 15 and 22 (Fig. 1a,b). Notably, a light pulse (55 lx, 15 min) induced a ~3-fold increase of eIF4E phosphorylation at subjective night (CT 15 and CT 22), but not during the subjective day (CT 6) (Fig. 1a,b). The photic induction of eIF4E phosphorylation was rapid (within 30 min) and lasted for about 2 h (Fig. 1c). These results demonstrate that light stimulates phosphorylation of eIF4E in the SCN and that the effect is phase dependent, suggesting that eIF4E phosphorylation may be involved in photic entrainment of the circadian clock.

eIF4E is phosphorylated by MNKs through the p38 and p42/44 (ERK) MAPK signaling cascades<sup>16</sup>. Notably, previous studies have revealed a prominent role for the ERK MAPK pathway in synaptic plasticity and photic entrainment of the circadian clock<sup>19,20</sup>. We detected activities of the MAPK-MNK-eIF4E pathway by western blotting for phosphorylated ERK (p-ERK, at Thr202 and/or Tyr204), MNK1 (at Thr250) and eIF4E (at Ser209) in the SCN. ERK, MNK1, MNK2 and eIF4E were all abundantly expressed in the SCN (Fig. 1d). Consistent with previous results<sup>19</sup>, a light pulse at night led to a marked increase of ERK phosphorylation in the SCN (Fig. 1d). Light also induced a modest increase of MNK1 phosphorylation and a robust increase in the phosphorylation of eIF4E (Fig. 1d and Supplementary Fig. 1a,b). These results indicate that the MAPK/MNK pathway is activated by light in the SCN.

To investigate whether photic induction of eIF4E phosphorylation is dependent on activation of the ERK/MNK pathway, we infused the MEK inhibitor U0126 (ref. 21) or the MNK inhibitor CGP57380 (ref. 22) into the SCN before light exposure. Light-induced p-ERK expression was blocked by U0126, but not by CGP57380 (Fig. 1e). In contrast, photic induction of p-eIF4E was abolished by both U0126 and CGP57380, indicating that phosphorylation of eIF4E occurs downstream of sequential activation of ERK and MNKs. These results suggest that the ERK-MNK pathway couples light to the eIF4E phosphorylation.

We next studied whether activities of the MAPK-MNK-eIF4E pathway are under circadian control. Consistent with previous results<sup>19</sup>, we detected rhythmic ERK phosphorylation in the SCN (P < 0.0001, Bonferroni-corrected rhythmity analysis incorporating non-parametric methods (RAIN); Fig. 1f,g). Moreover, the levels of p-MNK1 and p-eIF4E also exhibited significant rhythmicity (P < 0.0001, Bonferroni-corrected RAIN) with peaks at CT10 during a 24-h period (Fig. 1f,g). The expression of p-MNK1 and p-eIF4E followed a temporal pattern similar to that of p-ERK. These data indicate that the circadian clock controls the activity of the MAPK-MNK1-eIF4E pathway. We also observed significant circadian day and night variations in the levels of MNK1 (P < 0.01) and eIF4E (P < 0.005), but not ERK (P = 0.355, Bonferroni-corrected RAIN; Supplementary Fig. 1c). p-ERK and p-eIF4E exhibited similar temporal patterns when animals were placed in 12-h/12-h LD conditions as compared with DD conditions (Supplementary Fig. 1d). Together, these results indicate that a light- and circadian clock-regulated MAPK-MNK pathway leads to eIF4E phosphorylation in the SCN.

**Figure 1** Light and circadian clock–regulated phosphorylation of eIF4E in the SCN. (a) Representative microscopic images of coronal SCN sections show light-induced phosphorylation of eIF4E (at Ser209) in the SCN. Mice were exposed to light (55 lx, 15 min) during the day (CT6) or at night (CT15 and CT22) and killed 30 min after light. Scale bar represents 100 μm. (b) Quantitation of intensities of p-eIF4E labeling (Online Methods). *P < 0.05 versus no light (one-way ANOVA, F = 2.97, P = 0.044). (c) Time course of light-induced p-eIF4E expression in the SCN. A light pulse (55 lx, 15 min) was applied at CT15 and mice were killed at indicated time points. *P < 0.05 versus 0 min (one-way ANOVA, F = 10.141, P = 0.001). (d) Light-induced phosphorylation of ERK, MNK1 and eIF4E in the SCN. Western blots on SCN lysates before and 30 min after light exposure (55 lx, 15 min) at CT15 are shown. The blots of ERK, MNK1 and eIF4E were also shown as control. Full-length blots and gels are presented in Supplementary Figure 6. (e) Light-induced p-eIF4E and p-ERK expression in the SCN. MEK inhibitor U0126 (10 mM), MNK1 inhibitor CGP57380 (30 mM) or vehicle DMSO was infused into the lateral ventricle 30 min before light exposure. *P < 0.05 versus DMSO (one-way ANOVA, F = 103.220, P < 0.001). (f) Phosphorylation of ERK, MNK1 and eIF4E in the SCN over a 24-h period. Full-length blots and gels are presented in Supplementary Figure 6. (g) Phosphorylated proteins were quantified and normalized according to the levels of total proteins. *P < 0.05 versus CT2 (one-way ANOVA, F = 18.964, P < 0.001). The data are presented as the mean ± s.e.m.
**Figure 2** Light-induced phase shift of circadian behavior is decreased in the eIF4E KI mice. (a) Light-induced phosphorylation of eIF4E in the SCN. Entrained and dark-adapted mice were exposed to light (55 lx, 15 min) at CT15. Mice were killed 30 min after light and SCN sections were immunolabeled for p-eIF4E. Scale bar represents 100 μm. (b,c) Representative actograms of wheel-running behavior demonstrating light-induced circadian phase delay (b) and phase advance (c). For these experiments, animals were entrained to a 12-h/12-h light/dark (LD) cycle for 7 d and released into constant dark (DD) for 8 d before a light pulse (55 lx, 15 min) was applied at CT15, as indicated by the red asterisk (b). Yellow shades indicate light periods. 14 d after the first light pulse, a second light pulse (55 lx, 15 min) was applied at CT22, as indicated by the red asterisk (c). The black arrows indicate the day when a light pulse was applied. Note that light at CT15 induced a marked phase delay (b) and light at CT22 induced a modest phase advance (c) in the WT mice (left). Compared with the WT mice, both the phase delay and the phase advance were decreased in the KI mice (right). (d) Quantitation of light-induced phase shifts is shown. *P < 0.05 versus WT (Student’s t test; CT15, F = 13.100, P = 0.002; CT22, F = 21.702, P < 0.001). (e) Quantitation of circadian period in DD. The numbers of animals in each group are indicated on the histograms. *P < 0.05 versus WT (Student’s t test; days 1–8, F = 33.165, P = 0.000; days 9–22, F = 17.843, P < 0.001; days 23–28, F = 21.726, P < 0.001). The data are presented as the mean ± s.e.m.

eIF4E phosphorylation facilitates clock phase resetting

To further corroborate the function of eIF4E phosphorylation in the circadian clock, we used the non-phosphorylatable eIF4E KI mice. Notably, the level of total eIF4E is unchanged in the mice and they do not exhibit any gross defects (18). As expected, light-induced and basal phosphorylation of eIF4E was eliminated in the SCN of the KI mice (Fig. 2a). Next, we studied circadian behavior of the mice by monitoring their wheel-running activities. Mice were entrained to a 12-h/12-h LD cycle for 7 d and then transferred to DD. Both the KI mice and their wild-type (WT) littersmates were entrained to the LD cycle and exhibited free-running rhythms in DD (Fig. 2b,c). On the eighth day in DD, mice were exposed to a light pulse (55 lx, 15 min) in the early night (CT15, 3 h after the onset of wheel-running behavior). As expected, the light pulse induced a phase delay in the onset of wheel-running in the WT mice. Significantly, however, light-induced phase delay was decreased by ~50% in the KI mice (P < 0.05 versus WT, Student’s t test; Fig. 2b,d). A second light pulse (55 lx, 15 min) was applied to the same mice in the late night (CT22, 10 h after the onset of wheel running) 14 d after the light pulse at CT15. At this time, light induced a modest phase advance in the WT mice. As was the case with the phase delay, the phase advance was attenuated by ~50% in the KI mice (P < 0.05 versus WT; Student’s t test; Fig. 2c,d).

To determine whether the entrainment pathway upstream of eIF4E phosphorylation was compromised in the KI mice, we examined light-induced ERK phosphorylation and c-Fos expression, both of which are sensitive markers of photic input to the SCN. Light-induced ERK phosphorylation and c-Fos expression in the SCN were not different in the KI mice compared with the WT mice (P > 0.05, ANOVA; Supplementary Fig. 2), indicating that the entraining pathway upstream of eIF4E is intact in these mice. Lastly, the free-running period of the KI mice was ~0.2 h shorter than that of the WT mice (P < 0.05, ANOVA; Fig. 2e). Together, these results demonstrate that phosphorylation of eIF4E facilitates photic phase resetting and modulates the period of circadian behavior.

eIF4E phosphorylation is critical for T-cycle entrainment

To further assess the role of eIF4E phosphorylation in photic phase resetting of the clock, we exposed the mice to various non-24-h LD cycles (T-cycles). As the endogenous period of mice is ~24 h, entrainment to a non-24-h T-cycle requires large daily phase shift of the circadian clock, and can therefore be used as a rigorous test of the entraining capacity and range of a circadian clock (23). Mice were first placed in LD or DD for 10 d and then transferred to a T21 (10.5-h/10.5-h LD), T22 (11-h/11-h LD), T26 (13-h/13-h LD) or T27 (13.5-h/13.5-h LD) cycle for at least 14 d. Although all mice retained circadian rhythmicity, as assessed by X2 analysis in all lighting conditions, light entrainment was distinctly impaired. To assess this, we calculated the circadian period of the wheel-running rhythm in each T-cycle using activity onset in the first 5 d, and determined the difference between the period of the activity rhythm and the period of the T-cycle. Representative actograms revealed that the WT mice were stably entrained to the T22 cycle, whereas the KI mice failed to stably entrain. In the KI mice, the circadian rhythms of activity immediately detached from the imposed T22 cycle (Fig. 3a).

For T21, T26 and T27, representative actograms (Supplementary Fig. 3) also showed more stable entrainment in the WT mice as compared with the KI mice. On average, the difference in the period between the activity rhythm and the T-cycle was larger in the KI
mice than in the WT mice (P < 0.05, ANOVA; Fig. 3b), indicating compromised entrainment in the KI animals. 13 of 15 WT mice were entrained to T22 by day 14. In stark contrast, only 7 of 26 KI mice were entrained to T22 (P < 0.05 versus WT, X^2^ test; Fig. 3c). 5 of 9 WT mice were entrained to a T21 cycle, whereas only 2 of 14 KI mice were entrained (P = 0.052, KI versus WT, X^2^ test; Fig. 3c and Supplementary Fig. 3). Similarly, 6 of 9 WT mice were entrained to a T27 cycle, whereas only 3 of 14 KI mice were entrained (P < 0.05, KI versus WT, X^2^ test; Fig. 3c and Supplementary Fig. 3). However, all the WT and KI mice were entrained to T26 (P > 0.05, KI versus WT, X^2^ test; Fig. 3c and Supplementary Fig. 3). As the range of T-cycles to which the KI mice can be entrained was narrowed, the limit of clock entrainment was decreased in these mice. These results further demonstrate that eIF4E phosphorylation is important for photic entrainment of the circadian clock.

eIF4E phosphorylation increases abundance of PER proteins

Per1 and Per2 are essential components of the autoregulatory negative feedback loop in the core clock machinery^4^, Furthermore, they are the only core clock genes that can be induced by light in the SCN^8^–^10^.

It is thought that induction of Per gene expression underlies the clock resetting process^10^,^24^,^25^, Given the marked role of eIF4E phosphorylation in clock entrainment and a pivotal role of eIF4E in translation initiation, we reasoned that phosphorylation of eIF4E might regulate circadian entrainment through its control of PER protein synthesis. To address this question, we first determined the levels of PER1 and PER2 in the SCN by immunostaining using validated antibodies^15^,^23^,^26^, Consistent with previous reports^26^, the levels of PER1 and PER2 proteins in the SCN exhibited circadian oscillations and reached a peak at around CT14 in both the WT and KI mice. Notably, however, PER1 and PER2 levels were significantly decreased in the SCN of KI mice compared with in the WT mice (P < 0.05 at CT14 versus WT, ANOVA; Fig. 4a,b and Supplementary Fig. 4a,b). In a second experiment, a light pulse (15 min, 55 lx) given at CT15 induced a significant increase in PER1 and PER2 at CT19 (4 h after the light pulse) in the WT mice (P < 0.05, ANOVA; Fig. 4c–f), consistent with published results^27^,^28^, Notably, this effect was reduced by ~50% in the KI mice (P < 0.05 versus WT, ANOVA).

Serum shock induces Per gene expression and is widely used to synchronize cellular clocks in vitro^29^, To test whether phosphorylation of eIF4E regulates PER induction in peripheral tissue, we used horse serum to induce PER proteins in mouse embryonic fibroblasts (MEFs). For western blotting, we used validated antibodies to PER1 and PER2 reported previously^30^, We found that serum-stimulated expression of PER1 and PER2 proteins was attenuated by ~50% in the KI cells (P < 0.05 versus WT, ANOVA, Fig. 4g–i), consistent with the decreased photic induction of PER in the SCN of KI mice (Fig. 4c–f).

To determine the period of molecular PER oscillations, we crossed the KI mice with the PER2::Luciferase (PER2::LUC) reporter mice and monitored the PER2 bioluminescence rhythms of SCN explants as reported^15^, Consistent with the behavioral data, KI explants exhibited a shorter period (25.8 ± 0.30 versus 24.7 ± 0.09, WT versus KI, P < 0.05, Student’s test; Supplementary Fig. 4c–e). To complement the genetic approach, we applied the MNK inhibitor CGP57380 to KI explants (P < 0.05 versus WT, Student’s t test; Supplementary Fig. 4d,f). consistent with decreased PER levels in the SCN of KI mice (Fig. 4a,b). On the other hand, CGP57380 increased period length in both WT and KI explants (Supplementary Fig. 4d,e), possibly as a result of its effects on other MNK targets independent of eIF4E phosphorylation^16^, Together, these results demonstrate that phosphorylation of eIF4E increases the abundance of basal and induced PER proteins, consistent with its role in facilitating circadian clock entrainment.

eIF4E phosphorylation promotes Period mRNA translation

To pursue the biochemical mechanisms by which eIF4E phosphorylation regulates PER abundance, we used forebrain lysates (because of the limited amount of material from the SCN). We first examined the levels of canonical clock protein expression, including PER1, PER2, CLOCK, BMAL1, CRY1 and CRY2, in the brain by western blotting, PER1 and PER2 levels were decreased in the KI brain in a gene dosage–dependent manner, with the heterozygotes (Het) showing intermediate levels between WT and KI mice (PER1, F = 130.360, P < 0.001; PER2, F = 135.16, P < 0.001 versus WT, n = 3 mice for each group, one-way ANOVA; Fig. 5a,b). The levels of other clock
proteins were not changed (CLOCK, F = 4.154, P = 0.074; BMAL1, F = 4.160, P = 0.074; CRY1, F = 2.395, P = 0.172; CRY2, F = 0.979, P = 0.429; p-eIF4E, F = 511.440, P < 0.001 versus WT, n = 3 for each group, one-way ANOVA; Fig. 5a,b), indicating specific regulation of PER levels by eIF4E phosphorylation. Notably, total eIF4E level was not changed in the KI brains (Fig. 5a), which is consistent with global protein synthesis in the KI mice not being altered (Supplementary Fig. 5a,b).

To investigate whether phosphorylation of eIF4E controls translation of Per1 and Per2 mRNAs through an mRNA 5′ UTR-dependent mechanism, we created Per1 and Per2 mRNA 5′ UTRs fused to a luciferase reporter and transfected them into MEFs (Fig. 5c). The translation activities of the Per1 and Per2 reporters were reduced by ~70% in the KI MEFs (Per1, F = 294.000, P < 0.001; Per2, F = 291.84, P < 0.001 versus WT, n = 3 for each group, Student’s t test; Fig. 5d), which indicates that phosphorylation of eIF4E facilitates the translation of Per1 and Per2 5′ UTR reporters. As a control, expression of a capped mRNA luciferase reporter without a 5′ UTR was not changed (F = 0.521, P = 0.510 versus WT, n = 3 for each group, Student’s t test; Fig. 5d). These results demonstrate that the 5′ UTRs are essential for control of Per mRNA translation by eIF4E phosphorylation. To complement the genetic approach, we used the MNK inhibitor cecroporamide to decrease eIF4E phosphorylation in WT MEFs. Cecroporamide inhibited phosphorylation of eIF4E and decreased the levels of PER1 and PER2 proteins in WT, but not KI, cells (Fig. 5c and Supplementary Fig. 5c). Consistent with the western blot data, translation of Per1 and Per2 mRNA 5′ UTR reporters was suppressed by cecroporamide in a dose-dependent manner in WT, but not KI, cells (Per1, F = 126.016, P < 0.001; Per2, F = 42.225, P < 0.001; control, F = 1.091, P = 0.407 versus 0 µM, n = 3 for each group, one-way ANOVA; Fig. 5f and Supplementary Fig. 5d).

Lastly, we performed polysome profiling to analyze mRNA translation of Per1 and Per2 in the brain. Forebrain lysates were fractionated by sucrose density gradient centrifugation. Actively translated mRNAs are associated with polysomes and sediment at the heavy-density fractions (Fig. 5g). The polysome/monosome ratio was similar in KI mice compared with WT brains (KI, 2.78 ± 0.24; WT, 2.92 ± 0.11; n = 3, F = 0.231, P = 0.656; Student’s test), indicating that translation of most mRNAs was not affected. The amount of Per1, Per2 and Actb mRNAs in each gradient fraction was quantified by real-time quantitative reverse transcription-PCR (qRT-PCR), and the distribution of the mRNAs was compared between the WT and KI brains. In the KI brains, Per1 and Per2 mRNA distribution shifted toward the lighter density fractions, as compared with WT brains (Fig. 5h). Notably, the levels of total Per1 and Per2 mRNAs were similar (KI versus WT: Per1, 0.32 ± 0.06 versus 0.3 ± 0.08, F = 0.211, P = 0.67; Per2, 0.21 ± 0.11 versus 0.22 ± 0.1, F = 0.015, P = 0.908; Actb, 0.65 ± 0.1 versus 0.7 ± 0.09, F = 0.383, P = 0.960; n = 3 for each group, Student’s test), demonstrating depressed Per1 and Per2 mRNA translation in the KI brain and no detectable changes in transcription. The effect on Per1 and Per2 mRNA translation was specific, inasmuch as the distribution was not changed for mRNAs of Actb and other clock genes, including Clock, Bmal1, Crys1 and Cry2 (Fig. 5h and Supplementary Fig. 5e). These data are consistent with decreased protein levels of PER1 and PER2, but not β-actin and other clock proteins in the brains of KI mice (Fig. 5a,b). Together, these results demonstrate that phosphorylation of eIF4E specifically facilitates Per1 and Per2 gene expression by promoting their mRNA translation.
DISCUSSION

We found that the abundance of the clock proteins PER1 and PER2 is controlled at the mRNA translation initiation step through MAPK/MKNK-dependent phosphorylation of the cap-binding protein eIF4E. In the SCN, eIF4E phosphorylation is regulated by light and exhibits circadian rhythmicity under constant conditions. Thus, during the clock-resetting process, when upregulation of PER proteins is required, eIF4E phosphorylation may function as a trigger to boost PER mRNA translation and increase PER protein levels. Under constant conditions, rhythmic phosphorylation of eIF4E may contribute to the precision of the circadian clock through timely modulation of PER protein synthesis.

The SCN clock is continuously entrained by the external light/dark cycle, and light-induced gene expression is thought to be the molecular basis of resetting circadian behavior. Mechanistically, light stimulates melanopsin-expressing retinal ganglion cells and evokes neurotransmitter release at the terminals of retinohypothalamic tract. The neurotransmitters bind to the postsynaptic receptors and activate second messenger–mediated signal transduction cascades in the SCN neurons. These pathways ultimately impinge on the core clock machinery and regulate clock gene expression. The level of PER proteins is a critical state variable of the primary autoregulatory feedback loop that generates circadian oscillations. An increase in the PER level disrupts the dynamic balance of the feedback loops and leads to phase resetting of the SCN clock.

Light regulates Per gene expression in the SCN through multiple mechanisms. Previous studies have focused mostly on inducible gene transcription. For example, the transcription factor CREB is a key nexus at which multiple intracellular signal transduction pathways converge, and CREB is activated by phosphorylation and in turn binds to CREs to promote CRE-mediated gene expression. Notably, CREs have been identified in the promoter regions of Per genes. CREB-dependent transcription is also regulated by the CREB coactivator CRTC (CREB-regulated transcription coactivator), which is also known as TORC (transducer of regulated CREB).

Much less is known about the mechanisms of post-transcriptional regulation in the clock-resetting process. Recent work has revealed a role for MAPK signaling–mediated microRNA expression, which modulates Per1 gene transcription and Per2 protein stability. In addition, light activates the mTOR pathway, which in turn activates S6Ks and inhibits 4E-BPs. We recently reported that 4E-BP1 regulates SCN network synchrony by repressing Vip mRNA translation. Here we found that, as a downstream kinase of the ERK MAPK pathway, light-activated MNK phosphorlates eIF4E, promotes Per1 and Per2 mRNA translation, thereby facilitating photic entrainment of the circadian clock. Phosphorylation of eIF4E is an integral part of the molecular entrainment pathway that couples light to mRNA translation in the core clock machinery in the SCN. The mechanism enables the SCN to respond sufficiently to a phase-resetting light.

Figure 5 Phosphorylation of eIF4E promotes Per1 and Per2 mRNA translation. (a-b) Representative western blots of canonical clock proteins in the forebrain. Quantitation is shown in b. **p < 0.05 versus WT. (c) Schematic diagrams of the Firefly (Fluc) and Renilla (Rluc) luciferase reporter mRNAs. (d) Luciferase assay indicating translation levels of the reporter mRNAs in MEFs. Fluc mRNA was co-transfected with Fluc mRNAs as a transfection control. Error bars represent s.e.m. of three independent experiments. *p < 0.05 versus WT. (e) Western blots indicating that the MNK1 inhibitor cercosporamide decreased eIF4E phosphorylation and inhibited PER expression in MEFs. For a and e, full-length blots and gels are presented in Supplementary.
pulse at night and to entrain to a wide range of non-24-h light cycles, which might represent an evolutionary advantage.

Under clock-free–running conditions, Per gene expression is also controlled at multiple levels. The production and degradation rate of PER proteins determine the length of a circadian cycle. At the transcriptional level, rhythmic Per transcription is driven by the CLOCK:BMAL1 complexes through the E-box enhancers. At the post-transcriptional level, Per mRNA processing is regulated by methylation and PER cycling is also governed by post-translational modifications such as phosphorylation. Recent work has highlighted the important role of translational control for clock gene expression. For example, the RNA-binding proteins Ataxin-2 (Atx2) and Twenty-four (Tfy) interact to activate Per translation in pacemaker neurons to sustain robust behavioral rhythms in Drosophila. We found that Per1 and Per2 mRNA translation is controlled by rhythmic phosphorylation of the cap-binding protein eIF4E in mammals. All of these mechanisms contribute to the dynamic regulation of the PER level and precise timekeeping of the circadian clock.

The mRNA cap-binding protein eIF4E serves as a pivotal node for regulation of protein synthesis in the mammalian circadian clock by several signaling pathways. The MAPK and mTORC1 pathways interact to activate robust behavioral rhythms in as phosphorylation. Recent work has highlighted the important role of translational control for clock gene expression. For example, the RNA-binding proteins Ataxin-2 (Atx2) and Twenty-four (Tfy) interact to activate Per translation in pacemaker neurons to sustain robust behavioral rhythms in Drosophila. We found that Per1 and Per2 mRNA translation is controlled by rhythmic phosphorylation of the cap-binding protein eIF4E in mammals. All of these mechanisms contribute to the dynamic regulation of the PER level and precise timekeeping of the circadian clock.

The mRNA cap-binding protein eIF4E serves as a pivotal node for regulation of protein synthesis in the mammalian circadian clock by several signaling pathways. The MAPK and mTORC1 pathways converge on eIF4E to regulate circadian clock function by controlling protein synthesis. In our previous work, we found that rhythmic mTORC1 regulates eIF4E activity by phosphorylating and inhibiting the eIF4E repressor protein 4E-BP1. 4E-BP1 repression the translation of Vip mRNA and decreases the abundance of Vip. Thus, mTORC1 promotes Vip translation and facilitates entrainment and synchrony of SCN cell network. Here, we found that MAPK pathway leads to eIF4E phosphorylation through MNKs in the photo-recipient SCN cells and facilitated light-induced PER protein synthesis. In addition, circadian clock–regulated eIF4E phosphorylation promotes basal PER protein synthesis. Thus, the highly regulated eIF4E activity is important for timely protein synthesis and proper function of the circadian clock.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.C., S.A. and N.S. designed the study. R.C., N.D.Z., C.G.G., I.D.B., Y.T., A.Y. and H.X. performed the experiments. C.L. and K.-F.S. contributed reagents and analytic tools. R.C., I.D.B., C.G.G., A.Y. and H.X. analyzed the data. R.C., A.C.L., S.A. and N.S. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. The eIF4E5208A/5208A (KI) mice and the littermates on a C57BL/6 background were maintained in the animal facility at McGill University in accordance with institutional guidelines. All procedures were approved by the Institutional Animal Care and Use Committee at McGill University.

Photic stimulation. Adult (8–10 week old) C57BL/6 mice were entrained to a 12-h LD cycle for at least 2 weeks and then transferred to constant darkness for two consecutive 24-h cycles. After dark adaptation, animals received a light pulse (55 lx, 15 min) at one of three time points: the middle of the subjective day (CT 6), early subjective night (CT 15) or late subjective night (CT 22). CTs were calculated based on Zeitgeber time (ZT) and the tau of the mice (approximately 23 h 45 min) under free-running conditions, with ZT 0 denoting light on and ZT 12 denoting light off. The ‘no light’ control animal groups underwent the same handling conditions at the same time points.

Cannulation and infusions. Mice were cannulated in the lateral ventricles using the techniques as described. The following coordinates were used to place the tip of a 24-gauge guide cannula into the lateral ventricle: posterior, 0.34 mm from bregma; lateral, 0.90 mm from the midline; dorsoventral, −2.15 mm from bregma. To disrupt MEK activity, U0126 (10 mM, 2 µl; Cell Signaling Tech) was infused through the cannula. To disrupt MK1 activity, CGP57380 (30 mM, 2 µl; Cell Signaling Tech) was infused. Control animals were infused with the same volume of DMSO.

Brain tissue processing, immunostaining and microscopic imaging analysis. Under indicated conditions, mice were killed and brain tissue was harvested and processed. SCN coronal sections were immunostained for p-eIF4E, p-ERK, PER1 or PER2 as described. The antibody information is shown in Supplementary Table 1. Bright-field microscopy images were captured using a digital camera mounted on an inverted Zeiss microscope. The staining intensity in micrographs was quantified using Adobe Photoshop software (Adobe Systems) as described. Briefly, images of the SCN were acquired (10×), 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 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. The lowest value in an experiment is normalized to be ‘1’.

Circadian behavioral assay. 8–10-week-old male KI mice or WT littermates were individually housed in cages equipped with running wheels. Wheel rotation was recorded using the VitalView program (Mini Mitter). The light pulse experiments, mice were entrained to a 12-h/12-h LD cycle (55 lx) for 7 d and released into constant dark (DD) for 8 d. On the ninth day in DD, a light pulse (15 min, 55 lx) was applied at CT15. 14 d after the first light pulse, a second light pulse (15 min, 55 lx) was applied at CT22. Animals were kept in DD for another 14 d after the second light pulse. The CTs were calculated for each mouse based on their individual actograms before the light pulse.

For the T-cycle experiments, mice were first entrained in a 12-h/12-h LD for 9 d and put in DD for 14 d. Next, mice were transferred to T21 (10.5-h/10.5-h LD), T22 (11-h/11-h LD), T26 (13-h/13-h LD) or T27 (13.5-h/13.5-h LD) for 14 d followed by 10 d in DD. The actograms of wheel-running activities were analyzed using ActiView (Mini Mitter) and ClockLab software (Actimetrics). Circadian period is determined by the chi-square periodogram using the ClockLab software. For unstably entrained animals, period is determined from the actograms of the first 5 d in a T cycle. Stable entrainment is defined if animals show a consistent phase angle of entrainment (the time gap between light onset and activity onset) for at least five consecutive days.

Explant culture, kinetic bioluminescence recording and data analysis. The eIF4E KI mice were crossed with mPER2::LUC transgenic reporter mice to obtain the eIF4E KI: mPER2::LUC mice. Explants of WT and KI SCN tissue were dissected and cultured as reported. CGP57380 (10 µM) was applied to the explants without changing the medium. Real-time circadian reporter assays were performed using a Lumicycle luminometer (Actimetrics) as described. Baseline-subtracted data (counts per second) were plotted against time (days) in culture. For comparison, the first peak was aligned in the plotted data. The LumiCycle Analysis program (version 2.31, Actimetrics) was used to analyze rhythm parameters. For period length analysis, raw data were baseline fitted, and the baseline-subtracted data were fitted to a sine wave (damped), from which the period was determined. All samples showed persistent rhythms and goodness-of-fit of >90% was achieved. For amplitude analysis, baseline-subtracted data (polynomial order = 1; 3–6 d of recording data) were fitted to a sine wave, from which the amplitude was determined using Sin Fit.

Protein extraction and western blotting analysis. The SCN tissue was excised under a dissection microscope and frozen on dry ice. SCN tissue was pooled from 10 mice per condition. Brain tissue was homogenized with a pestle grinder (Fisher Scientific) and lysed. Western blotting analysis was performed as described. The antibody information is shown in Supplementary Table 1.

Preparation of the 5′ UTR reporter mRNAs and luciferase assay. PCR products encoding a T7 promoter followed by the 5′ UTR of mouse Per1 or Per2 were generated using a DNA library derived from MEFs, and inserted into pGAL3-Basic vector (Promega) between HindIII and NcoI sites. Resultant plasmids (pGAL3-Per1-5′UTR and pGAL3-Per2-5′UTR) were used as templates for in vitro transcription. Luciferase reporter mRNAs were generated using MAXIScript T7 in vitro Transcription kit (Ambion) according to the manufacturer’s protocol in the presence of the cap analog, and Poly (A) tail was added using the Poly (A) Tailing Kit (Ambion). WT and KI MEFs were seeded in a 24-well plate and maintained at 37 °C in 5% CO2 atmosphere in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% FCS (vol/vol), 100 U ml−1 penicillin and 100 µg ml−1 streptomycin. Cells were transfected with firefly luciferase reporter mRNAs together with renilla luciferase mRNA as transfection control using Lipofectamine (Invitrogen). Different doses of cescopospamide (Sigma-Aldrich) were added to the culture medium after transfection. Cells were incubated and lysed for luciferase activity assay using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instruction.

Brain polysome profiling and qRT-PCR. Brain polysome profiling was performed as described. The polysome to monosome ratio was calculated as the area under the A254 absorbance curve, using the absorbance values processed with the definite integral command in MATLAB. RNA extraction and qRT-PCR were performed as reported. The primer information is shown in Supplementary Table 2.

Statistical analysis. The data were collected and processed randomly. Data collection and analysis were not performed blind to the conditions of the experiments. The values are presented as the mean ± s.e.m. or percentage. Data distribution was assumed to be normal but this was not formally tested. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications. Statistical analysis was performed using SPSS software (SPSS). Mean values from multiple groups were compared via one-way ANOVA, followed by the Student-Newman-Keuls test for individual comparisons. Mean values from two groups were compared via Student’s t test. Ratios of entrainment (%) of WT and KI mice were compared via the non-parametric X2 test. Circadian rhythmicity of protein abundance was determined using RAIN27 performed in R (v.3.1.1, R Foundation for Statistical Computing) and Bonferroni correction was applied. P < 0.05 was considered to be statistically significant. A Supplementary Methods Checklist is available.