Circadian clock regulation of mRNA translation through eukaryotic elongation factor eEF-2

Stephen Z. Caster[a,b], Kathrina Castillo[b,c], Matthew S. Sachs[c], and Deborah Bell-Pedersen[a,b,c,1]

[a]Genetics Program, Texas A&M University, College Station, TX 77843; [b]Center for Biological Clocks Research, Texas A&M University, College Station, TX 77843; and [c]Department of Biology, Texas A&M University, College Station, TX 77843

Edited by Joseph S. Takahashi, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, and approved July 6, 2016 (received for review December 21, 2015)

The circadian clock has a profound effect on gene regulation, controlling rhythmic transcript accumulation for up to half of expressed genes in eukaryotes. Evidence also exists for clock control of mRNA translation, but the extent and mechanisms for this regulation are not known. In *Neurospora crassa*, the circadian clock generates daily rhythms in the activation of conserved mitogen-activated protein kinase (MAPK) pathways when cells are grown in constant conditions, including rhythmic activation of the well-characterized p38 osmosensing (OS) MAPK pathway. Rhythmic phosphorylation of the MAPK OS-2 (P-OS-2) leads to temporal control of downstream targets of OS-2. We show that osmotic stress in *N. crassa* induced the phosphorylation of a eukaryotic elongation factor-2 (eEF-2) kinase, radiation sensitivity complementing kinase-2 (RCK-2), and that RCK-2 is necessary for high-level phosphorylation of eEF-2, a key regulator of translation elongation. The levels of phosphorylated RCK-2 and phosphorylated eEF-2 cycle in abundance in wild-type cells but not in cells deleted for OS-2 or the core clock component FREQUENCY (FRQ). Translation extracts from cells grown in constant conditions show decreased translational activity in the late subjective morning, coincident with the peak in eEF-2 phosphorylation, and rhythmic translation of glutathione *S*-transferase (GST-3) from constitutive mRNA levels in vivo is dependent on circadian regulation of eEF-2 activity. In contrast, rhythms in phosphorylated eEF-2 levels are not necessary for rhythms in accumulation of the clock protein FRQ, indicating that clock control of eEF-2 activity promotes rhythmic translation of specific mRNAs.

circadian clock | eEF-2 | translation | translation elongation | *Neurospora crassa*

Circadian rhythms are the outward manifestation of an endogenous clock mechanism common to nearly all eukaryotes. Depending on the organism and tissue, nearly half of an organism's expressed genes are under control of the circadian clock at the level of transcription (1–4). However, mounting evidence indicates a role for the clock in controlling posttranscriptional mechanisms (5), including translation initiation (6, 7), whereas clock control of translation elongation has not been investigated.

The driver of circadian rhythms in *Neurospora crassa* is an autoregulatory molecular feedback loop composed of the negative elements FREQUENCY (FRQ) and FRQ RNA-interacting helicase (FRH), which inhibit the activity of the positive elements WHITE COLLAR-1 (WC-1) and WC-2 (8–11). WC-1 and WC-2 heterodimerize to form the white collar complex (WCC), which activates transcription of *frequency* (*frq*) (8, 12, 13) and activates transcription of a large set of downstream target genes important for overt rhythmicity (2, 14). One gene directly controlled by the WCC is *os-4*, which encodes the mitogen-activated protein kinase kinase kinase (MAPKKK) in the osmotically sensitive (OS) MAPK pathway (15). Rhythmic transcription of *os-4* leads to rhythmic accumulation of the phosphorylated active form of the downstream p38-like mitogen-activated protein kinase (MAPK) OS-2 (16). In *Saccharomyces cerevisiae*, the homolog of OS-2, Hog1, directly phosphorylates and activates the MAPK-activated protein kinase (MAPKAPK) Rck2 after acute osmotic stress (17, 18). Activated Rck2 phosphorylates EF-2 and, as a result, represses translation elongation for most mRNAs (19–21). Therefore, we hypothesized that circadian clock control of OS-2 activity in *N. crassa* leads to temporal control of translation elongation through rhythmic activation of RCK-2.

In support of this hypothesis, we show that clock control of OS-2 leads to rhythmic phosphorylation of RCK-2 and eEF-2 in cells grown in constant conditions. Consistent with the peak in phosphorylated eEF-2 during the subjective morning, translation elongation rates are reduced in extracts prepared from cells harvested at this time of the day. Furthermore, clock-controlled translation of GST-3 protein from constitutive mRNA levels in vivo is dependent on rhythmic eEF-2 activity, whereas rhythms in accumulation of FRQ are not dependent on rhythms in phosphorylated eEF-2. Together, these data support clock regulation of translation elongation of specific mRNAs as a mechanism to control rhythmic protein accumulation.

Results

Phosphorylation of RCK-2 Protein Requires OS-2. Total Rck2 kinase levels, and Rck-2 phosphorylation by Hog1, increase following osmotic stress in *S. cerevisiae* cells (19). Rck2 then phosphorylates and inactivates EF-2, leading to general repression of translation (19, 22). To investigate if a similar mechanism exists for regulation of translation in *N. crassa*, we identified *N. crassa* RCK-2 and eEF-2 through sequence homology (Fig. S1 *A* and *B*). To determine if *N. crassa* RCK-2 functions in the osmotic stress response pathway, RCK-2 protein levels and phosphorylation state were measured in WT or Δ*os-2* cells containing a C-terminal hemagglutinin (HA)-tagged RCK-2 protein (RCK-2::HA) grown in the dark for 24 h (DD24) and then subjected to acute

Significance

The circadian clock controls daily rhythms in genes involved in a wide range of biological processes, including signal transduction, cell division, metabolism, and behavior. The primary focus on understanding clock control of gene expression has been at the level of transcription. However, in many systems, there are examples of proteins that accumulate rhythmically from transcripts that are constitutively expressed. These data suggested that the clock regulates translation, but the underlying mechanisms were largely unknown. We show that the clock in *Neurospora crassa* controls the activity of translation elongation factor-2 (eEF-2) and that regulation of translation elongation leads to rhythmic translation in vitro and in vivo. These studies uncover a mechanism for controlling rhythmic protein accumulation.

Author contributions: S.Z.C., M.S.S., and D.B.-P. designed research; S.Z.C. and K.C. performed research; S.Z.C., M.S.S. and D.B.-P. analyzed data; and S.Z.C. and D.B.-P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: dpedersen@bio.tamu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1525268113/-/DCSupplemental.

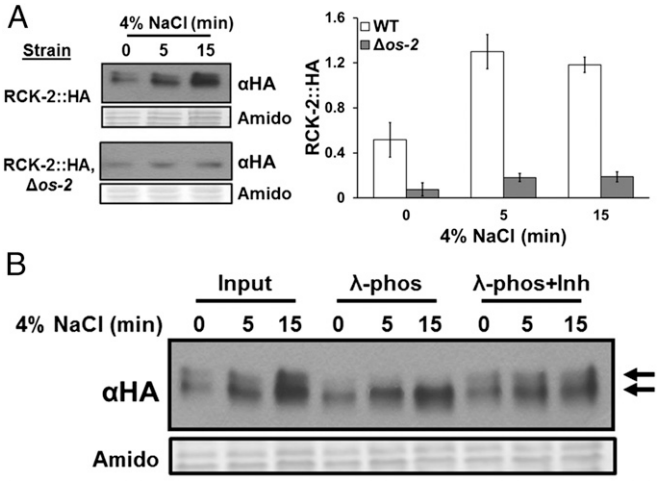

Fig. 1. RCK-2 protein is induced by osmotic shock. (*A*) Representative Western blot of protein isolated from RCK-2::HA and RCK-2::HA, Δ*os-2* cells grown in the dark (DD) for 24 h (DD24) and incubated with 4% (vol/vol) NaCl for the indicated times and probed with HA antibody (αHA). Amido-stained proteins served as loading controls. The data are plotted on the *Right* (mean ± SEM, $n = 3$). (*B*) Western blot of protein from RCK-2::HA cells grown in the dark for 24 h and incubated with 4% (vol/vol) NaCl for the indicated times and given no further treatment (Input), treated with λ-phosphatase (λ-phos), or treated with λ-phosphatase plus phosphatase inhibitors (λ-phos+inh) and probed with HA antibody. The upper arrow points to the phosphorylated form of RCK-2 and the lower arrow to the unphosphorylated protein.

osmotic stress. As in yeast, *N. crassa* RCK-2::HA protein levels increased following osmotic stress, and this induction required OS-2 (Fig. 1*A*). Two forms of RCK-2::HA protein were observed by SDS/PAGE in WT cells, but not in Δ*os-2* cells (Fig. 1*A*). Consistent with the idea that the slower migrating form of RCK-2::HA was phosphorylated, the band disappeared when protein extracts were treated with λ-phosphatase but persisted in extracts treated with both λ-phosphatase and phosphatase inhibitors (Fig. 1*B*). In yeast, phosphorylated Rck2 is observed in osmotically stressed but not in unstressed cells (19). In contrast, phosphorylated RCK-2::HA (P-RCK-2) was already present in unstressed *N. crassa* cells, suggesting that OS-2–dependent phosphorylation of RCK-2 was directed by different input signals to the OS MAPK pathway, possibly signals from the circadian clock.

eEF-2 Phosphorylation Is Regulated by RCK-2. To determine if *N. crassa* eEF-2 activity is altered following an acute osmotic stress, the levels of phosphorylated eEF-2 (P-eEF-2) were examined using an antibody that recognizes P-eEF-2 phosphorylated at the conserved threonine 56 (Fig. 2*A*). P-eEF-2 levels increased within 5 min of addition of 4% (vol/vol) NaCl to cultures at DD24, whereas total eEF-2 levels, detected using an antibody generated to recognize all forms of eEF-2, were similar in untreated and salt-treated samples. To verify the specificity of the P-eEF-2 antibody, salt-treated samples were incubated with λ-phosphatase (Fig. 2*B*). As expected, eEF-2 was not detected with P-eEF-2 antibody in the λ-phosphatase–treated samples but was detected in samples treated with λ-phosphatase plus phosphatase inhibitors.

In *S. cerevisiae*, Rck-2 directly phosphorylates eEF-2 (19). Immunoprecipitation of *N. crassa* RCK-2::HA with HA antibody resulted in coimmunoprecipitation of eEF-2 (Fig. S2), suggesting that similar to *S. cerevisiae*, RCK-2 may directly phosphorylate eEF-2 in response to osmotic stress. Consistent with this idea, P-eEF-2 levels were significantly lower in Δ*rck-2* cells compared with WT cells following osmotic stress, such that P-eEF-2 levels were reduced up to 80% in Δ*rck-2* cells subjected to 5 min of salt stress (Figs. 2*C* and S3*A*). However, low-level phosphorylation of eEF-2 persisted in the absence of RCK-2, indicating that in addition to RCK-2, other kinase(s) can phosphorylate eEF-2. One possible candidate kinase was the MAPK OS-2. We examined the levels of P-eEF-2 in Δ*rck-2*, Δ*os-2*, and double Δ*rck-2,* Δ*os-2* mutant cells and found that the levels of P-eEF-2 in Δ*os-2* and the double Δ*rck-2*, Δ*os-2* mutant strain were similar to that observed in Δ*rck-2* cells treated with NaCl for 5 or 15 min (Figs. 2*C* and S3 *B* and *C*). Furthermore, levels of total eEF-2 in Δ*rck-2*, Δ*os-2*, and double Δ*rck-2*, Δ*os-2* mutant cells were similar to levels in WT cells (Fig. S3 *A*–*C*), indicating that unidentified kinases phosphorylate eEF-2 following osmotic stress in the absence of RCK-2 and OS-2.

RCK-2 Phosphorylation Is Regulated by the Circadian Clock. Based on clock control of the OS MAPK pathway (15, 23) and OS-2–dependent phosphorylation of RCK-2 (Fig. 1), we predicted that the circadian clock regulates the accumulation of P-RCK-2 in constant environmental conditions. An antibody to detect P-RCK-2 was not available; however, P-RCK-2 could be separated from RCK-2 using PhosTag (Fig. S4 *A* and *B*). P-RCK-2 levels cycled, and accumulation peaked during the late subjective night (DD28–32) (Fig. 3*A*), slightly earlier than the peak accumulation of P-OS-2 (DD32–36) (15, 23). The earlier peak time in P-RCK-2 levels may be due to experimental error from using 4-h time points or may reflect increased P-OS-2 activity during

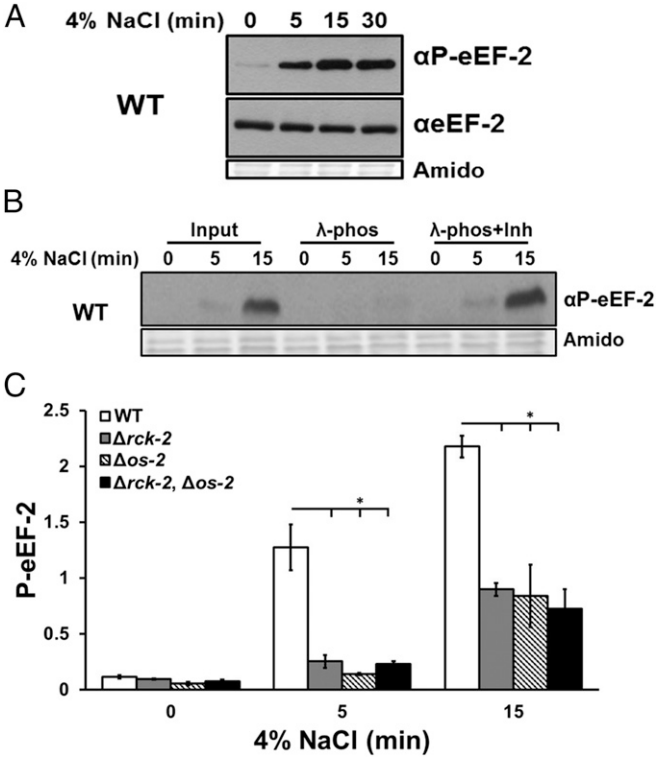

Fig. 2. Osmotic stress-induced phosphorylation of eEF-2 is reduced in Δ*rck-2* cells. (*A*) Western blots of protein extracted from WT cells grown in DD24 and treated with 4% (vol/vol) NaCl for the indicated times and probed with phospho-specific eEF-2 antibody (αP-eEF-2) or total eEF-2 antibody (αeEF-2). The film was exposed for 15 s. (*B*) Western blot of protein from WT cultures grown in DD24 and incubated with 4% (vol/vol) NaCl for the indicated times and given no further treatment (Input), treated with λ-phosphatase (λ-phos), or treated with λ-phosphatase plus phosphatase inhibitors (λ-phos+inh) and probed with αP-eEF-2. The film was exposed for 1.5 s. (*C*) Plot of eEF-2 levels from WT, Δ*rck-2*, Δ*os-2*, and double mutant Δ*rck-2*, Δ*os-2* cells grown in DD24 and incubated with 4% (vol/vol) NaCl for the indicated times (mean ± SEM, $n = 3$). The asterisks indicate a statistical difference ($P < 0.05$, Student's t test). The Western blots are shown in Fig. S3.

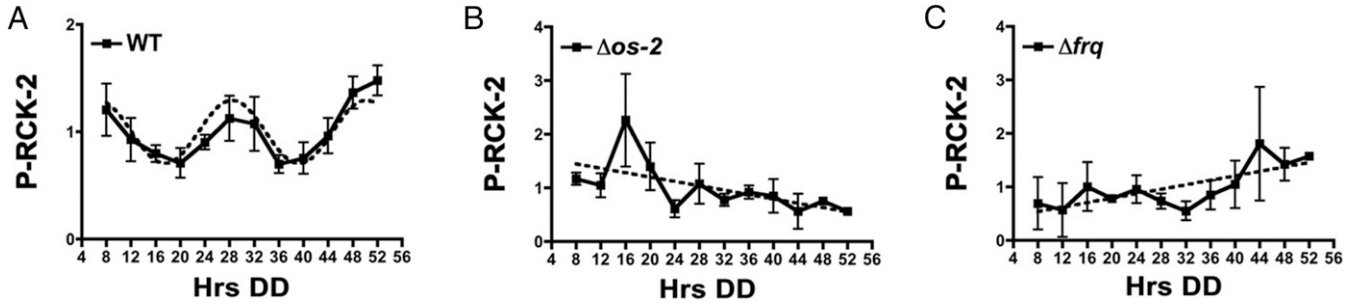

Fig. 3. Rhythmic RCK-2 phosphorylation is regulated by clock signaling through the OS-2 pathway. Shown are plots of levels of phosphorylated RCK-2 protein (P-RCK-2). Protein was extracted from the indicated cultures grown in DD and harvested every 4 h over 2 d (Hrs DD). (*A*) RCK-2::HA (WT); (*B*) RCK-2::HA, Δ*os-2* (Δ*os-2*); (*C*) RCK-2::HA, Δ*frq* (Δ*frq*). P-RCK-2 levels were determined using PhosTag gels (Fig. S4 *A* and *B*). The plots represent the average P-RCK-2 signal normalized to total protein for each genotype and thus do not reflect differences in P-RCK-2 levels in the strains. Rhythmicity of P-RCK-2 in WT cells was determined using *F* tests of fit of the data to a sine wave (dotted black line; $P < 0.0001$, $n = 4$). The levels of P-RCK-2 were arrhythmic in Δ*os-2* ($n = 3$) and Δ*frq* ($n = 2$) cells as indicated by a better fit of the data to a line (dotted black lines).

its accumulation. RCK-2 and P-RCK-2 levels were significantly reduced in Δ*os-2* cells harvested at DD24 (Fig. S4*C*). The reduced levels of RCK-2 in Δ*os-2* cells in the dark suggested that in addition to control of RCK-2 phosphorylation, the OS pathway is necessary for normal expression of RCK-2. This regulation was not further investigated in this study. Consistent with clock regulation of P-RCK-2 levels through the OS pathway, P-RCK-2 accumulation was low and arrhythmic in Δ*os-2* cells and arrhythmic in Δ*frq* cells (Fig. 3 *B* and *C* and Fig. S4*A*).

eEF-2 Phosphorylation Is Regulated by RCK-2 and the Circadian Clock. RCK-2 is required for normal induction of P-eEF-2 following acute osmotic stress (Fig. 2*C*), and the clock controls rhythms in the levels of P-RCK-2 (Fig. 3*A*). Therefore, we predicted that eEF-2 phosphorylation would be clock-controlled. Consistent with this idea, the levels of P-eEF-2, but not eEF-2, cycled over the course of the day, with peak P-eEF-2 accumulation in the subjective morning (DD36) (Fig. 4*A* and Figs. S5 and S6), slightly lagging the peak in P-RCK-2 levels (Fig. 3*A*). This delay may be due to experimental error introduced by using 4-h time points or due to the activity of phosphatases. P-eEF-2 levels fluctuated over time, but circadian rhythmicity was abolished in Δ*rck-2*, Δ*os-2*, and Δ*frq* cells (Fig. 4 *B*–*D* and Fig. S5). P-eEF-2 could be detected in both Δ*rck-2* (Fig. 4*E*) and Δ*os-2* cells (Fig. S5) in DD, consistent with additional kinases phosphorylating eEF-2. However, P-eEF-2 levels in Δ*rck-2* cells at DD36 (the time of peak P-eEF-2 levels in WT cells) were low and at levels similar to the trough levels of P-eEF-2 in WT cells at DD24 (Fig. 4*E*). Together, these data confirmed that clock signaling through the OS MAPK pathway and RCK-2 are necessary for rhythmic accumulation of P-eEF-2.

The extent of clock control of P-eEF-2 levels was measured by comparing the amount of P-eEF-2 from WT and Δ*rck-2* cells harvested at the trough (DD24) and peak (DD36) of P-eEF-2 accumulation (Fig. 4*E*). A twofold increase in P-eEF-2 was observed at DD36 compared with DD24 in WT cells but not Δ*rck-2* cells. At DD36, P-eEF-2 levels were reduced by 60% in Δ*rck-2* cells compared with WT cells, indicating that clock signaling through RCK-2 is responsible for over half of the total amount of P-eEF-2.

The Circadian Clock Controls Translation Elongation Rates in Vitro, and Rhythmic Protein Levels in Vivo, Through Phosphorylation of eEF-2. We predicted that mRNA translation would be highest when P-eEF-2 levels are lowest during the subjective evening (DD24) and lowest when P-eEF-2 accumulation peaks in the subjective morning (DD36) (Fig. 4*A*). To test this idea, we examined translation of *luciferase* (*luc*) mRNA in cell-free translation extracts isolated from WT and mutant cells harvested at DD24 and DD36. Consistent with this hypothesis, translation of *luc* mRNA in WT extracts was significantly higher at DD24 than at DD36 (Fig. 5*A*). P-eEF-2 levels in Δ*rck-2* cells at DD24 and DD36 were similar to the trough levels at DD24 in WT cells (Figs. 2*C* and 4*E*),

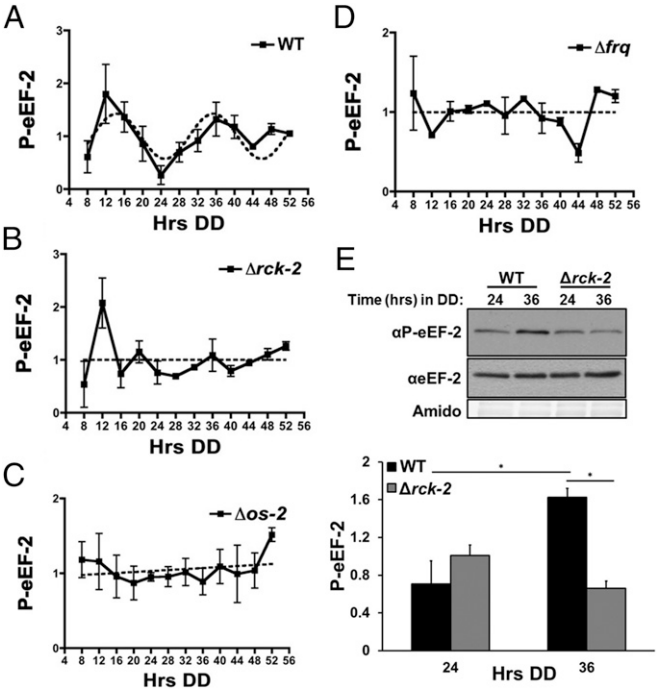

Fig. 4. Clock control of eEF-2 phosphorylation requires signaling through the OS MAPK pathway and RCK-2. Shown are plots of the abundance of P-eEF-2. Protein was extracted from the indicated strains grown in DD and harvested every 4 h over 2 d (Hrs DD): (*A*) wild type (WT), (*B*) Δ*rck-2*, (*C*) Δ*os-2*, and (*D*) Δ*frq*. Protein was probed with αP-eEF-2 (Fig. S5). Plots of the data are shown (mean ± SEM, $n = 3$) and represent the average P-eEF-2 signal normalized to total protein for each genotype as in Fig. 3. Rhythmicity of P-eEF-2 in WT cells was determined using *F* tests of fit of the data to a sine wave (dotted black line; $P < 0.0001$), whereas P-eEF-2 levels were arrhythmic in Δ*rck-2* ($n = 3$), Δ*os-2* ($n = 3$), and Δ*frq* ($n = 2$) cells as indicated by a better fit of the data to a line (dotted black lines). (*E*) Representative Western blot of protein extracted from the indicated strains grown in DD for 24 and 36 h and probed with αP-eEF-2 and αeEF-2. The film was exposed for 10 s. Amido-stained proteins served as loading controls. The data are plotted below (mean ± SEM, $n = 3$). The asterisks indicate a statistical difference ($P < 0.05$, Student's *t* test).

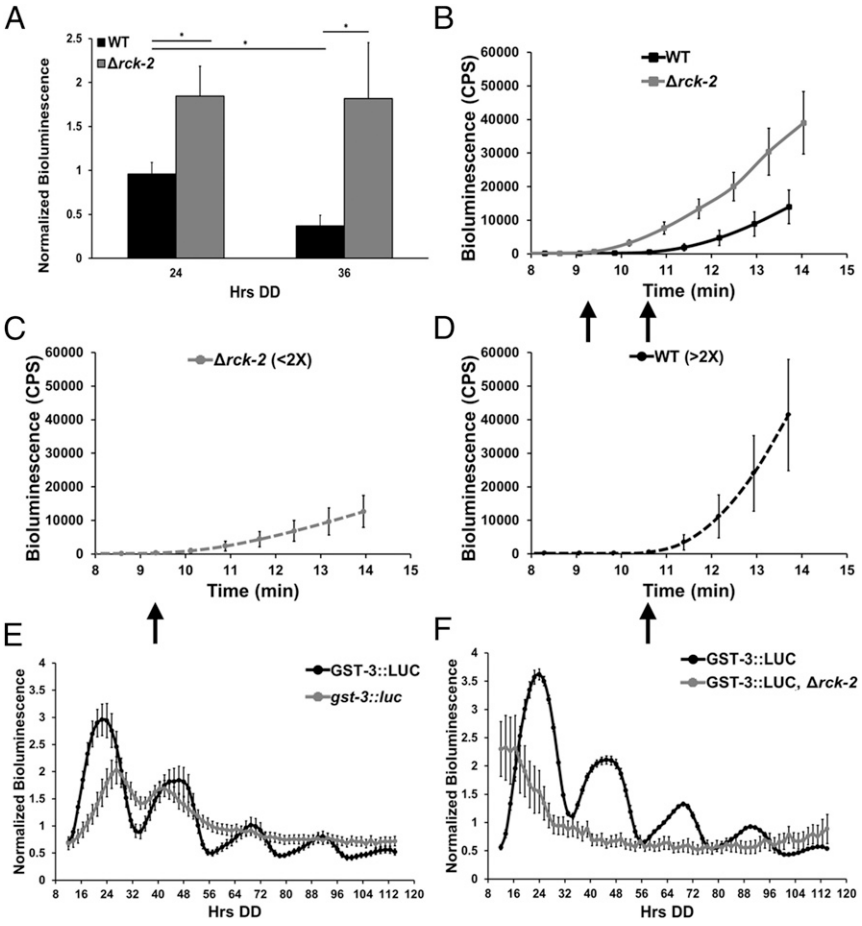

Fig. 5. The circadian clock regulates translation elongation through RCK-2 and P-eEF-2. (*A*) In vitro translation assay of *luciferase* (*luc*) mRNA using *N. crassa* cell-free extracts from wild-type (WT) and Δ*rck-2* strains grown in DD and harvested at 24 or 36 h (Hrs DD). The average (mean ± SEM) bioluminescence signal from WT ($n = 4$) and Δ*rck-2* ($n = 4$) are plotted. An asterisk represents a statistical difference ($P < 0.05$, Student's *t* test). (*B*) In vitro translation assay of *luc* mRNA using *N. crassa* cell-free extracts from WT and Δ*rck-2* strains grown in DD for 36 h. The average amount (mean ± SEM, $n = 3$) of bioluminescence counts per second (CPS) from WT and Δ*rck-2* extracts are plotted. The average amount (mean ± SEM, $n = 3$) of bioluminescence from Δ*rck-2* cell-free extract programmed with a twofold decrease (<2×) of *luc* mRNA (*C*), and WT cell-free extract programmed with twofold more (>2×) *luc* mRNA (*D*). The TFA of LUC is indicated with an arrow in *B*–*D*. (*E*) Luciferase assay using GST-3::LUC translational ($n = 15$) and *gst-3::luc* transcriptional ($n = 24$) fusion strains kept in DD for 4.5 d. The average amount (mean ± SEM) of bioluminescence is plotted. (*F*) Luciferase assay using GST-3::LUC translational ($n = 20$) and GST-3::LUC, Δ*rck-2* ($n = 11$) fusion strains kept in DD for 4.5 d. The average (mean ± SEM) bioluminescence signal is plotted.

and *luc* mRNA translation was high in Δ*rck-2* translation extracts at both times of the day (Fig. 5*A*). These data are consistent with (*i*) reduced P-eEF-2 levels increasing translation extract activity and (*ii*) the necessity for clock signaling through RCK-2 to observe time-of-day–specific differences in the extracts' capacity to translate *luc* mRNA. The levels of *luc* mRNA translation were higher in Δ*rck-2* cell extracts at both times of the day compared with the peak in WT extracts at DD24, despite finding that the steady-state levels of P-eEF-2 were similar to the WT level at DD24 (Fig. 4*E*). This result suggested that RCK-2 regulates additional factors in cells that reduce mRNA translation, possibly by altering levels or activities of ribosomes, tRNAs, or translation factors. The nature of this regulation will be investigated in future studies.

To determine if rhythmic P-eEF-2 levels affect translation elongation rates, we used an *N. crassa* cell-free translation protocol (24, 25) that accurately reflects protein synthesis in vivo (24, 26). Firefly *luc* mRNA was used as the template in the cell-free system to determine the time of first appearance (TFA) of the luminescence signal. TFA is a measure of the time needed for the ribosome to complete protein synthesis and is indicative of translation elongation rate (25, 26). Changes in translation elongation rates as a function of eEF-2 phosphorylation were examined in cell-free extracts isolated from WT and Δ*rck-2* cells harvested at the time of peak P-eEF-2 levels in WT cells (DD36). As predicted, TFA was detected earlier in Δ*rck-2* than in WT extracts (Fig. 5*B*), in line with reduced P-eEF-2 levels and increased elongation rates in Δ*rck-2* cells compared with WT cells. In accordance with increased translation elongation rates in Δ*rck-2* extracts, the slope of LUC accumulation over time was twofold higher in Δ*rck-2* extracts compared with WT extracts (Fig. 5*B*). To determine TFA under conditions where LUC synthesis rates were overall similar, we examined the consequences of varying the concentration of mRNA used to program extracts. The rate of accumulation of LUC in Δ*rck-2* was comparable to that of WT when Δ*rck-2* was programmed with twofold less mRNA than WT (Fig. 5*C*). Importantly, the TFA was still earlier than in WT extracts, consistent with an increased elongation rate in Δ*rck-2* extracts. Similarly, when WT extracts were programmed with twofold more mRNA than Δ*rck-2* (Fig. 5*D*), the accumulation of LUC after TFA correspondingly increased to levels similar to Δ*rck-2*, but TFA was still delayed relative to Δ*rck-2*.

To examine if rhythmic accumulation of P-eEF-2 provides a mechanism to rhythmically control mRNA translation elongation in vivo, we assayed rhythmicity of a GST-3 (encoding GST)::LUC translational fusion in WT and Δ*rck-2* cells. This gene was chosen based on constitutive *gst-3* mRNA accumulation (2) and evidence for rhythms of GST activity in mammalian cells (27). Consistent with the transcriptome data (2), a *gst-3* promoter::*luc* fusion was constitutively expressed in WT cells (Fig. 5*E*). In contrast, GST-3::LUC protein levels cycled in WT but not in Δ*rck-2* or Δ*frq* cells (Fig. 5 *E* and *F* and Fig. S7), demonstrating that clock signaling through RCK-2 and eEF-2 provides a mechanism to control translation elongation and protein accumulation. However, not all mRNAs are affected by rhythmic accumulation of P-eEF-2. We found that the levels and cycling of the core clock protein FRQ were not altered in Δ*rck-2* cells (Fig. S8 *A* and *B*).

Discussion

Increasing evidence points to the importance of the circadian clock in controlling mRNA translation, including rhythmic activation of cap-dependent translation factors (6, 7, 28–30), and

specific RNA binding proteins that control the translation of core clock genes (31–35). Although the initiation of translation has long been considered to be the primary control step in translation (36), a growing body of evidence points to translation elongation being regulated (37, 38), with phosphorylation and reduction in activity of eEF-2 being a central point in this control. Here, we showed that the *N. crassa* circadian clock, through rhythmic control of the OS MAPK pathway and downstream effector protein RCK-2, generates a rhythm in P-eEF-2 levels, peaking in the subjective day. This regulation leads to decreased translation activity and decreased translation elongation rates during the day in vitro.

N. crassa RCK-2 most closely resembles mammalian eEF-2 kinase MAPKAPK2. Similar to RCK-2 in fungi, mammalian MAPKAPK2 activity is controlled by the stress-induced p38 MAPK pathway (39, 40). MAPKAPK2 phosphorylates and activates eEF-2 kinase (eEF-2K), which in turn phosphorylates and inactivates eEF-2 (41). In mammals, several different signaling pathways converge on eEF-2K to activate or repress its activity and ultimately control the levels of P-eEF-2. In response to environmental stress, hypoxia, and nutrient status, p38 MAPK and mTOR signaling pathways inhibit eEF-2K (42–44), whereas AMP-kinase and cAMP-dependent protein kinase A signaling activate eEF-2K (45–48). *N. crassa* cells lack an obvious homolog of eEF-2K. Although our data showing that eEF-2 and RCK-2 coimmunoprecipitate in total protein extractions is consistent with a direct interaction between the two proteins, we cannot exclude the possibility that phosphorylation of eEF-2 by RCK-2 may be indirect and mediated through an unidentified kinase that functions similarly to eEF-2K. However, because low levels of P-eEF-2 were observed in Δ*rck-2*, Δ*os-2*, and Δ*rck-2,* Δ*os-2* double mutants following osmotic stress (Fig. 2*C* and Fig. S3), it appears that RCK-2 is the major pathway leading to phosphorylation of eEF-2, although other kinases, such as AMPK, PKA, and/or TOR pathway-directed kinases, may also have minor roles. Importantly, p38, as well as TOR, AMPK activity, and cAMP levels, are clock-controlled in higher eukaryotes (49–54), suggesting that clock control of translation elongation through rhythmic eEF-2 phosphorylation may be conserved. Furthermore, recent studies have shown that eEF-2 levels cycle in abundance in mouse liver (55, 56); however, it remains to be determined if rhythms in mammalian eEF-2 levels affect its activity.

Our studies have uncovered a mechanism for circadian clock control of protein abundance through the effects of rhythmic accumulation of P-eEF-2 on protein synthesis. These data lead to additional questions: (*i*) To what extent does clock control of translation elongation affect rhythmic protein levels, and (*ii*) what is the advantage of clock control of translation elongation to the organism? Recent ribosome profiling in mammalian cells revealed that ∼10% of mRNAs with rhythmic ribosome occupancy lacked corresponding rhythmic mRNA levels (56, 57). In the mouse liver, rhythmic ribosome occupancy on ∼500 constitutively accumulating mRNAs was enriched for mRNAs containing 5′-Terminal Oligo Pyrimidine (TOP) tracts, or Translation Initiator of Short 5′-UTR (TISU) elements (58). The clock controlled the amplitude and phase of ribosome occupancy of TISU-containing mRNAs, whereas rhythmic ribosome occupancy of mRNAs containing TOP elements was primarily controlled by rhythmic feeding. Thus, in mammalian cells, both the circadian clock and feeding cycles can contribute to rhythmic translation of specific mRNAs. Consistent with clock control of eEF-2 activity promoting rhythmic translation of specific mRNAs, rather than providing a mechanism to globally control translation, we show that RCK-2, which is needed for rhythms in P-eEF-2 levels, is not necessary for rhythms in accumulation of the clock protein FRQ (Fig. S7), whereas rhythms in GST-3 protein levels require RCK-2 (Fig. 5). Although there may be several ways to achieve specificity, we speculate that a slowdown in translation elongation rate during the day could reduce the expression of mRNAs for which elongation is rate-limiting, whereas the expression of mRNAs for which initiation is rate-limiting would either not be affected or would show relatively increased translation rates as a result of increased availability of initiation factors.

The clock plays a major role in controlling metabolism (59), including energy metabolism (60) and its major currency, ATP (61–63). As translation is one of the most energy-demanding processes in the cell, it makes sense to coordinate protein translation to times of day when energy levels are highest. In *N. crassa*, clock-controlled mRNAs generally peak at two phases, dawn and dusk, with dusk phase enriched for genes involved in ATP-requiring anabolic processes and dawn phase enriched for genes involved in ATP-generating catabolism (1, 2). Based on our data showing that the levels of P-eEF-2 peak during the day, we predict increased rates of mRNA translation at night, directly following the peak production of ATP during the day, and in coordination with other anabolic processes, including lipid and nucleotide metabolism. In addition, environmental stress to the organism peaks during the day (27). Linking translation elongation control to stress-induced and clock-controlled MAPK signaling, as well as to nutrient sensing pathways, likely provides an adaptive mechanism to partition the energy-demanding processes of translation to times of the day that are less stressful to the organism.

Materials and Methods

Vegetative growth conditions and crossing protocols were as previously described (15, 64). Strains used in the study are listed in Table S1. Protein extraction for Western blotting of FRQ, RCK-2 (65), and eEF-2 (66) were essentially as described. Western blotting using anti-HA primary antibody (#11867423001, Roche Diagnostics), anti–eEF-2 primary antibody (#2332S, Cell Signaling Technology), and rabbit anti–phospho-eEF-2 (Thr56) primary antibody (#2331S, Cell Signaling Technology) was carried out according to the manufacturer's instructions. Separation of RCK-2 forms was accomplished using Phos-tag acrylamide (#300–93523, Wako Chemicals USA, Inc.). Phosphatase treatment (#P0753S, New England Biolabs) and coimmunoprecipitation using GammaBind G Sepharose beads (#17–0885-01, GE Healthcare Bio-Sciences) were as recommended by the manufacturer. In vitro translation of *luc* mRNA (67, 68), in vivo luciferase assays (69), and statistical analysis of rhythmic data (15) were accomplished as previously described. Detailed methods can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Cheng Wu for technical assistance and for providing the *luc* mRNA template. This work was supported by the National Institutes of Health Grants R01-NIGMS-GM58529 and GM467721.

1. Sancar C, Sancar G, Ha N, Cesbron F, Brunner M (2015) Dawn- and dusk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in Neurospora. *BMC Biol* 13:17.
2. Hurley JM, et al. (2014) Analysis of clock-regulated genes in Neurospora reveals widespread posttranscriptional control of metabolic potential. *Proc Natl Acad Sci USA* 111(48):16995–17002.
3. Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB (2014) A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proc Natl Acad Sci USA* 111(45):16219–16224.
4. Nagel DH, Kay SA (2012) Complexity in the wiring and regulation of plant circadian networks. *Curr Biol* 22(16):R648–R657.
5. Beckwith EJ, Yanovsky MJ (2014) Circadian regulation of gene expression: At the crossroads of transcriptional and post-transcriptional regulatory networks. *Curr Opin Genet Dev* 27:35–42.
6. Jouffe C, et al. (2013) The circadian clock coordinates ribosome biogenesis. *PLoS Biol* 11(1):e1001455.
7. Lipton JO, et al. (2015) The circadian protein BMAL1 regulates translation in response to S6K1-mediated phosphorylation. *Cell* 161(5):1138–1151.
8. Cheng P, He Q, He Q, Wang L, Liu Y (2005) Regulation of the Neurospora circadian clock by an RNA helicase. *Genes Dev* 19(2):234–241.
9. He Q, et al. (2006) CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop. *Genes Dev* 20(18):2552–2565.
10. Schafmeier T, Káldi K, Diernfellner A, Mohr C, Brunner M (2006) Phosphorylation-dependent maturation of Neurospora circadian clock protein from a nuclear repressor toward a cytoplasmic activator. *Genes Dev* 20(3):297–306.
11. Heintzen C, Liu Y (2007) The Neurospora crassa circadian clock. *Adv Genet* 58:25–66.

MICROBIOLOGY

12. Froehlich AC, Liu Y, Loros JJ, Dunlap JC (2002) White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. *Science* 297(5582):815–819.
13. Froehlich AC, Loros JJ, Dunlap JC (2003) Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. *Proc Natl Acad Sci USA* 100(10):5914–5919.
14. Smith KM, et al. (2010) Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for neurospora white collar complex. *Eukaryot Cell* 9(10):1549–1556.
15. Lamb TM, Goldsmith CS, Bennett L, Finch KE, Bell-Pedersen D (2011) Direct transcriptional control of a p38 MAPK pathway by the circadian clock in Neurospora crassa. *PLoS One* 6(11):e27149.
16. Vitalini MW, et al. (2007) Circadian rhythmicity mediated by temporal regulation of the activity of p38 MAPK. *Proc Natl Acad Sci USA* 104(46):18223–18228.
17. Zhang Y, Lamm R, Pillonel C, Lam S, Xu JR (2002) Osmoregulation and fungicide resistance: The Neurospora crassa os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. *Appl Environ Microbiol* 68(2):532–538.
18. Bilsland-Marchesan E, Ariño J, Saito H, Sunnerhagen P, Posas F (2000) Rck2 kinase is a substrate for the osmotic stress-activated mitogen-activated protein kinase Hog1. *Mol Cell Biol* 20(11):3887–3895.
19. Teige M, Scheikl E, Reiser V, Ruis H, Ammerer G (2001) Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. *Proc Natl Acad Sci USA* 98(10):5625–5630.
20. Swaminathan S, Masek T, Molin C, Pospisek M, Sunnerhagen P (2006) Rck2 is required for reprogramming of ribosomes during oxidative stress. *Mol Biol Cell* 17(3): 1472–1482.
21. Warringer J, Hult M, Regot S, Posas F, Sunnerhagen P (2010) The HOG pathway dictates the short-term translational response after hyperosmotic shock. *Mol Biol Cell* 21(17):3080–3092.
22. Bilsland E, Molin C, Swaminathan S, Ramne A, Sunnerhagen P (2004) Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. *Mol Microbiol* 53(6):1743–1756.
23. de Paula RM, Lamb TM, Bennett L, Bell-Pedersen D (2008) A connection between MAPK pathways and circadian clocks. *Cell Cycle* 7(17):2630–2634.
24. Wei J, Zhang Y, Ivanov IP, Sachs MS (2013) The stringency of start codon selection in the filamentous fungus Neurospora crassa. *J Biol Chem* 288(13):9549–9562.
25. Wang Z, Fang P, Sachs MS (1998) The evolutionarily conserved eukaryotic arginine attenuator peptide regulates the movement of ribosomes that have translated it. *Mol Cell Biol* 18(12):7528–7536.
26. Yu CH, et al. (2015) Codon usage influences the local rate of translation elongation to regulate co-translational protein folding. *Mol Cell* 59(5):744–754.
27. Tuñón MJ, González P, López P, Salido GM, Madrid JA (1992) Circadian rhythms in glutathione and glutathione-S transferase activity of rat liver. *Arch Int Physiol Biochim Biophys* 100(1):83–87.
28. Cao R, et al. (2015) Light-regulated translational control of circadian behavior by eIF4E phosphorylation. *Nat Neurosci* 18(6):855–862.
29. Cao R, et al. (2013) Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling. *Neuron* 79(4):712–724.
30. Cornu M, et al. (2014) Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21. *Proc Natl Acad Sci USA* 111(32): 11592–11599.
31. Lim C, Allada R (2013) ATAXIN-2 activates PERIOD translation to sustain circadian rhythms in Drosophila. *Science* 340(6134):875–879.
32. Zhang Y, Ling J, Yuan C, Dubruille R, Emery P (2013) A role for Drosophila ATX2 in activation of PER translation and circadian behavior. *Science* 340(6134):879–882.
33. Bradley S, Narayanan S, Rosbash M (2012) NAT1/DAP5/p97 and atypical translational control in the Drosophila Circadian Oscillator. *Genetics* 192(3):943–957.
34. Morf J, et al. (2012) Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. *Science* 338(6105):379–383.
35. Kojima S, et al. (2007) LARK activates posttranscriptional expression of an essential mammalian clock protein, PERIOD1. *Proc Natl Acad Sci USA* 104(6):1859–1864.
36. Aitken CE, Lorsch JR (2012) A mechanistic overview of translation initiation in eukaryotes. *Nat Struct Mol Biol* 19(6):568–576.
37. Richter JD, Coller J (2015) Pausing on polyribosomes: Make way for elongation in translational control. *Cell* 163(2):292–300.
38. Liu B, Qian SB (2014) Translational reprogramming in cellular stress response. *Wiley Interdiscip Rev RNA* 5(3):301–315.
39. Zarubin T, Han J (2005) Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 15(1):11–18.
40. Sudo T, Kawai K, Matsuzaki H, Osada H (2005) p38 mitogen-activated protein kinase plays a key role in regulating MAPKAPK2 expression. *Biochem Biophys Res Commun* 337(2):415–421.
41. Kaul G, Pattan G, Rafeequi T (2011) Eukaryotic elongation factor-2 (eEF2): Its regulation and peptide chain elongation. *Cell Biochem Funct* 29(3):227–234.
42. Knebel A, Morrice N, Cohen P (2001) A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta. *EMBO J* 20(16):4360–4369.
43. Redpath NT, Foulstone EJ, Proud CG (1996) Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway. *EMBO J* 15(9): 2291–2297.
44. Wang X, et al. (2001) Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J* 20(16):4370–4379.
45. Althausen S, et al. (2001) Changes in the phosphorylation of initiation factor eIF-2alpha, elongation factor eEF-2 and p70 S6 kinase after transient focal cerebral ischaemia in mice. *J Neurochem* 78(4):779–787.
46. Browne GJ, Finn SG, Proud CG (2004) Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J Biol Chem* 279(13):12220–12231.
47. Horman S, et al. (2002) Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr Biol* 12(16):1419–1423.
48. Redpath NT, Proud CG (1993) Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity. *Biochem J* 293(Pt 1):31–34.
49. Williams JA, Su HS, Bernards A, Field J, Sehgal A (2001) A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. *Science* 293(5538): 2251–2256.
50. Hayashi Y, Sanada K, Hirota T, Shimizu F, Fukada Y (2003) p38 mitogen-activated protein kinase regulates oscillation of chick pineal circadian clock. *J Biol Chem* 278(27):25166–25171.
51. Pizzio GA, Hainich EC, Ferreyra GA, Coso OA, Golombek DA (2003) Circadian and photic regulation of ERK, JNK and p38 in the hamster SCN. *Neuroreport* 14(11): 1417–1419.
52. Cao R, Anderson FE, Jung YJ, Dziema H, Obrietan K (2011) Circadian regulation of mammalian target of rapamycin signaling in the mouse suprachiasmatic nucleus. *Neuroscience* 181:79–88.
53. Nikaido SS, Takahashi JS (1998) Day/night differences in the stimulation of adenylate cyclase activity by calcium/calmodulin in chick pineal cell cultures: Evidence for circadian regulation of cyclic AMP. *J Biol Rhythms* 13(6):479–493.
54. Lamia KA, et al. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* 326(5951):437–440.
55. Robles MS, Cox J, Mann M (2014) In-vivo quantitative proteomics reveals a key contribution of post-transcriptional mechanisms to the circadian regulation of liver metabolism. *PLoS Genet* 10(1):e1004047.
56. Janich P, Arpat AB, Castelo-Szekely V, Lopes M, Gatfield D (2015) Ribosome profiling reveals the rhythmic liver translatome and circadian clock regulation by upstream open reading frames. *Genome Res* 25(12):1848–1859.
57. Jang C, Lahens NF, Hogenesch JB, Sehgal A (2015) Ribosome profiling reveals an important role for translational control in circadian gene expression. *Genome Res* 25(12):1836–1847.
58. Atger F, et al. (2015) Circadian and feeding rhythms differentially affect rhythmic mRNA transcription and translation in mouse liver. *Proc Natl Acad Sci USA* 112(47): E6579–E6588.
59. Bass J (2012) Circadian topology of metabolism. *Nature* 491(7424):348–356.
60. Froy O (2013) Circadian aspects of energy metabolism and aging. *Ageing Res Rev* 12(4):931–940.
61. Burkeen JF, Womac AD, Earnest DJ, Zoran MJ (2011) Mitochondrial calcium signaling mediates rhythmic extracellular ATP accumulation in suprachiasmatic nucleus astrocytes. *J Neurosci* 31(23):8432–8440.
62. Yamazaki S, Ishida Y, Inouye S (1994) Circadian rhythms of adenosine triphosphate contents in the suprachiasmatic nucleus, anterior hypothalamic area and caudate putamen of the rat–negative correlation with electrical activity. *Brain Res* 664(1-2): 237–240.
63. Marpegan L, et al. (2011) Circadian regulation of ATP release in astrocytes. *J Neurosci* 31(23):8342–8350.
64. Loros JJ, Denome SA, Dunlap JC (1989) Molecular cloning of genes under control of the circadian clock in Neurospora. *Science* 243(4889):385–388.
65. Garceau NY, Liu Y, Loros JJ, Dunlap JC (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. *Cell* 89(3):469–476.
66. Jones CA, Greer-Phillips SE, Borkovich KA (2007) The response regulator RRG-1 functions upstream of a mitogen-activated protein kinase pathway impacting asexual development, female fertility, osmotic stress, and fungicide resistance in Neurospora crassa. *Mol Biol Cell* 18(6):2123–2136.
67. Zeenko VV, et al. (2008) An efficient in vitro translation system from mammalian cells lacking the translational inhibition caused by eIF2 phosphorylation. *RNA* 14(3): 593–602.
68. Wang Z, Sachs MS (1997) Arginine-specific regulation mediated by the Neurospora crassa arg-2 upstream open reading frame in a homologous, cell-free in vitro translation system. *J Biol Chem* 272(1):255–261.
69. Larrondo LF, Olivares-Yañez C, Baker CL, Loros JJ, Dunlap JC (2015) Circadian rhythms. Decoupling circadian clock protein turnover from circadian period determination. *Science* 347(6221):1257277.
70. Honda S, Selker EU (2009) Tools for fungal proteomics: Multifunctional neurospora vectors for gene replacement, protein expression and protein purification. *Genetics* 182(1):11–23.
71. Gooch VD, et al. (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the Neurospora clock. *Eukaryot Cell* 7(1):28–37.
72. Davis RH, de Serres FJ (1970) Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol* 17(Pt A):79–143.
73. Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* 282(1):158–161.
74. Bennett LD, Beremand P, Thomas TL, Bell-Pedersen D (2013) Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in Neurospora crassa. *Eukaryot Cell* 12(1):59–69.