Circadian clock control of eIF2α phosphorylation is necessary for rhythmic translation initiation

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The circadian clock in eukaryotes controls transcriptional and posttranscriptional events, including regulation of the levels and phosphorylation state of translation factors. However, the mechanisms underlying clock control of translation initiation, and the impact of this potential regulation on rhythmic protein synthesis, were not known. We show that inhibitory phosphorylation of eIF2α (P-eIF2α), a conserved translation initiation factor, is clock controlled in *Neurospora crassa*, peaking during the subjective day. Cycling P-eIF2α levels required rhythmic activation of the eIF2α kinase CPC-3 (the homolog of yeast and mammalian GCN2), and rhythmic activation of CPC-3 was abolished under conditions in which the levels of charged tRNAs were altered. Clock-controlled accumulation of P-eIF2α led to reduced translation during the day in vitro and was necessary for the rhythmic synthesis of select proteins in vivo. Finally, loss of rhythmic P-eIF2α levels led to reduced linear growth rates, supporting the idea that partitioning translation to specific times of day provides a growth advantage to the organism. Together, these results reveal a fundamental mechanism by which the clock regulates rhythmic protein production, and provide key insights into how rhythmic translation, cellular energy, stress, and nutrient metabolism are linked through the levels of charged versus uncharged tRNAs.

eIF2α | cpc-3 | translation initiation | circadian clock | *Neurospora crassa*

Circadian clocks regulate physiology and behavior through the rhythmic control of gene expression to optimize the timing of resource allocation for improved fitness (1). Remarkably, up to 50% of the eukaryotic genome is under control of the clock at the level of rhythmic mRNA abundance (2–8). In addition, mounting evidence supports circadian posttranscriptional regulation, including clock control of mRNA capping, splicing, polyadenylation, and deadenylation (3, 9–14). Rhythmic proteomic analysis in mammalian cells revealed that up to 50% of rhythmic proteins arise from noncycling mRNAs (15–18). Similar results were observed in the well-established circadian model organism *Neurospora crassa* where 41% of the rhythmic proteome arose from arrhythmic mRNAs (18). These data suggested that cycling protein accumulation is driven by temporal protein degradation and/or mRNA translation. In support of clock control of translation, the levels and modification of several translation initiation factors accumulate rhythmically in *N. crassa* (18) and mammals (19, 20), including rhythmic accumulation of translation initiation factor eIF2α levels in mouse liver and brain (21), and cycling phosphorylated eIF2α (P-eIF2α) levels in the mouse suprachiasmatic nucleus (22). Furthermore, the activity of translation elongation factor eEF-2 is controlled by the *N. crassa* clock through rhythmic activation of the p38 MAPK pathway and the downstream eEF-2 kinase RCK-2 (23). However, the mechanisms and extent of clock regulation of translation initiation are not fully understood. Therefore, we investigated the connection between the *N. crassa* clock and translation initiation.

One of the first steps in translation initiation is binding of eIF2 to GTP and the methionyl-initiator tRNA to form the ternary complex (24, 25). The ternary complex associates with the 40S ribosomal subunit to form the 43S preinitiation complex (PIC), which binds to the mRNA cap to form the 48S PIC. The PIC scans the mRNA as an open complex, and upon choosing a start codon in a preferred context, becomes a closed complex with the start codon paired to the initiator tRNA anticodon (26, 27). In the process, eIF2-GDP is released. The 60S ribosomal subunit then joins the 40S subunit to form a functional 80S ribosome for protein synthesis. eIF2-GDP is recycled to eIF2-GTP by the guanine nucleotide exchange factor eIF2B to enable reconstitution of the ternary complex for another round of translation (25).

A central mechanism for translational control is phosphorylation of the α-subunit of eIF2 (25, 28). In mammalian cells, eIF2α can be phosphorylated by four different kinases (GCN2, HRI, PERK, and protein kinase A) in response to different types of extracellular and intracellular stresses (29–31). Among these kinases, GCN2 is conserved in fungi and mammals (32–34). GCN2 is activated by chemical and genetic perturbations that lead to amino acid starvation, and other stresses, which result in the accumulation of uncharged tRNAs (35). Uncharged tRNA binds to the histidyl-tRNA synthetase-like (HisRS) domain and interacts with the C-terminal domain (CTD) of GCN2 to activate the kinase domain (11, 33, 36, 37). In yeast and mammalian cells, GCN1 is required for GCN2 activation (38). GCN1 interacts with ribosomal protein S10 in the ribosomal A site and is thought to transfer uncharged tRNA to activate GCN2 kinase (39, 40). Active GCN2 phosphorylates a conserved serine of eIF2α in fungi and mammals, which inhibits GDP/GTP exchange by two other known mechanisms of eIF2α phosphorylation. In mammalian cells, eIF2α phosphorylation is mediated by the GCN2 homologs HRI and PERK, and PKA, which can be activated by different stresses (41). This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Significance

Circadian clock control of mRNA translation, which contributes to the daily cycling of at least 50% of the proteins synthesized in eukaryotic cells, is understudied. We show that the circadian clock in the model fungus *Neurospora crassa* regulates rhythms in phosphorylation and activity of the conserved translation initiation factor eIF2α, with a peak in phosphorylated eIF2α levels during the daytime. This leads to reduced mRNA translation of select messages during the day and increased translation at night. We demonstrate that rhythmic accumulation of phosphorylated eIF2α requires increased uncharged tRNA levels during the day to activate the eIF2α kinase, coordinating rhythmic translation initiation and protein production with nutrient and energy metabolism.

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eIF2B (28). This reduces translation of many mRNAs, while selectively enhancing the translation of mRNAs that encode proteins required to cope with the stress, including genes encoding key amino acid biosynthetic enzymes (41). Because P-eIF2α is a competitive inhibitor of eIF2B, and because eIF2α is present in excess of eIF2B, small changes in the levels of P-eIF2α in cells are enough to substantially alter protein synthesis (30, 42).

Stimulation for all or any single amino acid, as well as too much of any one amino acid, leads to an amino acid imbalance, alterations in the levels of charged tRNAs, activation of GCN2, and synthesis of all 20 amino acids to relieve the imbalance (43–46). This general amino acid control (30), originally called cross-pathway control in *N. crassa* (46), leads to the activation of GCN2 kinase, phosphorylation of eIF2α, and translation of the bZIP transcription factors CPC-1 in *N. crassa*, and Gcn4 in yeast (30, 32). Both cpc-1 and Gcn4 contain upstream open reading frame (uORF) in the 5’t mRNA leader sequence that control translation of the main ORF in response to amino acid imbalance and the accumulation of P-eIF2α (30, 47–49).

The critical role for eIF2α in cap-dependent translation initiation led us to examine if, and how, the *N. crassa* circadian clock regulates translation initiation by regulating the phosphorylation state and activity of eIF2α. We show that ~30% of available *N. crassa* eIF2α is phosphorylated during the subjective day under control of the circadian clock. CPC-3 rhythmic activity, which was altered by chemical and/or genetic perturbation of amino acid levels and the levels of uncharged tRNA, was necessary for rhythmic accumulation of P-eIF2α. This daytime peak in P-eIF2α levels corresponded with increased levels of uncharged tRNA during the day, and to reduced translation in cell-free translation assays prepared from those cells. Furthermore, while the core clock component FREQUENCY (FRQ) accumulated rhythmically in Δcpc-3 cells, indicating the circadian oscillator was not impacted by P-eIF2α levels, we confirmed that one gene whose expression was predicted to be controlled at the level of translation by P-eIF2α levels, ALG-11, had protein rhythms that were dependent on CPC-3 and rhythmic P-eIF2α levels in vivo. These data suggested that clock regulation of P-eIF2α levels by CPC-3 drives rhythmic translation of specific mRNAs, rather than controlling global rhythmic translation, uncovering potential clock control of select mRNA translation by conserved mechanisms.

**Results**

eIF2α Phosphorylation Is Clock Controlled. To determine whether the *N. crassa* circadian clock regulates translation initiation, rhythms in phosphorylation of eIF2α (NCU08277) were investigated. Protein extracts were isolated from *N. crassa* cells grown in constant dark (DD) over a 2-d circadian time course. The protein extracts were used to assay the levels of total and P-eIF2α with anti-mammalian eIF2α antibodies directed against conserved epitopes (Fig. 1 and SI Appendix, Fig. S1A). eIF2α is essential; therefore, the specificity of the antibodies could not be confirmed using a deletion of eIF2α. Instead, antibody directed against total eIF2α was confirmed to cross-react with *N. crassa* eIF2α expressed and purified from bacteria with a band corresponding to the appropriate molecular weight (36 kDa) (SI Appendix, Fig. S1B). The specificity of the phosphospecific eIF2α antibody (P-eIF2α antibody) was confirmed by the signal being dependent on CPC-3 kinase (NCU01187) (Fig. 2A), and by stimulation of the signal during histidine starvation using 3-amino-1,2,4-triazole (3-AT) treatment (50) (see Fig. 4/4).

In wild-type (WT) cells, P-eIF2α levels (Fig. L4 and SI Appendix, Fig. S1C), but not total eIF2α levels (Fig. 1B and SI Appendix, Fig. S1D), cycled with a daily rhythm, reaching peak levels in the subjective late morning (DD16 and DD40). The levels of P-eIF2α and total eIF2α fluctuated in clock mutant Δfrq cells, but rhythmicity of P-eIF2α accumulation was abolished (Fig. 1C). These data demonstrated that the rhythm in P-eIF2α accumulation is controlled by the circadian clock.

CPC-3 Is Required for Phosphorylation of eIF2α. To determine whether CPC-3 kinase is required for phosphorylation of eIF2α in *N. crassa*, the levels and phosphorylation status of eIF2α were examined in Δcpc-3 cells grown in a circadian time course in DD (Fig. 2). No P-eIF2α was detected in Δcpc-3 cells in DD (Fig. 2A), whereas eIF2α was detected at all time points (Fig. 2B). Furthermore, complementation of Δcpc-3 cells with a WT copy of cpc-3 rescued P-eIF2α to a level similar to that observed in WT cells in DD (Fig. 2C). These data supported that CPC-3 kinase is necessary for eIF2α phosphorylation when cells are grown in DD.

Because eIF2α is a general translation initiation factor that may be critical for expression of components of the molecular circadian oscillator, we examined whether Δcpc-3 cells retain a functional clock by assaying canonical FRQ protein rhythms (51) from the same extracts used to examine P-eIF2α levels (Fig. 2A). FRQ protein levels were rhythmic in both WT and Δcpc-3 cells (Fig. 2D and SI Appendix, Fig. S1E). In addition, Δcpc-3 had no significant effect on the period of the circadian rhythm of development in strains carrying the ras-1Δd mutation (Fig. 2E), which slows growth rate and clarifies the rhythm in assexual spore development (52). However, the linear growth rate in ras-1Δd, Δcpc-3 was slower compared to ras-1Δd cells. The growth rate of Δcpc-3 was also slower than WT cells grown in DD (Fig. 4C). Thus, CPC-3 is necessary for phosphorylation of eIF2α, and for normal linear growth rate in DD. However, neither CPC-3 nor rhythmic P-eIF2α levels are required for a functional circadian oscillator.

Rhythmic Phosphorylation of eIF2α Is Not Dependent on Rhythmic CPC-3 Levels. Phosphorylation of eIF2α is rhythmic and requires CPC-3 in DD. Rhythmic control of eIF2α phosphorylation might be due to clock control of the levels and/or activity of CPC-3 kinase. To first establish whether the clock controls the levels of cpc-3 mRNA and protein, WT and Δfrq cells were transformed with either a cpc-3 promoter::luciferase transcriptional fusion (Pcpc-3::luc), or a Cpc-3::V5 translational fusion. Ppcpc-3::luc and Cpcpc-3::V5 were expressed in DD and examined in Δcpc-3 cells. The growth rate in Δcpc-3 was slower than WT cells grown in DD (Fig. 4C). Thus, CPC-3 is necessary for phosphorylation of eIF2α, and for normal linear growth rate in DD. However, neither CPC-3 nor rhythmic P-eIF2α levels are required for a functional circadian oscillator.

Constitutive Activation of CPC-3 Abolished Rhythmic P-eIF2α Levels. In Saccharomyces cerevisiae, GCN2 kinase is activated upon binding of uncharged tRNA to the regulatory domains (33, 36, 37). In addition, activation of GCN2 by uncharged tRNA requires the transacting positive effector protein GCN1 (54). The regulatory domains of *S. cerevisiae* GCN2 are conserved in *N. crassa* (28). This reduces translation of many mRNAs, while selectively enhancing the translation of mRNAs that encode proteins required to cope with the stress, including genes encoding key amino acid biosynthetic enzymes (41). Because P-eIF2α is a competitive inhibitor of eIF2B, and because eIF2α is present in excess of eIF2B, small changes in the levels of P-eIF2α in cells are enough to substantially alter protein synthesis (30, 42).
crassa CPC-3 (34); therefore, we hypothesized that CPC-3 is similarly activated by uncharged tRNA and GCN1 (NCU05803). To first establish whether CPC-3 is activated by amino acid starvation, 3-AT, a competitive inhibitor of imidazoleglycerol-phosphate dehydratase enzyme necessary for histidine production (55), was added to N. crassa cultures 8 h prior to harvest at the peak (DD40) and trough (DD28) of P-eIF2α levels (Fig. L4). As a result of clock control, the levels of P-eIF2α were twofold higher in untreated WT cells at DD40 compared to DD28 (Fig. 4A). However, in 3-AT–treated cells, the time-of-day difference in P-eIF2α levels was abolished, and the overall levels of P-eIF2α were approximately threefold higher than the peak at DD40 in WT cells. As expected, no significant change in total eIF2α levels was observed in 3-AT–treated versus untreated cells at either time point. These data demonstrated that up to 30% of eIF2α is phosphorylated by the clock during the day (DD40) compared to maximum levels of P-eIF2α during amino acid starvation. Consistent with the requirement for binding of uncharged tRNA for activation, CPC-3 required GCN1 for activation of the kinase domain to phosphorylate eIF2α, but deletion of GCN1 had no effect on total eIF2α levels (Fig. 4B and SI Appendix, Fig. S3A), or on FRO::LUC protein rhythms (SI Appendix, Fig. S3B). However, compared to WT cells, the linear growth rate of Δgcnl-1 was reduced to a level comparable to Δcpc-3 cells (Fig. 4C). These data are consistent with the idea that, similar to S. cerevisiae GCN2, N. crassa CPC-3 requires uncharged tRNA and GCN1 for activation. Therefore, we predicted that the corresponding mutations known to constitutively activate GCN2 in S. cerevisiae would constitutively activate N. crassa CPC-3, and provide the tool needed to examine whether rhythmic CPC-3 activity is required for cycling P-eIF2α levels. In S. cerevisiae, the F835L mutation leads to constitutive activation of GCN2, independent of uncharged tRNA binding, dimerization, association with ribosomes, and association with GCN1 (56). The homologous mutation generated in N. crassa CPC-3, cpc-3, led to a greater than threefold increase in P-eIF2α levels but had no significant effect on total eIF2α levels compared to WT cells grown for 28 h in DD (Fig. 4D). To validate that the cpc-3 mutation bypasses the requirement for activation by binding of uncharged tRNA, we showed that P-eIF2α levels were similar to WT levels in Δgcn1: cpc-3′ cells (Fig. 4B). These data supported that, in cpc-3′ mutant cells, CPC-3 is constitutively active, and this activity is independent of the requirement for GCN1 and uncharged tRNA.

Next, to determine whether P-eIF2α rhythmicity requires rhythmic activation of CPC-3, we examined P-eIF2α levels in cpc-3′ strains in a circadian time course in DD. If clock control of the activity of CPC-3 is necessary for rhythmic P-eIF2α accumulation, then the levels of P-eIF2α should be high and arrhythmic in cpc-3′ cells. Indeed, in the cpc-3′ mutant, the levels of P-eIF2α were higher compared to WT (Fig. 4D) and arrhythmic (Fig. 4E), with no corresponding change in the levels of total eIF2α (Fig. 4D and SI Appendix, Fig. S3C). Furthermore, the clock functioned normally in the mutant, as demonstrated by robust FRQ::LUC protein rhythms in cpc-3′ cells (SI Appendix, Fig. S3B). Similar to the reduced growth in Δcpc-3 and Δgcn1 that lack P-eIF2α accumulation, constitutive activation of CPC-3, and the resulting high and arrhythmic accumulation of P-eIF2α, also led to a reduction in linear growth rate compared to WT cells (Fig. 4C). Thus, CPC-3 activity is regulated by the clock, and this regulation is necessary for the rhythmic accumulation of P-eIF2α and normal linear growth rate.
Clock control of CPC-3 activity could be through rhythms in uncharged tRNA levels, and/or GCN1 levels. To test these ideas, we first examined whether GCN1 protein levels cycle by generating a GCN1::LUC translational fusion. The levels of GCN1::LUC cycled in WT cells, peaking in the early subjective night (SI Appendix, Fig. S4A). The observed nighttime peak in GCN1:LUC levels would not

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be expected if GCN1 directly controlled rhythmic CPC-3 activity, given that CPC-3 activity peaks during the subjective day. However, to rule out the possibility that the daytime accumulation of GCN1 might be sufficient for rhythmic CPC-3 activity, GCN1 was tagged with the HA epitope, and GCN1::HA was constitutively expressed from Ptcu-1 (53). In the presence of the Ptcu-1 inducer BCS, GCN1::HA levels were arrhythmic (SI Appendix, Fig. S4B), but the loss of rhythmic activation GCN1::HA had no effect on cycling P-eIF2α levels (SI Appendix, Fig. S4C). Therefore, while these data cannot rule out clock control of the activity of GCN1, we suspected that rhythms in the levels of uncharged tRNA accounts for rhythmic activation of CPC-3 during the subjective day.

Rhythms in uncharged tRNA levels could arise from rhythms in amino acid levels or aminoacyl-tRNA synthetase (aaRS) levels. Metabolic analysis of free amino acid levels in N. crassa WT cells did not reveal a significant rhythm for any of the 19 amino acids detected under our growth conditions (SI Appendix, Fig. S5), despite reported circadian rhythms in amino acid biosynthetic enzymes and their corresponding mRNAs (2, 18, 57). However, several aaRSs, including valyl-tRNA synthetase (ValRS) (NCU01965), were reported to cycle in abundance at the mRNA and/or protein levels (18, 57), which could lead to a rhythm in the balance of uncharged versus charged tRNAs. A translational fusion of ValRS to LUC (ValRS::LUC) confirmed that ValRS protein levels are rhythmic in WT, but not in clock mutant Δfrq cells, with a peak in the subjective night (SI Appendix, Fig. S64). We predicted that the trough in ValRS during the subjective day would lead to increased levels of uncharged tRNA Val and activation of CPC-3 during the subjective day. To first test this prediction, P-eIF2α levels were assayed in the temperature-sensitive ValRS mutant un-3ts 9, which leads to an ∼50% reduction in ValRS activity when cells are grown at the permissive temperature (25 °C) (58). Consistent with a role for uncharged tRNA in activating CPC-3, the levels of P-eIF2α were high and arrhythmic in the un-3ts mutant over a circadian time course (Fig. 5A), whereas FRQ protein level cycling was unaffected in the mutant (Fig. 5B and SI Appendix, Fig. S6B). Complementation of un-3ts with a WT copy of valRS rescued P-eIF2α rhythmicity (SI Appendix, Fig. S6C). Next, the levels of uncharged ValRS were measured over a circadian time course. The levels of uncharged ValRS cycled with a low amplitude, with a peak during the subjective day in WT cells (Fig. 5C), but were arrhythmic in clock mutant Δfrq (Fig. 5D) and un-3ts (SI Appendix, Fig. S6D) cells. The levels of total tRNA Val did not cycle (SI Appendix, Fig. S6E). Together, these data supported that circadian rhythms in the ratio of charged versus uncharged tRNAs drive rhythmic CPC-3 activity, which in turn directs the daytime peak in P-eIF2α levels.

Clock Control of P-eIF2α Activity Is Required for Rhythmic Translation of Select Transcripts In Vitro and In Vivo. Phosphorylation of eIF2α leads to an overall decrease in protein synthesis (28), and the clock regulates rhythms in the levels of P-eIF2α in N. crassa. Thus, we predicted that translation of some mRNAs would be reduced during the subjective day when the levels of P-eIF2α are high, and that translation would be increased during the subjective night when the levels of P-eIF2α are low. As an initial test of this prediction, we carried out in vitro translation assays using cell-free extracts isolated from WT, Δfrq, and Δcpc-3 cells harbored at the peak (DD40) and trough (DD28) of rhythmic P-eIF2α levels, that were programmed with capped polyadenylated mRNA encoding firefly luciferase (LUC). Translation was monitored by quantitating LUC activity (Fig. 6A). As predicted, LUC translation was higher when P-eIF2α levels were
low during the subjective evening (DD28), and translation was reduced at the peak of P-eIF2α levels in the subjective day (DD40). In Δfrq cells, LUC translation was similar to WT DD28 at both times of the day, supporting that the clock is necessary for increased P-eIF2α during the day. In Δcpc-3 extracts, LUC translation was significantly higher at both times of day, reflecting the absence of P-eIF2α under these growth conditions (Fig. 2). These data supported that clock control of the levels of P-eIF2α provides a mechanism to regulate rhythmic translation initiation.

To investigate a role for rhythmic P-eIF2α accumulation on mRNA translation in vivo, we examined rhythmicity of alg-11 (NCU06779) mRNA and ALG-11 protein levels in WT, Δfrq, and Δcpc-3 cells. ALG-11, a mannosyl transferase involved in N-linked glycosylation of proteins, was chosen based on its conserved role in posttranslational modifications important in cell wall stability, signaling, and endoplasmic reticulum protein quality control (59). In addition, our preliminary RNA-seq and ribosome profiling data showed that while alg-11 mRNA levels are arrhythmic, the clock controls rhythms in ribosome occupancy on alg-11 mRNA. These data suggested that alg-11, which contains a uORF (SI Appendix, Fig. S7), is regulated at the translational level by the clock. In WT cells, an ALG-11::LUC translational reporter fusion was rhythmic in DD, peaking during subjective night when P-eIF2α levels are low, whereas an alg-11 promoter luc fusion (Palg-11::luc, a transcriptional reporter) was arrhythmic (Fig. 6B). Consistent with translation regulation by the clock and rhythmic eIF2α activity, ALG-11::LUC was arrhythmic in Δfrq and Δcpc-3 cells (Fig. 6 C and D), as well as in un-3ts cells (SI Appendix, Fig. S7C). These data demonstrated that ALG-11 protein rhythms, which arise from constant mRNA levels, require a functional clock and rhythmic eIF2α activity.

**Discussion**

Translation initiation is a tightly regulated process that requires several translation initiation factors. One of the main targets of translational control is eIF2α. We established that the N. crassa clock regulates the activity of eIF2α (Fig. 1), with inhibitory P-eIF2α levels peaking during the subjective day and active eIF2α levels peaking during the subjective night. The nighttime activity of eIF2α parallels the peak activity of eEF-2 (23), suggesting coordinate control of mRNA translation initiation and elongation at night.

Consistent with the idea of coordinate circadian control of initiation and elongation, translation peaked at night in vitro when P-eIF2α (Fig. 6A) and PeEF-2 (23) levels are low. Similarly, the level of ALG-11 protein expression, which requires CPC-3 for rhythmic mRNA translation, peaked at night (Fig. 6B). In independent studies, mass spectroscopy analyses of rhythmic protein accumulation in N. crassa revealed that about 27% of the identified proteome accumulated with a circadian rhythm, with an approximately twofold increase in rhythmic protein accumulation at night compared to the day (2). In contrast, the peak in rhythmic mRNA levels was biphasic, with most transcripts peaking during the late night to early morning (2, 18, 57, 60).

Interestingly, N. crassa clock-controlled genes involved in catabolism generally peak during the day to provide energy for anabolic functions that occur at night (2, 57). As protein synthesis is energetically costly, it makes sense for the organism to synchronize most translation to the night when energy resources are at their maximum to support growth. Indeed, disruption of rhythmic P-eIF2α levels in N. crassa led to a significant reduction in linear growth rate (Fig. 4C). Conversely, some proteins accumulate to high levels during the day under control of the clock (18). A daytime peak in protein levels may be, in part, due to only up to one-half of available eIF2α and eEF-2 reaching peak
phosphorylation levels under control of the clock in constant environmental conditions (Fig. 4A and ref. 23), allowing some mRNAs to escape the daytime inhibition of translation. Moreover, phosphorylation of eIF2α selectively enhances translation of some target mRNAs, particularly those with uORFs in the leader sequence (49, 61, 62). Similar situations are observed in mice, in which some neuronal mRNAs involved in memory processing have increased translation when eEF-2 is hyperphosphorylated (63), and in Aplysia neurons, where eEF-2 phosphorylation promotes the translation of some messages, while repressing others (64). Taken together, these data support the notion that certain mRNAs are more sensitive to increased daytime phosphorylation (P-eIF2α) than repressing others (64). Taken together, these data support the notion that certain mRNAs are more sensitive to increased daytime phosphorylation (P-eIF2α) than repressing others (64).

Although our data show that rhythmic P-eIF2α does not significantly alter the core clock mechanism in N. crassa, a recent study of the mammalian circadian clock found that a similar daytime peak in levels of P-eIF2α in the suprachiasmatic nucleus (SCN) promoted the translation of Atf4, which in turn, activated transcription of the core clock gene Per2 (22). As a result, mutations or drugs that decreased P-eIF2α levels in the SCN lengthened circadian period, whereas mutations or drugs that increased P-eIF2α levels shortened the period. Despite differences in the effect of rhythmic P-eIF2α on the clock between N. crassa and mice, these data demonstrate a significant and conserved role for the regulation of translation initiation through eIF2α activity by the clock and its impact on the physiology of the organism.

Rhythmic CPC-3 activity (Fig. 4E), but not cycling CPC-3 levels (Fig. 3C and D), is necessary for the daytime peak in P-eIF2α levels. Activation of GCN2 requires binding of uncharged tRNA to the HisRS domain and the CTD, leading to a conformational change, activation of GCN2 protein kinase domain, and phosphorylation of eIF2α (25, 36, 37, 66). Because the HisRS domain is conserved in all GCN2 homologs (37), we speculated that CPC-3 in N. crassa is similarly activated by rhythmic binding of uncharged tRNA. Consistent with this idea, histidine starvation, induced by the addition of 3-AT to N. crassa cells, abolished the time-of-day difference in P-eIF2α levels (Fig. 4A). Amino acid levels accumulate rhythmically in the mouse brain under control of the clock (67), and in N. crassa, genes involved in amino acid biosynthesis are clock controlled (2, 18, 57). However, we did not observe a rhythm in free amino acid levels when N. crassa cells were cultured in DD. Alternatively, several aaRS mRNAs and proteins were reported in genome-wide studies to be clock controlled (2, 18, 57), and we validated that ValRS protein levels are rhythmic, peaking at night (SI Appendix, Fig. S6A). We found that reduced activity of ValRS led to high and arrhythmic P-eIF2α levels (Fig. 5A). In addition,
Although surprisingly little is known about the regulation of phosphatases in N. crassa. We are currently testing whether the homologous phosphatases are active at night to remove the phosphate with high EEF-2 levels. As a result, during the day, mRNA translation would be generally low, and protein catabolism would rise. Nighttime peaks in aaRSs would lead to increased charged tRNA levels, low CPC-3 activity and inhibitory P-eIF2α levels, and enhanced mRNA translation at night. While our data cannot rule out the possibility that rhythmic GCN1 activity might be required for cycling CPC-3 activity, the finding that CPC-3 and GCN1 levels cycle, but that rhythmic accumulation is not necessary for the daytime peak in CPC-3 activity and P-eIF2α levels, suggest that cycling CPC-3 and GCN1 levels may have additional functions in the cell and/or increase the robustness of CPC-3 activity rhythms.

Reprogramming protein production through CPC-3/GCN2 activity provides a conserved mechanism for organisms to quickly adapt to significant changes in the levels of amino acids, energy, and the ratio of charged versus uncharged tRNAs, such as during acute nutrient deprivation. Control of the activity of initiation and elongation factors and their kinetics by the clock under normal physiological conditions adds a further level of regulation to allow integration of nighttime anabolic and daytime catabolic cellular pathways with protein translation to effectively utilize available energy resources, and to contend with predictable daily stress.

### Materials and Methods

**N. crassa Strains and Growth Conditions.** A list of strains, key reagents, and oligonucleotides are listed in *SI Appendix, Table S1*. Vegetative growth conditions and crossing protocols were as previously described (72). All strains containing the hph construct were maintained on Vogel’s minimal media (72), supplemented with 200 μg/mL hygromycin B. Strains containing the bar cassette were maintained on Vogel’s minimal media lacking NH₄NO₃ and supplemented with 0.5% proline and 200 μg/mL Basta. Race tube assays to monitor developmental rhythms in strains carrying the ras-1α mutation, and linear growth rates, were accomplished using 1x Vogels salt, 0.1% glucose, 0.17% arginine, and 50 μg/mL biotin media as previously described (73).

To assay cpc-3 mRNA rhythms, a Ppc3-3::lac transcriptional fusion was generated by first amplifying a 1.5-kb promoter fragment upstream of the cpc-3 coding region using primers LUCF1 and LUCR1 containing NotI and Ascl restriction sites. The resulting 1.5-kb fragment was cloned into pRMPS7 containing a N. crassa codon-optimized luciferase gene (26), linearized with Ndel, and transformed into WT cells (Fungal Genetics Stock Center #4200 [FGSC#4200]), and transformants were assayed for luciferase activity. Ppc3-3::lac transformants were crossed with WT (FGSC#2489) and Δfrq::bar (DBP1228) strains to generate Ppc3-3::lac (DBP2439) and Ppc3-3::lac, Δfrq::bar (DBP2442) homokaryons.

A GCN1::LUC translational fusion was generated by three-way PCR with 1.5 kb of gcni (73) ORF (gcni F1 and R1 primers), 1.65 kb of N. crassa codon-optimized luciferase gene (gcni F2 and R2 primers), 1.0 kb of 3′ gcni (gcni F3 and R3), and cotransformed into WT cells (FGSC#4200) with plasmid for pBARGEM7-2 (74) for Basta selection. The Δfrq::bar::Ptcu-1::cpc-3::v5::hph construct was generated by three-way PCR containing 1.5 kb of the 5′ cpc-3 ORF (primers tcu1F1 and tcu1R1), bar::Ptcu-1 from plasmid DBP450 (primers tcu1F2 and tcu2R2), and 1 kb of 3′ of cpc-3 (primers tcu1F3 and tcu3R3) into DBP2717 strain. Following validation of integration by PCR (primers tcu1F4 and tcu4R), a bar::Ptcu-1::cpc-3::v5::hph homokaryon (DBP2742) was obtained by microcolony filtration (75).

For overexpression of GCN1::HA from the tcu1 promoter, 5′ and 3′ integrating fragments of DNA, consisting of 5′ flank gcni::3′ bar (primers gcni1F1 and barR), and 5′ bar::Ptcu-1::gcni ORF (primers barF and gcni1R1, respectively, were transformed into WT cells (FGSC#4200), creating DBP3473. Colonies were selected on 250 μg/mL fluorosinate ammonium, and proper integration was confirmed by PCR using primers gcni1S1 and gcni1S2.

A GCN1::HA translational fusion was created by three-way PCR with 1.8 kb of the gcni ORF (primers gcni1F1 and gcni1R1), 0.183 kb of 3′ glycine linker-VA-hygromycin B resistance gene (hph) (primers VSF2 and VSR2), and 1 kb of 3′ of cpc-3 (primers VF3 and VSIR). PCR was used to verify endogenous integration of the construct into the cpc-3 locus using VSF4 and VSIR primers, and expression of cpc-3::V5 was validated by Western blot using anti-V5 antibody. A cpc-3::v5::hph homokaryon (DBP2717) was generated by transforming WT with cpc-3::v5::hph, followed by crossing with WT (FGSC#2489). For overexpression of CPC-3::V5, a bar::Ptcu-1::cpc-3::hph strain was generated by transforming a three-way PCR product containing 1.5 kb of the 5′ cpc-3 ORF (primers tcu1F1 and tcu1R1), bar::Ptcu-1 from plasmid DBP450 (primers tcu1F2 and tcu2R2), and 1 kb of 3′ of cpc-3 (primers tcu1F3 and tcu3R3) into DBP2717 strain. Following validation of integration by PCR (primers tcu1F4 and tcu4R), a bar::Ptcu-1::cpc-3::v5::hph homokaryon (DBP2742) was obtained by microcolony filtration (75).
pBARGEM7-2 (74) into Δcpc3 cells (FGSC#10697). Hygromycin or Basta-resistant transformants were screened for luciferase activity and homology extension into the Δcpc3 genome (primers aln-11Fs and aln-11Rs). To generate the Palg-1::lac transcripational fusion, a 1.3-kb promoter region of alg-11 was amplified with primers alg-11F1 and alg-11R1 containing Xmal restriction sites. The PCR product was digested with Xmal and cloned into plasmid pRMP5 containing the codon-optimized luciferase gene (73). The resulting plasmid was linearized by digestion with PciI, cotransformed with hygR pBPl5 (77) into WT (FGSC2489) cells, and hygromycin-resistant transformants were screened for luciferase activity.

To assay FrQ::LUC protein rhythms, strains FGSC#14201, FGSC#10697, or DBP8290, were crossed to strains containing a FRQ::LUC translational fusion linked to bar (78). Hygromycin and Basta-resistant progeny were screened for luciferase activity associated with frq::luc, cpc-3cF3 and cpc-3cR3 primers followed by restriction digestion with BciI.

Circadian Time Courses. Circadian time course experiments for Western blots and amino acid analyses were accomplished according to published methods (79) as follows. Mycelial mats in Vogel’s minimal media containing 2% glucose (pH 6.0) were synchronized to the same time of day by a shift from 30 °C light (LL) to 25 °C dark (DD). The cultures were grown in LL for a minimum of 4 h and transferred to DD on day 1 (for collection at day 1, -44, -48, -52), day 2 (for collection at DD12, -16, -20, -24, -28, -32), day 3 (for collection at DD8), and harvested either at 9:00 AM (DD12, -16, -20, -36, -40, -44) or 5:00 PM (DD8, -24, -28, -32, -48, -52) on day 3. Harvested tissue was immediately frozen in liquid N2. For constitutive expression of bar::Frq::cpc3::v5::hph, cells were grown in Vogel’s medium containing 30 μM of the copper chelator BCS to induce the tcu-1 promoter (83).

Complementation of Δcpc3 and un-3th. Complementation of Δcpc3 was done to validate that CPC-3 was required for P-eIF2α rhythms. A WT copy of cpc-3 was amplified from the genome by PCR (primers cpc-3F1 and cpc-3R1) using Phusion Hot Start High-Fidelity DNA polymerase. The primer pair amplified a 1.5 kb upstream of the cpc-3 coding region, the 5.8 kb cpc-3 ORF, and 1 kb downstream of the cpc-3 coding region. This PCR product was cotransformed with hygR pBPl5 (77) into Δcpc3 (FGSC#10697). Transformants were selected for Basta resistance and validated for having a WT copy of cpc-3 by PCR using primers cpc-3F2 and cpc-3R2. Complementation of un-3th was done to validate that valRS was required for P-eIF2α rhythms. A WT copy of valRS was amplified from WT genomic DNA by PCR (primers un-3F3 and un-3R3) using Phusion Hot-Start High-Fidelity DNA polymerase. The primer pair amplified a 1.2 kb upstream of the valRS coding region, the 5.9-kb cpc-3 ORF, and 1.4 kb downstream of the valRS coding region. The construct was cotransformed with hygR pBPl5 (77) into the un-3th strain (FGSC#81). Hygromycin-resistant transformants were selected and validated for having a WT copy of valRS by growth at 30 °C and for rhythmic P-eIF2α levels.

3-AT Treatment. To determine whether CPC-3 is activated by amino acid starvation, germinating conidia were treated with 3-AT. Conidia (1 × 10⁷) from WT and Δcpc3 strains were inoculated in 500 mL of Vogel’s minimal media containing 2% glucose. Conidia were germinated in LL at 25 °C for 4 h, and then transferred to DD at 25 °C. A final concentration of 9 mM 3-AT was added to the cultures 8 h before harvesting at the indicated time points for Western blotting.

Protein Extraction and Western Blotting. Protein was extracted as previously described (80) with the following modifications: the extraction buffer contained 100 mM Tris pH 7.0, 1% SDS, 10 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1× aprotinin, 1× leupeptin, hemisulfate salt, and 1× pepstatin A. Protein concentration was determined by the Bradford assay. Protein samples (50 μg) were separated on 8% SDS/PAGE gels and blotted to an Immobilon-P nitrocellulose membrane according to standard methods.

The levels of P-eIF2α were detected using rabbit monoclonal anti-EIF2S1 antibody diluted 1:5,000 in 5% BSA, 1× TBS, 0.1% Tween, and anti-rabbit IgG HRP secondary antibody diluted 1:10,000. Total eIF2α levels were detected using rabbit polyclonal anti-EIF2S1 antibody diluted 1:5,000, and anti-rabbit IgG HRP secondary antibody diluted 1:10,000. CPC-3-5VS was detected using mouse monoclonal anti-α-anti-V5 antibody diluted 1:5,000 in 5% milk, 1× TBS, 0.1% Tween, and anti-mouse IgG HRP secondary antibody diluted 1:20,000. FRQ protein was detected using mouse monoclonal anti-α-FRQ antibody diluted 1:200 in 7.5% milk, 1× TBS, 0.1% Tween, and anti-mouse IgG HRP secondary antibody diluted at 1:10,000. All proteins except FRQ were detected using chemiluminescence SuperSignal West Pico Substrate. FRQ was detected using SuperSignal West Femto Maximum Sensitivity Substrate. Densitometry was performed using NIH ImageJ software (81) and normalized to protein loading using amido black-stained protein.

Purification of eIF2α. To validate the specificity of total eIF2α antibody, eIF2α was first amplified from N. crassa cDNA with primers eIF2F1 and eIF2R1 containing restriction sites for Ndel and NotI. The PCR product and pET30b vector were digested with Ndel and NotI prior to ligation to create pDBP607, which creates an IPTG inducible eIF2α-His purification plasmid. Expression in Escherichia coli pDBP607 was transformed to E. coli (L21 cells and transformed cells were grown at 37 °C overnight in 5 mL of Luria broth (LB), supplemented with 30 μg/mL kanamycin for selection. For purification of eIF2α, 1 mL of the overnight culture was inoculated into 50 mL of LB at 37 °C with shaking at 250 rpm until an OD of 0.63 was reached. A final concentration of 1 mM IPTG was added to induce expression, and cells were chilled and harvested 3 h after IPTG addition. Total eIF2α was purified by batch binding of a guanidinium buffer lysate to a Ni-NTA column and eluting with denaturing elution buffer according to published methods (82). After purification, the protein was dialyzed against multiple volumes of 10 mM Tris-Cl, pH 8.0, 0.1% Triton X-100, and then brought up to a final concentration of 20 ng/mL in 10% glycerol, 100 mM NaCl, and 0.1 mM EDTA. Purified protein was visualized by Western blot using total eIF2α antibody.

Luciferase Assays. To examine bioluminescence rhythms arising from strains containing luciferase fusions, 1 × 10⁷ conidia were inoculated into 96-well microtiter plates containing 150 μL of 1 μg Voldemort’s salts, 0.01% glucose, 0.03% arginine, 0.1% quinic acid, 1.5% agar, and 25 μM firefly luciferin, pH 6. After inoculation of conidia (1 × 10⁷ conidia), the microtiter plate was incubated at 30 °C in LL for 24 h and transferred to DD at 25 °C to obtain bioluminescence recordings with EnVision Xcite Multilabel Reader, with recordings taken every 90 min over 4 to 5 d. Raw luciferase activity data were analyzed for period, phase, and amplitude using BioDARE (83). Raw reads were normalized to the mean to graph the data.

Amino Acid Analysis. Free amino acid levels were measured in WT and Δfrq cells over a circadian time course in Vogel’s 2% glucose medium. Cells were washed with cold water and harvested using vacuum filtration, followed by flash freezing in liquid nitrogen. Frozen tissue was crushed into a thin powder using a mortar and pestle. Crushed cells (0.35 g) were boiled in 600 μL of distilled water for 20 min, followed by centrifugation for 15 min at 14,000 rpm. Supernatant (500 μL) was transferred to a VIVASPIN 500 column concentrator with a molecular weight cutoff of 5,000 Da, followed by centrifugation at 4° for 45 min at 14,000 rpm. The samples were analyzed for free amino acid levels by HPLC in the Protein Chemistry Lab Core Facility, Texas A&M University. Ten microliters of the sample were used for amino acid quantification.

In Vitro Translation. In vitro translation of luc mRNA was accomplished as previously described (23, 84).

tRNA Charging Assay. To examine rhythms in the levels of tRNAval, a tRNA charging assay was performed (85). Total RNA was extracted from ground tissue obtained from circadian time courses of WT (FGSC#42000) and un-3th (FGSC#81) strains. The pH was maintained at 4.5 throughout the RNA isolation to prevent deacylation. Two micrograms of RNA was treated with 12.5 mM NaIO4 or 12.5 mM NaCl in sodium acetate buffer (pH 4.5) in the dark for 20 min, and then quenched with 0.25 M glucose for 10 min at 25 °C. Each sample was spiked with 7.3 ng of deacylated yeast tRNAval and processed using Microspin G-25 columns to remove the salt. Desalted RNA was subjected to deacylation by resuspension in 50 mM Tris-HCl (pH 9.0), incubation at 37 °C for 45 min, followed by precipitation with cold 100% ethanol. Four hundred nanograms of tRNA was ligated to a 5′-adenylated linker (primer tRNAqPCR-linker) using T4 RNA ligase 2 truncated. An oligo (primer tRNAqPCR-GSP) complementary to the linker was used to generate cDNA with SuperScript RT III First-Strand Synthesis System. cDNA was diluted 1:10 and used as a template for quantitative PCR to detect val-specific tRNA using the corresponding primer (primers tRNAval-F and sc-tRNAval-R) and N. crassa tRNAval (primers nc-tRNAval-F and sc-tRNAval-R). The data were normalized to yeast tRNAval, and the uncharged tRNAval fraction was calculated by subtracting the charged fraction (NaIO4-treated) from total tRNAval (NaCl-treated).
1. V. K. Sharma, Adaptive significance of circadian clocks. Chronobiol. Int. 20, 901–919 (2003).

2. J. M. Hurley et al., Analysis of clock-regulated genes in Neurospora reveals widespread posttranscriptional control of metabolic potential. Proc. Natl. Acad. Sci. U.S.A. 111, 16995–17002 (2014).

3. N. Koike et al., Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 339, 434–467 (2012).

4. J. S. Menet, J. Rodriguez, K. C. Abruzzi, M. Rosbash, Nascent-seq reveals novel features of mouse circadian transcriptional regulation. eLife 1, e00112 (2012).

5. C. L. Partch, C. B. Green, J. S. Takahashi, Molecular architecture of the mammalian circadian clock. Trends Cell Biol. 24, 90–99 (2014).

6. M. W. Vitalini, R. M. de Paula, W. D. Park, D. Bell-Pedersen, The rhythms of life: when compared to the circadian clock, 466, 547–559 (2015).

7. A. G. Hinnebusch, Structural insights into the mechanism of scanning and start codon recognition in the filamentous fungus Neurospora. J. Biol. Chem. 280, 1927–1929 (2005).

8. J. L. Hilton, P. C. Kearney, B. N. Ames, Mode of action of the herbicide, 3-amino-1,2,4-triazole (amitrole): Inhibition of an enzyme of histidine biosynthesis. Arch. Biochem. Biophys. 112, 544–547 (1965).

9. H. Qi, J. Hu, D. G. Hinnebusch, Mutations that bypass TRNA binding activate the intrinsically defective kinase domain in GCN2, Genes Dev. 16, 1271–1280 (2002).

10. C. Sancar, G. Sancar, N. H. F. Cosbrun, M. Brunner, Darn- and dsk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in Neurospora. BMC Biol. 13, 17 (2015).

11. J. P. Ivanov, A. G. Hinnebusch, Separate domains in GCN1 for binding protein kinase activity. J. Biol. Chem. 280, 469–476 (2015).
60. D. Bell-Pedersen, M. L. Shinohara, J. J. Loros, J. C. Dunlap, Circadian clock-controlled genes isolated from Neurospora crassa are late night- to early morning-specific. Proc. Natl. Acad. Sci. U.S.A. 93, 13096–13101 (1996).

61. T. D. Baird, R. C. Wei, Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv. Nutr. 3, 307–321 (2012).

62. P. D. Lu, H. P. Harding, D. Ron, Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J. Cell Biol. 167, 27–33 (2004).

63. S. Park et al., Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGlur-LTD. Neuron 59, 70–83 (2008).

64. D. B. Weatherill et al., Compartment-specific, differential regulation of eukaryotic elongation factor 2 and its kinase within Aplysia sensory neurons. J. Neurochem. 117, 841–855 (2011).

65. Y. Liu, N. Y. Garceau, J. J. Loros, J. C. Dunlap, Thermally regulated translational control of FRQ mediates aspects of temperature responses in the Neurospora circadian clock. Cell 89, 477–486 (1997).

66. S. Zhu, A. Y. Sobolev, R. C. Wei, Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2. J. Biol. Chem. 271, 24989–24994 (1996).

67. J. M. K. S. Fustin, S. Karakawa, H. Okamura, Circadian profiling of amino acids in the SCN and cerebral cortex by laser capture microdissection-mass spectrometry. J. Biol. Rhythms 32, 609–620 (2017).

68. S. L. Spurgeon, W. H. Matchett, Inhibition of aminoacyl-transfer ribonucleic acid synthetases and the regulation of amino acid biosynthetic enzymes in Neurospora crassa. J. Bacteriol. 129, 1303–1312 (1977).

69. A. Cmokovic, D. Vargas-Rodriguez, D. Soll, Plasticity and constraints of tRNA aminoacylation define directed evolution of aminoacyl-tRNA synthetases. Int. J. Mol. Sci. 20, E2294 (2019).

70. R. C. Wei, J. F. Cannon, T. E. Dever, A. G. Hinnebusch, Truncated protein phosphatase GLC7 restores translational activation of GCN4 expression in yeast mutants defective for the eIF-2 alpha kinase GCN2. Mol. Cell. Biol. 12, 5700–5710 (1992).

71. V. Cherkasova, H. Qiu, A. G. Hinnebusch, Snf1 promotes phosphorylation of the α subunit of eukaryotic translation initiation factor 2 by activating Gcn2 and inhibiting phosphatases Glc7 and Sit4. Mol. Cell. Biol. 30, 2862–2873 (2010).

72. R. H. Davis, F. J. de Serres, Genetic and microbiological research techniques for Neurospora crassa. Methods Enzymol. 27A, 79–143 (1970).

73. V. D. Guroch et al., Fully codon-optimized luciferase uncovers novel temperature characteristics of the Neurospora clock. Eukaryot. Cell 7, 28–37 (2008).

74. M. L. Palli, J. P. Brunelli, A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. Fungal Genet. Rep. 40 (1993).

75. D. J. Ebbole, M. S. Sachs, A rapid and simple method for isolation of Neurospora crassa homokaryons using microconidia. Fungal Genet. Newsl. 37, 17–18 (1990).

76. N. Bardyia, P. K. Shiu, Cyclosporin A-resistance based gene placement system for Neurospora crassa. Fungal Genet. Biol. 44, 307–314 (2007).

77. A. K. Beasley, T. M. Lamb, W. K. Versaw, D. Bell-Pedersen, A ras-1bd Mauriceville strain for mapping mutations in Oak Ridge ras-1bd strains. Fungal Genet. Rep. 53, 30–33 (2006).

78. L. F. Larrondo, J. J. Loros, J. C. Dunlap, High-resolution spatiotemporal analysis of gene expression in real time: In vivo analysis of circadian rhythms in Neurospora crassa using a FREQUENCY-luciferase translational reporter. Fungal Genet. Biol. 49, 681–683 (2012).

79. T. M. Lamb, C. S. Goldsmith, L. Bennett, K. E. Finch, D. Bell-Pedersen, Direct transcriptional control of a p38 MAPK pathway by the circadian clock in Neurospora crassa. PLoS One 6, e27149 (2011).

80. C. A. Jones, S. E. Greer-Phillips, K. A. Borkovich, The response regulator RRG-1 functions upstream of a mitogen-activated protein kinase pathway impacting axonal development, female fertility, osmotic stress, and fungicide resistance in Neurospora crassa. Mol. Biol. Cell 18, 2123–2136 (2007).

81. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH image to image: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).

82. H. Liu, J. H. Naismith, A simple and efficient expression and purification system using two newly constructed vectors. Protein Expr. Purif. 63, 102–111 (2009).

83. T. Zielinski, A. M. Moore, E. Troup, K. J. Halliday, A. J. Millar, Strengths and limitations of period estimation methods for circadian data. PLoS One 9, e96462 (2014).

84. Z. Wang, M. S. Sachs, 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, 255–261 (1997).

85. J. Jiang et al., Promoter demethylation of the asparagine synthetase gene is required for ATF4-dependent adaptation to asparagine depletion. J. Biol. Chem. 294, 18674–18684 (2019).