Circadian Clock Control of Translation Initiation Factor eIF2α Activity Requires eIF2γ-Dependent Recruitment of Rhythmic PPP-1 Phosphatase in Neurospora crassa

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ABSTRACT  The circadian clock controls the phosphorylation and activity of eukaryotic translation initiation factor 2α (eIF2α). In Neurospora crassa, the clock drives a daytime peak in the activity of the eIF2α kinase CPC-3, the homolog of yeast and mammalian GCN2 kinase. This leads to increased levels of phosphorylated eIF2α (P-eIF2α) and reduced mRNA translation initiation during the day. We hypothesized that rhythmic eIF2α activity also requires dephosphorylation of P-eIF2α at night by phosphatases. In support of this hypothesis, we show that mutation of N. crassa PPP-1, a homolog of the yeast eIF2α phosphatase GLC7, leads to high and arrhythmic P-eIF2α levels, while maintaining core circadian oscillator function. PPP-1 levels are clock-controlled, peaking in the early evening, and rhythmic PPP-1 levels are necessary for rhythmic P-eIF2α accumulation. Deletion of the N terminus of N. crassa eIF2γ, the region necessary for eIF2γ interaction with GLC7 in yeast, led to high and arrhythmic P-eIF2α levels. These data supported that N. crassa eIF2γ functions to recruit PPP-1 to dephosphorylate eIF2α at night. Thus, in addition to the activity of CPC-3 kinase, circadian clock regulation of eIF2α activity requires dephosphorylation by PPP-1 phosphatase at night. These data show how the circadian clock controls the activity a central regulator of translation, critical for cellular metabolism and growth control, through the temporal coordination of phosphorylation and dephosphorylation events.

IMPORTANCE  Circadian clock control of mRNA translation contributes to the daily cycling of a significant proportion of the cellular protein synthesis, but how this is accomplished is not understood. We discovered that the clock in the model fungus Neurospora crassa regulates rhythms in protein synthesis by controlling the phosphorylation and dephosphorylation of a conserved translation initiation factor eIF2α. During the day, N. crassa eIF2α is phosphorylated and inactivated by CPC-3 kinase. At night, a clock-controlled phosphatase, PPP-1, dephosphorylates and activates eIF2α, leading to increased nighttime protein synthesis. Translation requires significant cellular energy; thus, partitioning translation to the night by the clock provides a mechanism to coordinate energy metabolism with protein synthesis and cellular growth.

KEYWORDS  eIF2α, PPP-1, phosphatase, translation initiation, circadian clock, eIF2γ, Neurospora crassa

The endogenous circadian clock is a conserved mechanism that allows organisms to anticipate daily environmental changes to maximize fitness (1–4). As such, it is linked to environmental sensing pathways that monitor external light, temperature, and nutrient availability. These input pathways provide information to modulate the clock. In turn, the clock utilizes feedback loops to sustain endogenous molecular oscillations and to generate rhythms in downstream output pathways, even in the absence of external cues (5–10). One of the most studied output pathways is rhythmic
transcription, with up to 50% of the eukaryotic genome regulated by clock at the transcriptional level (9, 11–17). Furthermore, several transcript-modifying processes (including mRNA capping, splicing, polyadenylation, and deadenylation) are under clock control (11, 18–21). While mRNA rhythms contribute to the generation of rhythmic protein abundance, approximately 40 to 50% of rhythmic proteins in both mouse liver and in the fungus \textit{N. crassa} derive from mRNAs that are not rhythmic (22–25), suggesting circadian regulation of protein stability and/or mRNA translation. In support of clock control of mRNA translation, the expression and/or phosphorylation of several translation factors are rhythmic in eukaryotic cells (22, 26–28), including rhythms in the phosphorylation and activity of the highly conserved translation initiation factor \(\text{eIF}2\alpha\) (29–31). Interestingly, many of the proteins in this class (rhythmic protein, arrhythmic mRNA) revealed a metabolic time of day partitioning, with daytime peaks in proteins involved in catabolism or energy utilization and nighttime peaks in anabolism or energy storage (25). These findings support that clock control of translation impacts the metabolic state of the cell. Thus, understanding the connection between the clock and control of the energetically expensive process of translation is crucial for a complete understanding of cellular growth control.

How the clock controls translation is just beginning to be unraveled, with recent studies revealing a conserved role for the clock in control of translation initiation (29–31). Translation initiation starts with the formation of the ternary complex, which contains initiation factor \(\text{eIF}2\alpha\), composed of \(\alpha\), \(\beta\) and \(\gamma\) subunits, Met-tRNA\(^{\text{Met}}\) and GTP (32, 33). There are multiple steps in the process, but initiation ends and elongation begins when \(\text{eIF}5\) mediates the hydrolysis of GTP-\(\text{eIF}2\alpha\) to GDP-\(\text{eIF}2\alpha\), which along with the other initiation factors, dissociates and allows 40S- and 60S-ribosomal subunit joining to create the translation-competent 80S ribosome (34, 35). To initiate another round of translation, the guanine nucleotide exchange factor (GEF) \(\text{eIF}2\beta\) must charge GDP-\(\text{eIF}2\alpha\) with GTP in a recycling step that is critical for controlling overall translation rates (32, 33). Ser51 phosphorylated \(\text{eIF}2\alpha\) (P-\(\text{eIF}2\alpha\)) inhibits \(\text{eIF}2\beta\) GEF activity by competitively binding to the limiting \(\text{eIF}2\beta\) (33), thus leading to reduced translation initiation of many mRNAs (32, 33), while also promoting translation of mRNAs with special motifs, including upstream ORFs (uORFs) (36). The levels of P-\(\text{eIF}2\alpha\) have been correlated with cell growth, cancer, memory and learning and are stimulated by the integrated stress response (ISR) and the mammalian target of rapamycin (mTOR) pathways (37–39). In mammals (29, 30) and \textit{N. crassa} (31), the circadian clock controls rhythms in P-\(\text{eIF}2\alpha\) abundance. Thus, studies examining the interplay between the clock and the known input pathways will help reveal the full range of translational regulation by \(\text{eIF}2\alpha\) phosphorylation.

The mechanism of clock control of \(\text{eIF}2\alpha\) phosphorylation is currently best understood in the fungus \textit{N. crassa} where the activity of the ISR responsive kinase CPC-3 (the homolog of yeast/mammalian GCN2) is thought to be modulated at different times of day via GCN1-dependent delivery of rhythmic levels of uncharged tRNAs (31). CPC-3 is required for Ser51 phosphorylation of \(\text{eIF}2\alpha\) (P-\(\text{eIF}2\alpha\)), and hyperactivation of CPC-3 kinase activity, either by pharmacological induction (3-AT) or by a constitutively active mutation (\textit{cpc-3c}), abolished P-\(\text{eIF}2\alpha\) rhythms. However, it is not known whether CPC-3 is sufficient to drive rhythms in P-\(\text{eIF}2\alpha\) accumulation (31). In particular, we were interested in learning how P-\(\text{eIF}2\alpha\) is converted back to the initiation competent dephosphorylated \(\text{eIF}2\alpha\) and whether a phosphatase might also contribute to the daily rhythms in \(\text{eIF}2\alpha\) activity.

Protein phosphatase 1 (PP1) dephosphorylates \(\text{eIF}2\alpha\) in yeast (40) and mammalian (41) cells. This activity requires the catalytic subunit GLC7 in yeast, as well as PP1\(\alpha\), PP1\(\beta\), or PP1\(\gamma\) isoforms in mammalian cells, and also requires one or more noncatalytic regulatory subunits to target PP1 to P-\(\text{eIF}2\alpha\) (42). In mammalian cells, the RVxF motif present on GADD34 (PPP1R15A) and CReP (PPP1R15B) recruits PP1 to dephosphorylate Ser51 on \(\text{eIF}2\alpha\) (43, 44). GADD34 and/or CReP homologs are present in chickens, frogs, and zebrafish, and a degenerate ortholog was identified in \textit{Drosophila} (45). In yeast
cells, however, there is no GADD34 or CReP homolog, but instead, an N-terminal extension of elf2γ contains an RVxF motif that recruits GLC7 to dephosphorylate Ser51 of elf2α (45). N. crassa PPP-1 (NCU00043) is the homolog of yeast GLC7 and is essential for survival (46). PPP-1 was previously shown to dephosphorylate FRQ protein to regulate the pace of the circadian clock (47). However, it was not known whether PPP-1 also functions to dephosphorylate P-elf2α and control rhythmic elf2α activity in N. crassa. In this study, we show that dephosphorylation of elf2α in vitro required PPP-1, that PPP-1 levels are clock-controlled with a peak during the subjective night, and that the rhythm in PPP-1 accumulation is necessary for cycling P-elf2α levels. Our study further revealed that the N terminus of elf2γ, which lacks a consensus RVxF motif, is required to recruit PPP-1 to dephosphorylate elf2α and maintain robust P-elf2α rhythmicity but is not required to maintain circadian clock function.

RESULTS

PPP-1 phosphatase reduces P-elf2α levels. To determine whether phosphatase PPP-1 regulates the levels of P-elf2α in N. crassa, the levels and phosphorylation status of elf2α were examined in ppp-1RIP mutant cells (47) from cultures grown in constant dark (DD) and harvested at 28 h (subjective night), which represents the low point of P-elf2α abundance in wild-type (WT) cells (31) (Fig. 1A). P-elf2α levels were significantly higher in ppp-1RIP mutant cells compared to WT cells harvested at 28 h, as well as in cells harvested in the subjective morning (DD40) (see Fig. S1A in the supplemental material) or grown in constant light (LL) (see Fig. S1B). The ppp-1RIP mutant was previously shown in vitro to reduce PPP-1 activity on P-phosphorylase by ~70% (47). P-elf2α abundance was ~2-fold higher in ppp-1RIP cells compared to WT cells, suggesting that PPP-1 promotes the dephosphorylation of P-elf2α. The abundance of total elf2α was not altered in the ppp-1RIP cells (Fig. 1A). Complementation of ppp-1RIP cells with a WT copy of ppp-1 inserted into the csr-1 locus (ppp-1RIP; csr-1::ppp-1) reduced P-elf2α levels to back to WT levels (Fig. 1A). These data support a role for PPP-1 in maintaining the low levels of P-elf2α present at subjective night.

To determine whether changes in PPP-1 protein abundance can control P-elf2α levels, ppp-1 was put under the control of the copper regulatable Ptcu-1 promoter (48), and PPP-1 levels were detected using a PPP-1-specific antibody (see Fig. S2A). Consistent with the idea that PPP-1 controls P-elf2α levels in vivo, copper sulfate (Cu) repression of Ptcu-1::ppp-1 led to low PPP-1 protein expression and high P-elf2α levels. Conversely, addition of the copper chelator bathocuproinedisulfonic acid (BCS), led to high PPP-1 protein expression and low P-elf2α levels compared to the control (U, untreated) (Fig. 1B). No significant changes were observed in elf2α levels in any of the conditions used. Together, these data support the idea that PPP-1 either directly and/or indirectly reduces P-elf2α levels in N. crassa.

To determine whether PPP-1 is responsible for dephosphorylating P-elf2α, the elf2 complex was purified from N. crassa cells containing a C-terminal V5-tagged elf2γ (elf2γ::V5) by coimmunoprecipitation with anti-V5 antibody (see Fig. S2B). To test whether there is a stable association of PPP-1 phosphatase with the elf2 complex, we first examined P-elf2α levels over time from the immunoprecipitated complex without addition of cell extract (Mock). We found that P-elf2α levels in the mock treatments were unchanged over time, suggesting that PPP-1, or other phosphatases, were not copurified in the elf2 complex (see Fig. S2C). Consistent with these data, PPP-1 was not detected in the elf2 complex using anti-PPP-1 antibody in Western blots. To examine whether addition of PPP-1 can dephosphorylate P-elf2α in the elf2 complex, total protein extracts containing endogenous PPP-1 from subjective evening (DD28) cells were added. To avoid potential rephosphorylation by the CPC-3 kinase, we utilized Δpcp-3 extracts (Fig. 1C). Cell extracts deficient in PPP-1 (ppp-1RIP; Δpcp-3) were also examined (Fig. 1C). Extracts from Δpcp-3 cells led to an ~50% reduction of P-elf2α levels after 120 min, while no significant dephosphorylation of elf2α was detected using extracts from ppp-1RIP; Δpcp-3 cells despite the ppp-1RIP mutant retaining some activity.
These data suggested that the residual PPP-1 activity in the \textit{ppp-1}\textsuperscript{RIP} mutant is not sufficient to dephosphorylate P-eIF2\textalpha at a level that is detectable in \textit{in vitro} assays. Taken together, these data support that \textit{in vitro} dephosphorylation of P-eIF2\textalpha depends on the transient presence of PPP-1.

In \textit{S. cerevisiae}, activation of the eIF2\textalpha kinase GCN2 \textit{in vivo} requires its association with ribosomes (49). Uncharged tRNAs are transferred from the ribosome to GCN2 by GCN1 to activate GCN2 (50–53). We found that \textit{N. crassa} PPP-1 associates with ribosomes (see Fig. S2D), suggesting the possibility that this interaction may facilitate direct access to its substrate P-eIF2\textalpha. Taken together, these results support the idea that PPP-1 promotes P-eIF2\textalpha dephosphorylation and are consistent with PPP-1 directly dephosphorylating eIF2\textalpha.

**PPP-1 phosphatase is required for clock control of P-eIF2\textalpha levels.** To determine whether PPP-1 phosphatase controls rhythmic P-eIF2\textalpha levels in \textit{N. crassa}, the phosphorylation status of eIF2\textalpha was examined in WT and \textit{ppp-1}\textsuperscript{RIP} cells grown in a circadian time course (Fig. 2). In WT cells, P-eIF2\textalpha, but not total eIF2\textalpha levels, were rhythmic, with

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**FIG 1** PPP-1 phosphatase reduces P-eIF2\textalpha levels and is critical for dephosphorylation of P-eIF2\textalpha \textit{in vitro}. (A) Western blot of protein extracted from WT, \textit{ppp-1}\textsuperscript{RIP} mutant, and \textit{ppp-1}\textsuperscript{RIP} \textit{csr1::ppp-1} complemented strains during the subjective night (DD28) and probed with anti-P-eIF2\textalpha and total eIF2\textalpha antibodies. The P-eIF2\textalpha/total eIF2\textalpha signal is plotted below for each strain (mean \pm the SEM, \textit{n}=3; *, \textit{P}<0.05 [Student’s \textit{t}-test]). (B) Western blot of protein from \textit{Ptcu1-ppp-1} cells grown in the presence of copper sulfate (Cu), BCS, or untreated (U); harvested at DD28; and probed with anti-PPP-1, anti-P-eIF2\textalpha, and anti-eIF2\textalpha antibodies. The graph below shows the average signal of P-eIF2\textalpha/total eIF2\textalpha (mean \pm the SEM, \textit{n}=3; *, \textit{P}<0.05 [Student’s \textit{t}-test]). (C) \textit{In vitro} dephosphorylation assay using cell extracts from \textit{Dcpc-3} and \textit{ppp-1}\textsuperscript{RIP}; \textit{Dcpc-3} cells incubated with P-eIF2\textalpha from \textit{elF2g::v5} cells for 0, 30, 60, or 120 min. P-eIF2\textalpha and total eIF2\textalpha levels were examined by Western blotting. The graph below shows the average signal of P-eIF2\textalpha normalized to total protein for each time point and normalized to the value at time zero (mean \pm the SEM, \textit{n}=4; *, \textit{P}<0.05 [Student’s \textit{t}-test compared to time zero]). In panels A to C, membranes were stained with amido black as a protein loading control.
a peak in the subjective late morning (Fig. 2A and B), consistent with our previous studies (31). While P-eIF2α and total eIF2α levels fluctuated in ppp-1RIP cells, P-eIF2α rhythms were abolished (Fig. 2C and D). Because the circadian clock was previously shown to be functional in ppp-1RIP cells (47), it seemed unlikely that P-eIF2α rhythms were abolished due to a clock defect in these cells. However, to confirm clock function in the mutant, FRQ::LUC protein rhythms were examined in WT and ppp-1RIP cells. Consistent with published data (47), FRQ levels oscillated robustly in ppp-1RIP cells, but with an ∼2 h shorter period compared to WT cells (Fig. 2E). Taken together, these data support the idea that the loss of P-eIF2α rhythms in ppp-1RIP cells is not due to loss of rhythmicity of the core oscillator, but instead results from disruption of downstream circadian regulation of P-eIF2α levels.

**Deletion of the N terminus of eIF2α alters eIF2α phosphorylation levels and the dephosphorylation rate of eIF2α in vitro.** The N terminus of *N. crassa* eIF2α (NCU02810) resembles the N terminus of the *S. cerevisiae* eIF2α in that it has an 80-amino-acid extension compared to eIF2α homologs in higher eukaryotes. In *S. cerevisiae* this region is required to recruit PPP-1 to eIF2α (45) (see Fig. S3A). We predicted that if the N terminus of *N. crassa* eIF2α functions analogously, the levels of P-eIF2α would be high in strains that have an N-terminal eIF2α deletion. To test this prediction, residues 2 to 62 were deleted from the endogenous eIF2α gene (here referred to as eIF2α2-62) (see Fig. S3A), and P-eIF2α levels were examined in a circadian time course (Fig. 3). As predicted, removal of this putative phosphatase-recruiting domain resulted in significantly higher P-eIF2α levels in eIF2α2-62 compared to WT cells. Furthermore, P-eIF2α levels in eIF2α2-62 cells were not significantly different than the high levels observed in ppp-1RIP cells (Fig. 3A). These results support a role for the N-terminal region of *N. crassa* eIF2α in recruiting PPP-1 phosphatase to P-eIF2α in vivo.

To determine whether the N terminus of eIF2α impacts dephosphorylation of eIF2α by PPP-1 in vitro, the eIF2 complex was purified from *N. crassa* eIF2α:V5 and eIF2α2-62::V5 cells by coimmunoprecipitation with anti-V5 antibody (see Fig. S3B). The eIF2 complex containing pulled down P-eIF2α with eIF2α:V5 was incubated with total cell extracts (containing PPP-1) from Δcpc-3 cells, and the eIF2 complex pulled down with eIF2α2-62::V5 was incubated with total cell extracts from eIF2α2-62::Δcpc-3 cells (Fig. 3B). Extracts from Δcpc-3 cells led to an ∼50% reduction of P-eIF2α levels after 120 min, consistent with dephosphorylation of P-eIF2α by PPP-1 and the data shown in Fig. 1C. However, extracts from eIF2α2-62::Δcpc-3 cells that lack the N terminus of eIF2α showed significantly reduced dephosphorylation of P-eIF2α levels compared to extracts from Δcpc-3 cells (Fig. 3B). In eIF2α2-62::Δcpc-3 cells, P-eIF2α levels were reduced up to 20% at 120 min compared to the 0-min time point, suggesting that additional regulatory subunits present in eIF2α2-62::Δcpc-3 extracts may recruit PPP-1 to dephosphorylate P-eIF2α, although less efficiently than eIF2α. These results, together with the lack of dephosphorylation of P-eIF2α in mutant PPP-1 extracts (Fig. 1C), support the idea that the N terminus of eIF2α recruits PPP-1 to dephosphorylate eIF2α in *N. crassa*.

**Deletion of the N terminus of eIF2α disrupts P-eIF2α level rhythms.** To determine whether the N-terminal extension of eIF2α is essential for circadian clock control of P-eIF2α, the levels of P-eIF2α were examined over a circadian time course in eIF2α2-62 cells. The levels of P-eIF2α increased over time, and P-eIF2α rhythms were severely dampened in eIF2α2-62 cells (Fig. 3C). When the data were detrended to account for the increasing levels of P-eIF2α over time, a rhythm with significantly reduced amplitude and period was detected (see Fig. 5A). This dampened P-eIF2α rhythm observed in eIF2α2-62 cells in vivo is consistent with residual PPP-1 phosphatase activity observed in vitro in eIF2α2-62::Δcpc-3 extracts (Fig. 3B). Total eIF2α levels in eIF2α2-62 cells were arrhythmic (Fig. 3D; see also Fig. 5A in the supplemental material). Unlike the short period FRQ::LUC rhythm observed in ppp-1RIP cells (Fig. 2E), the period of FRQ::LUC reporter rhythms was not significantly altered in eIF2α2-62 cells compared to WT cells (Fig. 3E). Therefore, it is likely that a different regulator is used to target PPP-1 to dephosphorylate FRQ. Also, PPP-1 protein levels were still rhythmic in eIF2α2-62 cells, suggesting the mutation did not impact PPP-1 protein expression (see Fig. S4D). Taken together,
these data support a role for the N terminus of eIF2γ in recruiting PPP-1 to P-eIF2α and promoting circadian clock control of P-eIF2α levels.

**Rhythmic phosphorylation of eIF2α requires rhythmic PPP-1 levels.** PPP-1 phosphatase and the N terminus of eIF2γ are necessary for circadian rhythms of P-eIF2α levels but not for core clock function. Thus, rhythmic control of eIF2α activity may be through clock control of the levels and/or activities of PPP-1 phosphatase and/or eIF2γ. Prior mass spectrometry proteomic studies suggested that PPP-1 protein, but not eIF2γ, could be rhythmic (25). To determine whether the circadian clock controls the levels of PPP-1 phosphatase and/or eIF2γ, PPP-1::luciferase (PPP-1::LUC) and eIF2γ::V5 C-terminal translational fusion constructs were generated and used to replace the corresponding endogenous loci. No change in P-eIF2α levels was observed in cells containing the V5-tagged version of eIF2γ::V5 compared to WT cells, indicating the tag does not alter the function of eIF2γ (see Fig. S5). PPP-1::LUC protein accumulated rhythmically in WT cells but not in control clock mutant Δfrq cells, (Fig. 4A), demonstrating that PPP-1 protein levels are clock-controlled. Consistent with PPP-1 functioning as an eIF2α phosphatase, the early evening peak (with phase CT [circadian time] 14, which corresponds to DD24) in PPP-1::LUC levels correlated with the trough of P-eIF2α levels.
Deletion of the amino terminal 60 amino acids of *N. crassa* eIF2γ alters P-eIF2α levels and rhythmicity. (A) Western blot of protein extracted from the indicated strains harvested at DD28 were probed with anti-P-eIF2α or total eIF2α antibodies. P-eIF2α/total eIF2α signals are plotted below (mean ± the SEM, n = 3; *, P < 0.05 [Student’s t-test]). (B) In vitro dephosphorylation assay using cell extracts from Δcpc-3 and eIF2γΔ2-62; Δcpc-3 cells incubated with pulled down P-eIF2α from eIF2γ::V5 and eIF2γΔ2-62::V5 cells, respectively, for 0, 30, 60, and 120 min. P-eIF2α and total eIF2α levels were examined by Western blotting. The graph below shows the average signal of P-eIF2α normalized to total protein for each time point and normalized to the value at time zero (mean ± the SEM, n = 5; *, P < 0.05 [Student’s t-test compared with time zero]). (C and D) Western blots of protein from eIF2γΔ2-62 cells grown in a circadian time course, harvested at the indicated times in DD (Hrs DD), and probed with anti-P-eIF2α (C) or anti-total eIF2α (D) antibody. Plots of the data (mean ± the SEM, n = 5) below display the average P-eIF2α (C) or eIF2α (D) signal normalized to total protein (solid line). Both P-eIF2α and total eIF2α in eIF2γΔ2-62 cells were arrhythmic determined by F tests of the fit to a line (dotted lines). Membranes were stained with amido black as a protein loading control. (E) Luciferase activity from a FRQ::LUC translational fusion expressed in WT (black line) and eIF2γΔ2-62 (gray line) cells grown in DD and recorded every 90 min over 6 days (Hrs DD). The average normalized bioluminescence signal is plotted (mean ± the SEM, n = 24 for WT and n = 32 for eIF2γΔ2-62). The period (h) (mean ± the SEM) is shown on the right.

(see Fig. 2A, DD24). Alternatively, eIF2γ::V5 levels did not cycle in WT cells (Fig. 4B). In addition, PPP-1::LUC rhythmicity was not altered in Δcpc-3 cells that are unable to phosphorylate eIF2α (31) (see Fig. S6), indicating that PPP-1 protein level rhythms arise from mechanisms that are independent of rhythmic eIF2α activity. Together, these data suggested the possibility that the nighttime peak in PPP-1 levels may be critical for P-eIF2α rhythms.
To determine whether rhythmic accumulation of PPP-1 is necessary for rhythms in P-eIF2α levels, protein from strains containing Ptcu-1::ppp-1 (Fig. 1B) grown in a circa-dian time course were isolated and examined by Western blotting with anti-PPP-1 antibody. In WT cells, PPP-1 protein levels were rhythmic, peaking in the subjective early evening (DD24), consistent with the PPP-1::LUC rhythms (Fig. 5A). In Ptcu1::ppp-1 cells grown in the presence of the activating chelator BCS, PPP-1 levels were high and non-cycling (Fig. 1B and 5B), and P-eIF2α levels were low and arrhythmic (Fig. 5C). In Ptcu1::ppp-1 cells grown in the presence of the repressive copper ion (Cu), PPP-1 protein levels were low (Fig. 1B), and P-eIF2α levels were high and arrhythmic (Fig. 5D). Thus, non-rhythmic PPP-1 expression at either low or high levels abolished P-eIF2α rhythms. These data demonstrated that the rhythmic accumulation of PPP-1 protein is necessary for circadian rhythms in P-eIF2α levels.

DISCUSSION

In N. crassa and mice, circadian clock regulation of eIF2α phosphorylation controls rhythmic mRNA translation and protein accumulation (30, 31). In N. crassa the eIF2α kinase, CPC-3, is necessary for the accumulation of P-eIF2α levels and a constitutively active allele causes arrhythmicity of P-eIF2α (31). Here, we show that protein phosphatase PPP-1, which peaks in levels during the subjective night, is also necessary for circadian rhythms in P-eIF2α levels. These data support a model whereby the circadian clock dynamically regulates both the phosphorylation, through the day-stimulated CPC-3 kinase, and dephosphorylation, by the night-peaking PPP-1 phosphatase, of eIF2α (Fig. 5E). The peak in activity of eIF2α at night, together with increased nighttime activity of translation elongation factor eEF-2 (28), provide a mechanism to explain increased rhythmic protein production at night in N. crassa (25).

While PPP-1 is necessary for rhythmic eIF2α activity, it is not sufficient to drive rhythms in P-eIF2α levels in strains with constitutively active CPC-3 (CPC-3C). In cpc-3C cells, P-eIF2α levels are high and arrhythmic (31), despite normal rhythmic PPP-1 levels in the mutant (see Fig. S7 in the supplemental material). This may be due the levels or activity of PPP-1 not being sufficient to dephosphorylate the constantly high levels of P-eIF2α present in this mutant. While we showed that P-eIF2α levels are directly related to PPP-1 levels in a strain with WT CPC-3 activity (Fig. 1B), after 2 h in vitro only up to 50% of P-eIF2α was dephosphorylated by PPP-1 indicating that the dephosphorylation step may be kinetically unfavorable (Fig. 1C). These data are consistent with the slow in vitro dephosphorylation rate of eIF2α observed in yeast extracts (45). A second possibility for why PPP-1 rhythms are not sufficient to drive P-eIF2α rhythms in the cpc-3C mutant is that PPP-1 may also regulate CPC-3 activity. This idea is supported...
by the presence of at least two phosphatases in *S. cerevisiae* known to target both P-eIF2α and P-GCN2. The 2A-related phosphatase SIT4, which responds to the Target of Rapamycin (TOR) pathway (54, 55) and dephosphorylates eIF2α (56), also controls Ser577 phosphorylation and activity of GCN2. The phosphatase PPZ1 also impacts GCN2-dependent phosphorylation of eIF2α by an unknown mechanism (57, 58). Thus, in addition to direct dephosphorylation of eIF2α, these data support a role for phosphatases controlling the activity of the eIF2α kinases. Experiments are under way to identify potentially rhythmic phosphorylation sites on CPC-3 that may be dephosphorylated by PPP-1. The presence of WT PPP-1 was necessary for dephosphorylation of P-eIF2α in vitro. Given the residual in vitro phosphatase activity in *elf2*yΔ2-62 cells (Fig. 3B), we cannot absolutely rule out that other PPP-1-dependent phosphatases in the extracts perform the dephosphorylation of P-eIF2α. This residual activity may also explain why the rhythms of P-eIF2α levels are severely diminished, and not completely abolished, in *elf2*yΔ2-62 cells (see Fig. S4). In any case, our data support that PPP-1 is recruited to P-eIF2α by the eIF2 subunit eIF2γ to directly dephosphorylate P-eIF2α.

**FIG 5** Clock control of PPP-1 is necessary for rhythmic P-eIF2α levels. (A to D) Western blots of protein extracted from WT (A) or *Pcu1::ppp-1* cells cultured with 50 μM BCS (B and C) or 250 μM copper sulfate (D) over a circadian time course, harvested at the indicated times in DD (Hrs DD), and probed with anti-PPP-1 (A and B) or anti-P-eIF2α (C and D) antibodies. Membranes were stained with amido black as a protein loading control. The normalized protein levels are plotted below the blots (mean ± the SEM, n = 3) (solid black line). PPP-1 levels in WT cells (A) were rhythmic based on best fit to a sine wave (dotted line, \( P < 0.001 \)), whereas PPP-1 and P-eIF2α (B to D) were arrhythmic as indicated by best fit to a line (dotted line). (E) Model of the mechanisms of clock coordination of the day-active eIF2α kinase CPC-3 and the night-active phosphatase PPP-1 controlling rhythmic eIF2α activity and translation initiation.
Kinases typically target specific substrates; however, phosphatases generally have a wide substrate range (45). In addition to dephosphorylation of eIF2α, S. cerevisiae GNC7, the catalytic subunit of PP1, dephosphorylates substrates that function in glycogen metabolism, glucose regulation, and cell division (59). Furthermore, PP1 requires one or more noncatalytic regulatory subunits to target it to different cellular compartments and for substrate specificity. More than 180 PP1 regulatory subunits have been identified in mammalian cells (60), and 17 regulatory subunits were discovered in S. cerevisiae (61). Most, but not all, PP1 regulatory subunits contain a conserved RVxF motif, which is typically flanked by basic residues at the N terminus, and by acidic residues at the C terminus (62). Regulatory subunits that recruit PP1 to eIF2α in mammalian cells, GADD34 and CReP, contain an RVxF motif (43, 44). In the PP1 regulatory subunit eIF2γ in S. cerevisiae, the RVxF motif is present in an N-terminal domain that extends beyond homology to mammalian eIF2γ (45), and deletion of the N terminus of eIF2γ does not affect yeast cell growth, indicating that the eIF2 complex is functional in translation (63). Although N. crassa eIF2γ lacks the conserved RVxF motif (see Fig. S3A), we show that the N terminus of eIF2γ is important for P-eIF2α levels (Fig. 3A), in vitro dephosphorylation (Fig. 3B), and rhythmicity (Fig. 3C). Because the levels of eIF2γ are not clock-controlled (Fig. 4B), we suggest that the interaction between the eIF2γ and eIF2α in the eIF2 complex provides a platform for eIF2γ to deliver PPP-1 at night, when it is at peak levels under the control of the clock (Fig. 4A). Furthermore, our data support the possibility that interactions between PPP-1 and eIF2, including eIF2γ and eIF2α subunits, as well as CPC-3, may be localized to the ribosome (see Fig. S2C), although additional experiments are needed to confirm this possibility.

Disruption of P-eIF2α rhythms, either by deletion or mutation of CPC-3 kinase in N. crassa, impacts the rhythmic translation of alg-11, but not FRQ (31) or PPP-1 (see Fig. S6) protein rhythms, or overt developmental rhythms (31). These data support that under constant environmental conditions, circadian translational regulation by the rhythmic activity of eIF2α is gene specific, as opposed to a global translational response (31). In ppp-1RIP cells, the period of FRQ::LUC accumulation rhythms is shorter compared to WT cells (47) (Fig. 2E). However, the short period FRQ::LUC rhythm in ppp-1RIP is not due to loss of P-eIF2α rhythms in the mutant because disruption of P-eIF2α rhythms in eIF2γL2-62 cells did not significantly alter the period of FRQ::LUC rhythmicity (Fig. 3E).

eIF2α phosphorylation regulates protein production to enable the organism to quickly respond to environmental stresses, including amino acid starvation. The circadian clock provides an additional layer of regulation of eIF2α activity to control the rhythmic translation of specific target genes. While the mechanisms underlying this specificity are not known, these data support the idea that temporal control of eIF2α activity provides organisms, from fungi to mammals, the ability to respond and adapt to internal and environmental stimuli (64). Because mRNA translation requires significant cellular energy, clock control of translation may provide a mechanism to coordinate energy metabolism with translation to partition translation to the times of day when energy levels are high.

MATERIALS AND METHODS

N. crassa strains and growth conditions. N. crassa vegetative growth conditions, transformation and crossing protocols were as described previously (65). Strains generated for use in this study are described in the supplemental materials and methods (see Text S1) and are listed in Table S1 in the supplemental material. The primers used in the generation and validation strains are listed in Table S2.

Circadian time courses. Circadian time course experiments for Western blots were done as previously described (65). For constitutive expression of bar::Picu-1::ppp-1, cells were grown in Vogel’s medium containing 50 μM copper chelator bathocuproinedisulfonic acid (BCS, B1125; Sigma-Aldrich, St. Louis, MO) or 250 μM copper sulfate (CuSO4; C7631; Sigma-Aldrich) to control the expression of the tcu-1 promoter (48).

Protein extraction and Western blotting. Protein extraction, protein concentration, and Western blot analyses were performed as previously described (28). Briefly, tissue was ground in liquid nitrogen with a mortar and pestle, and suspended in extraction buffer containing 100 mM Tris pH 7.0, 1% sodium dodecyl sulfate (SDS), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1× aprotinin, 1× leupeptin hemisulfate salt, and 1× pepstatin A. Protein
concentration was determined by NanoDrop (Thermo Fisher Scientific, Wilmington, DE). Protein samples (100 μg) were separated on 10% SDS-PAGE gels and blotted to Immobilon-P nitrocellulose membranes (catalog no. IPVH0010; Millipore Sigma, Burlington, MA) according to standard methods.

The levels of P-eIF2α were detected using rabbit monoclonal anti-EIF2S1 (phospho S51) antibody (catalog no. ab32157; Abcam, Cambridge, UK) diluted 1:1,000 in 7.5% milk, 1× Tris-buffered saline (TBS), 0.1% Tween, and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (catalog no. 1706515; Bio-Rad, Hercules, CA) diluted 1:10,000. Total eIF2α levels were detected using rabbit polyclonal anti-EIF2S1 antibody (catalog no. 47508; Abcam) diluted 1:5,000, and anti-rabbit IgG HRP secondary antibody diluted 1:10,000. P-eIF2α was detected using a custom rabbit polyclonal anti-PPP-1 antibody (peptide EVRGSRPGKQVQLLC as antigen; GenScript, Piscataway, NJ) diluted 1:1,000 in 7.5% milk, 1× TBS, 0.1% Tween, and anti-mouse IgG HRP- secondary antibody diluted 1:10,000. PPP-1 was visualized by Coomassie blue stain and total eIF2α was detected using rabbit polyclonal anti-EIF2S1 antibody (catalog no. R960-25; Invitrogen, Carlsbad, CA) diluted 1:5,000 in 5% milk, 1× TBS, 0.03% Tween, and anti-rabbit IgG HRP secondary antibody diluted 1:10,000. PPP-1 was detected using a custom rabbit polyclonal anti-PPP-1 antibody (peptide EVRGSRPGKQVQLLC as antigen; GenScript, Piscataway, NJ) diluted 1:1,000 in 7.5% milk, 1× TBS, 0.1% Tween, and anti-rabbit IgG HRP secondary antibody diluted 1:10,000. Signals were detected using chemiluminescence SuperSignal West Pico substrate (catalog no. 34077; Thermo Fisher Scientific). Densitometry was performed using NIH ImageJ software (66) and normalized to protein loading using amido black-stained protein.

Expression and purification of PPP-1::His6 protein in E. coli. To validate the specificity of PPP-1 antibody, the ppp-1 ORF was amplified with the primers PPP-1::His6F and PPP-1::His6R containing restriction sites for Ndel and NotI using N. cressa cdNA as the template. The pET30b vector (Invitrogen) and PCR fragment were digested with Ndel and NotI restriction enzymes and then ligated with T7 ligase (NEB). The ligated plasmids were transformed to E. coli DH5α cells and screened by kanamycin resistance and restriction digestion to get an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible PPP-1::His6 fusion plasmid. The plasmid was transformed into E. coli BL21 cells and grown in 400 ml of Luria-Bertani medium at 37°C with shaking at 250 rpm to an optical density of 0.6. PPP-1::His6 expression was induced by adding 1 mM IPTG 1 h before protein extraction. PPP-1::His6 protein was purified with Ni-NTA column following published methods (67). PPP-1::His6 protein was visualized by Coomassie blue stain and Western blotting with PPP-1 antibody.

In vivo luciferase assays. Luciferase assays to examine bioluminescence rhythms arising from strains containing luciferase fusions were performed as previously described (28). Briefly, 5 μl of 1 × 10^5 conidia/ml were inoculated into 96-well microtiter plates containing 150 μl of 1 × Vogel’s salts, 0.03% arginine, 0.1 M quinic acid, 1.5% agar, and 25 μl firefly luciferin (LUNCA-300; Gold Biotechnology, St. Louis, MO) (pH 6). After inoculation, the microtiter plate was incubated at 30°C in constant light (LL) for 24 h and transferred to DD 25°C to obtain bioluminescence recordings using an EnVision Xcite Multilabel Reader (Perkin-Elmer Life Science, Boston, MA), with recordings taken every 90 min over at least 5 continuous days. Raw luciferase activity data were analyzed for period using BioDARE (68). Raw reads were normalized to the mean to graph the data.

Statistical analysis. Circadian time course data were examined using F tests of the fit of the data to a sine wave or a line, as previously described (65, 69). A Student’s t-test was used to determine significance in changes in the levels of P-eIF2α and PPP-1. Error bars in all graphs represent the standard errors of the mean (SEM) from at least three independent experiments.

In vitro dephosphorylation assay. The eIF2 complex was isolated by anti-V5 coimmunoprecipitation from an eIF2α::V5 and eIF2α-D2-62::V5 protein extracts. The eIF2 complex was immobilized onto magnetic Dynabeads (catalog no. 10008D; Invitrogen) and washed with 2× phosphorylase buffer (100 mM HEPES, 200 mM NaCl, 2 mM dithiothreitol, 2 mM MnCl₂, 0.01% Brij-35) (45). Then, 500 μl of protein extracted from Δαpc-3 or ppp-1ΔΔ, Δαpc-3 or eIF2α-D2-62Δ, Δαpc-3 strains, harvested at DD28, was mixed with 200 μl of the immobilized eIF2-Dynabeads in 2× phosphorylase buffer. Reaction mixtures were incubated at 30°C with gentle rotation, and at each time point 48 μl of the reaction mix was transferred to a fresh tube and boiled for 5 min with 16 μl of 4× SDS loading buffer (250 mM [pH 6.8] Tris-Cl, 8% SDS, 0.2% bromophenol blue, 40% glycerol, 20% β-mercaptoethanol) to stop the reaction. P-eIF2α and total eIF2α levels were detected by Western blotting.

Sucrose gradient fractionation. Linear sucrose gradients (10 to 50% in 10 mM HEPES-KOH, 70 mM ammonium acetate, 5 mM magnesium acetate) were prepared in ultracentrifuge tubes by using a BIOCAMP gradient station (Fredericton, NB, Canada) and stored at 4°C before use. Extracts were prepared by adding polysome extraction buffer (100 mM KCl, 20 mM HEPES-KOH, 10 mM magnesium acetate, 15 mM β-mercaptoethanol, 100 μg/ml cycloheximide) to ground tissues and centrifuging the solution to remove cellular debris and lipids. Next, 400 μl of the extract containing 100 A₂₆₀ units/ml (1 A₂₆₀ unit corresponds to an absorbance of 1.0 at 260 nm) was added onto the sucrose gradient and centrifuged at 41,000 rpm for 2 h at 4°C. The samples were then divided into 14 fractions of approximately 1 ml each using the BIOCAMP. The absorbances at 260 nm were used as a proxy for RNA content and graphed against the fraction of the gradient. Disome, trisome, tetrosome, and pentasome fractions were pooled as the polysome fraction. Fractions representing the 40S (Δ45), 60S (Δ55), 80S (Δ65) ribosome and the pooled polysome fraction were boiled in SDS loading buffer (250 mM [pH 6.8] Tris-Cl, 8% SDS, 0.2% bromophenol blue, 40% glycerol, 20% β-mercaptoethanol), and 15 μl was separated on a 10% SDS-PAGE gel for Western blotting.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOXC file, 0.03 MB.

FIG S1, JPG file, 0.3 MB.
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