Regulation of the *Neurospora* Circadian Clock by the Spliceosome Component PRP5

Huan Ma,*,† Lin Zhang,*,† Xinyang Yu,*,† Yufeng Wan,*, Dongni Wang,*, Weirui Shi,† Meiyan Huang,*, Manhao Xu,*, Enze Shen,*, Menghan Gao,* and Jinhu Guo*,2

*State Key Laboratory of Biocontrol, Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China, †Zhuhai Interventional Medical Center, Zhuhai Precision Medical Center, Zhuhai People’s Hospital, Zhuhai Hospital Affiliated with Jinan University, Zhuhai, Guangdong 519000, China, and ‡School of Life Sciences, The Chinese University of Hong Kong, Hong Kong 999077, China

ORCID IDs: 0000-0002-5155-675X (Y.W.); 0000-0003-4405-3959 (J.G.)

ABSTRACT Increasing evidence has pointed to the connection between pre-mRNA splicing and the circadian clock; however, the underlying mechanisms of this connection remain largely elusive. In the filamentous fungus *Neurospora crassa*, the core circadian clock elements comprise White Collar 1 (WC-1), WC-2 and FREQUENCY (FRQ), which form a negative feedback loop to control the circadian rhythms of gene expression and physiological processes. Previously, we have shown that in *Neurospora*, the pre-mRNA splicing factors Pre-mRNA-processing ATP-dependent RNA helicase 5 (PRP5), protein arginine methyl transferase 5 (PRMT5) and snRNA gene U4-2 are involved in the regulation of splicing of *frq* transcripts, which encode the negative component of the circadian clock system. In this work we further demonstrated that repression of spliceosomal component sRNA genes, U5, U4-1, and prp5, affected the circadian conidiation rhythms. In a prp5 knockdown strain, the molecular rhythmicity was dampened. The expression of a set of snRNP genes including prp5 was up-regulated in a mutant strain lacking the clock component wc-2, suggesting that the function of spliceosome might be under the circadian control. Among these snRNP genes, the levels of prp5 RNA and PRP5 protein oscillated. The distribution of PRP5 in cytosol was rhythmic, suggesting a dynamic assembly of PRP5 in the spliceosome complex in a circadian fashion. Silencing of prp5 caused changes in the transcription and splicing of NCU09649, a clock-controlled gene. Moreover, in the clock mutant *frq*, the rhythmicity of frq I-6 splicing was abolished. These data shed new lights on the regulation of circadian clock by the pre-RNA splicing, and PRP5 may link the circadian clock and pre-RNA splicing events through mediating the assembly and function of the spliceosome complex.

KEYWORDS circadian clock *Neurospora crassa* PRP5 spliceosome alternative splicing

Most organisms possess circadian clocks to orchestrate their daily metabolic, physiologic and behavioral rhythmicities (Bell-Pedersen et al. 1996). In eukaryotes, circadian clocks are controlled by positive and negative components that constitute transcriptional-translational negative feedback loops (Bell-Pedersen et al. 1996). In recent decades, regulatory layers acting on circadian clock networks, including post-transcriptional, post-translational and epigenetic regulation, have been identified. All of these regulations are necessary for the coordination of appropriate circadian clock functions (Bell-Pedersen et al. 1996; Gallego and Virshup 2007; Vanselow and Kramer 2007; Gibois et al. 2010; Sanchez et al. 2010; Durgan et al. 2011; Koijima et al. 2011; Staiger and Green. 2011; Staiger and Köster 2011; Kusakina and Dodd 2012; Wang et al. 2013).

The filamentous fungus *Neurospora crassa* is an important model for circadian research. In the *Neurospora* circadian clock, WC-1 and WC-2 are two PAS (PER-ARNT-SIM) domain-containing proteins that form the White Collar Complex (WCC). WCC binds to the promoter of the frequency (*frq*) gene and consequently activates its transcription. As a negative element, FRQ forms the FRQ-FRH
complex (FFC) with FRH (FRQ-interacting RNA helicase) which inhibits the function of WCC. The inhibition of WCC by FRQ is relieved by darkened phosphorylation and degradation of FRQ. These positive and negative components constitute the transcription-translational negative feedback loop (TTFL) (Baker et al. 2012).

FRQ proteins contain small FRQ (s-FRQ) or large FRQ (l-FRQ) isoforms which are produced through the alternative splicing of intron 6 (1-6) of the frq pre-mRNA. The proportion of s-FRQ to l-FRQ is critical for the function of the clock (Liu et al. 1997). Higher temperatures induce more expression of l-FRQ and repress the expression of s-FRQ (Liu et al. 1997; Garceau et al. 1997; Colot et al. 2005; Diernfellner et al. 2005; Brunner and Diernfellner 2006). S-FRQ supports a longer circadian period and l-FRQ supports a shorter one (Liu et al. 1997; Brunner and Diernfellner 2006). FRQ isoforms also display differences in nucleocytoplasmic shuttling, in which l-FRQ accumulates in the nucleus (Cha et al. 2014). Recently, it has been shown that the catalytic subunit of exosome complex, RRP44, regulates the splicing of frq in addition to its mediating frq mRNA decay (Guo et al. 2009; Zhang et al. 2015). In Neurospora, the core nonsense-mediated RNA decay (NMD) factor - UPF1 - is also involved in controlling the splicing of frq-16 (Wu et al. 2017).

A growing body of evidence suggests that alternative splicing plays a critical role in the regulation of circadian clocks in multiple species (Smith et al. 1989; Liu et al. 1997; Liu et al. 1998; Majercak et al. 2004; Colot et al. 2005; Diernfellner et al. 2005; Brunner and Diernfellner 2006; Hong et al. 2010; James et al. 2012a; James et al. 2012b; Jones et al. 2012; Koike et al. 2012; Low et al. 2012; McGlincy et al. 2012). The splicing regulation of clock genes or clock-controlled genes has been reported in various organisms, including mammals (Koike et al. 2012; Na et al. 2012), insects (Majercak et al. 2004; Sanchez et al. 2010) and plants (Sanchez et al. 2010; Hong et al. 2010; Jones et al. 2012; Wang et al. 2013; Schlaen et al. 2015). Most of the reported regulators are either spliceosome components or spliceosome-associated factors. The regulation of splicing acts as a critical layer on top of the basic negative feedback loop of the circadian clock.

Though connections between the circadian clock and pre-mRNA splicing have been investigated, the mechanism underlying the regulation of the spliceosome by the circadian clock remains largely unknown. PRP5 is a DExD/H-box containing RNA-dependent ATPase required for the formation of pre-spliceosome during the nuclear pre-mRNA splicing (Kosowska et al. 2009). PRP5 has been previously shown to mediate the splicing of frq-16 (Zhang et al. 2015). We report that PRP5 is controlled by circadian clock, while in return also modulates the circadian oscillator and downstream alternative splicing events.

MATERIALS AND METHODS

Strains and growth conditions

The 301-5 (bd, a) strain was used as the wild type (WT) strain. The frqΔ strain bears a frameshift mutation in the frq ORF (Aronson et al. 1994), and the frq gene is deleted in the frq10 strain (Aronson et al. 1994). The 301-6-6 strain (bd, his-3, A) was used as the host strain for his-3 targeting constructs. Liquid cultures were incubated in minimal medium (1 × Vogel’s, 2% glucose). When quinic acid (QA) was used, liquid cultures were grown in 0.01 M or indicated concentrations of QA (pH 5.8), 1 × Vogel’s, 0.1% glucose, and 0.17% arginine. The race tube medium contained 1 × Vogel’s, 0.1% glucose (0% when QA was used), 0.17% arginine, 50 ng/mL biotin, and 1.5% agar.

To generate these knockout (KO) strains, the entire coding sequences of snRNA genes U5 and U4-1 were deleted by replacement with the hph gene (Colot et al. 2006). The Neurospora crassa unit (NCU) numbers of U5 and U4-1 are NCU02572 and NCU09547, respectively. The gene replacement cassette harboring hph was transformed into the bd, ku70RIP strain.

The dsprp5 strains were generated by introducing plasmids expressing RNA hairpins that were complementary to the gene to be inhibited into the WT strain 301-6-6 (Cheng et al. 2005), and this strain has been previously described (Zhang et al. 2015). The following primers containing specific restriction enzyme sites were used to generate the construct expressing RNA hairpins: forward: 5’-caggattcgcagttgaggatgattcag-3’; reverse: 5’-ataaatcttcgcgttgcgacctggagt-3’. The hairpin sequence in the amplified products was complementary to approximately 500 bp of the gene of interest downstream of the qa-2 promoter. The resulting plasmids were targeted to the his-3 locus by transformation into 301-6-6 (bd, his-3, A). Addition of QA induces the repression of prp5 expression in the dsprp5 strain.

The information of other primers used in this work is available in the supplemental primer list.

Luciferase assay for circadian rhythms

The bar-frq-luc-1 plasmid was transformed into the 301-5 (WT) and dsprp5 strains to monitor the real-time fluctuation of luciferase signal. Obtained transformants were screened using basta/ignite (200 µg/mL) resistance conferred by the bar gene (Gooch et al. 2008). To observe fluorescence the strains were inoculated on AFV (autoclaved FGS-Vogel’s medium that contained 1×FGS (0.05% fructose, 0.05% glucose, 2% sorbose), 1 × Vogel’s medium, 50 µg/L biotin, and 1.8% agar. Firefly luciferin (BioSytnt L-8200 D-luciferin firefly (synthetic) potassium salt) was added to the medium after autoclaving (final concentration of 50 µM). A LumicCycle high-throughput luminometer (Actimetrics, USA) was used for the luciferase assay as described previously (Gooch et al. 2008; Zhou et al. 2013).

RNA and protein analyses

For reverse transcription PCR (qRT-PCR) analysis, the total RNA samples were isolated and treated with RNase-Free DNase I (NEB, USA) and subjected to reverse transcription using M-MLV (Invitrogen, USA) and random primers. The PCR products were resolved on a 1% agarose gel. The information of primers used in this work is available in the supplemental primer list.

For quantitative reverse transcription PCR (qRT-PCR) analysis, the total RNA samples were isolated and treated in same way described above. The obtained cDNAs were amplified with SYBR Green Master Mix (Takara, Japan) using a LightCycler 480 (Roche, Germany).

Protein extraction, western blot analysis and immunoprecipitation assays were performed as previously described (Garceau et al. 1997). Equal amounts of total protein (40 µg) were loaded in each lane of an SDS-PAGE gel (7.5%, containing a ratio of 37.5:1 acrylamide/bisacrylamide). Diphosphorylation of the FRQ protein was achieved by λ-phosphatase treatment.

Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (Cao et al. 2018). The immunoprecipitation was performed with a WC-2 antibody. Each experiment was independently performed three times, and immunoprecipitation without the WC-2 antibody or with the wc-2KO extract was used as the negative control. The following primers were used in ChIP assay: forward, 5’-tgatcagctggagaagctggt-3’; reverse, 5’-ccagcttaggtaaactg-3’.

Sucrose fractionation analysis

Sucrose density gradients (10–30%) were prepared and 4 mg of total protein samples were loaded for each analysis. The gradients were
centrifuged at 175,000 x g for 18 h in a SW-40 rotor at 4°C. Twelve equal fractions were collected and 450 μl of each fraction was used for RNA analysis. The samples were treated with DNase I prior to the RT-PCR to determine the levels of U5. Western blot analysis was also used to determine the distribution of PRP5 (Wu et al. 2017).

Statistical analysis
Statistical significance was calculated using Student’s t-test. The values presented are the mean ± SD or SE as denoted. Significance values are *P < 0.05, **P < 0.01 and ***P < 0.001.

Data availability
The RNA sequencing data of Neurospora WT strain in constant darkness for 12 hr (DD12) and 20 hr (DD20) were deposited at Gene Expression Omnibus (GSE117118). Supplemental protocol for RNA-seq analysis and Tables S1-S5 are available at FigShare: https://doi.org/10.25387/g3.9790751.

RESULTS
Regulation of the circadian clock by PRP5
Spliceosome is one of the largest cellular complexes and comprises small nuclear ribonucleic acids (snRNAs), small nuclear ribonucleoprotein particles (snRNPs) and an additional group of non-snRNP proteins. Neurospora possesses 15 snRNA genes (belonging to the U1, U2, U5, and U4/U6 species) in total (Wan et al. 2015). To determine the effects of the spliceosomal components on the circadian clock, knockout strains of the snRNA genes U5 (U5KO) and U4-1 (U4-1KO) were created and validated (Figure 1A&B). These two knockout strains are heterokaryon that failed to cross and generate homokaryotic progeny, suggesting that appropriate pre-mRNA splicing is essential for sexual reproduction. To analyze the effects of the U4-1KO and U5KO heterokaryotic strains on the circadian rhythms, these two strains were inoculated inside and at one end of long glass tubes called race tubes. In a race tube assay, Neurospora grows toward the other end of the tube on a layer of solid media. During growth, Neurospora releases asexual conidia, and the circadian periods can be calculated by analyzing the interval time between the conidiation bands (Baker et al. 2012). From the race tube results, both U4-1KO and U5KO heterokaryotic strains exhibited a slight but significant decrease in their conidiation period lengths (Figure 1C-F).

Previously we generated a knockdown strain of the prp5 gene, which has been named dsprp5 (Zhang et al. 2015). We have obtained two transformants of dsprp5, which are named dsprp5#1 and dsprp5#2, and the dsprp5#1 strain has been previously described (Zhang et al. 2015). In these two transformants, QA induces the silencing of prp5 expression. Both of the dsprp5 strains display much slower growth rate compared to WT even without QA, and the presence of 0.01M QA resulted in a more dramatic decrease in growth and a reduction of aerial hyphae and conidia, which might...
be owing to a leakage effect of the qa promoter leakage. Despite the conferred growth, dsprp5 exhibited conidiation rhythms in the absence of QA, with a period shorter compared to that in WT strain. By contrast, the conidiation rhythms of dsprp5 were abolished in the presence of QA (Figure 1G-I), suggesting that knockdown of prp5 leads to influence on the circadian clock. We used dsprp5#1 for the following studies as it shows slighter leakage effect. Previously the repression of prp5 RNA in this strain was verified by northern blot, and here, we further validated them by western blot with PRP5 antiserum (Figure 1J).

In dsprp5 strain, alterations in the expression of the clock genes frq, wc-1 and wc-2 in constant light were observed. The RNA levels of frq and wc-2 were decreased while wc-1 increased in dsprp5. The changes in protein levels of these three genes were consistent with the RNA data (Figure 2A&B). These data suggest that the negative feedback loop of the circadian clock might be extensively affected upon prp5 knockdown. We next examined the expression of the frq mRNAs and FRQ proteins in constant dark (DD) for 48 hr, by qRT-PCR and western blot analysis, respectively. The qRT-PCR results showed that the frq mRNA levels oscillated and the period was ~2 h shorter in dsprp5 than that in dscontrol (Figure 2C). The western blot analysis revealed that FRQ proteins showed a peak at DD20 but the second peak was dampened (Figure 2D). The results of both frq RNA and FRQ protein showed that the phase of rising up on the first day after transition from LL to DD was advanced in the dsprp5 strain (Figure 2C, D). We next introduced a luciferase reporter construct under the control of the frq promoter into the WT and dsprp5 strains, to allow us to observe the molecular rhythms for a longer time, and the results showed that the rhythmicity of luciferase activity was severely dampened in the dsprp5 which disappeared within several days (Figure 2E). These results demonstrate that the spliceosome plays an important role in maintaining the robust circadian rhythms.

![Figure 2](image_url)
Circadian control of PRP5 gene expression

To assess whether the expression of spliceosomal genes is affected by the circadian clock, we compared the expression of spliceosomal genes in the *wc-2* knockout strain (*wc-2KO*) using qRT-PCR. The expression of all of the tested snRNA genes showed no significant changes, while the expression of most snRNP genes was increased, with the exception of that of *prp8*, *prp46*, *prp3* and *snu66* (Figure 3A, B). Despite the relatively low levels, the overall increase suggests that the gene expression and function of spliceosome components may be under the circadian control.

Sequence analysis indicated the existence of a putative C-box in the promoter region of *prp5* (Figure 3C). Flanking primers were synthesized, and a chromatin immunoprecipitation (ChIP) assay was conducted, and the results showed that WCC bound specifically to the C-box-like element in the *prp5* promoter and this binding might peak around DD14 (Figure 3C, D). These data suggest that the expression of PRP5 might be controlled by the circadian clock.

We next investigated *prp5* expression under constant dark conditions for 48 h. The results of the RT-PCR analyses showed that *prp5* RNA levels exhibited low but significant circadian rhythmicity in the WT strain. By contrast, the circadian rhythmicity of *prp5* RNA was abolished in the *frq9* strain (Figure 3E, F). In consistence, the western blot results using a PRP5 antibody demonstrated that the PRP5 levels oscillated with a period of approximately 24 h under constant dark in the WT strain but not in the *frq10* strain, in which the ORF region of *frq* gene was deleted (Figure 3G, H). These data confirmed the clock-controlled expression of *prp5* RNA and PRP5 protein.

**The circadian clock controls PRP5 assembly**

We next conducted sucrose fractionation assays to examine whether the circadian clock controls the assembly of PRP5 in the spliceosome complex, which can be reflected by the changes in PRP5 distribution in fractionated samples (Wu et al. 2017). We performed sucrose sedimentation assays in triplicate and compared the distribution of PRP5 in the sucrose gradient fractions as a function of time under constant dark conditions. A periodicity of approximately 24 h was observed in the WT strain while it was arrhythmic in *frq10* (Figure 4A, B). These data suggest that circadian clock governs the assembly of PRP5 in the spliceosome complex.
Regulation of the splicing rhythm by the spliceosome and clock

To identify the downstream genes whose splice variant proportions are controlled by the circadian clock, RNA-sequencing (RNA-seq) and bioinformatic analyses were carried out. The duplicate RNA samples from WT grown in constant darkness for 12 hr (DD12) and 20 hr (DD20) were used to generate the mRNA-seq library and RNA, each of which comprised equally pooled three independent samples (GSE117118). From this analysis, we identified hundreds of sites that were differentially spliced when DD12 and DD20 were compared (Supplemental Protocol and Tables S1-S5). We further conducted qRT-PCR in about thirty splicing sites in three set of samples harvested at DD12 to DD42 in increments of 6 h, however, only a few of which were confirmed to oscillate. This inconsistency suggests the differences between two DD12 and DD20 mostly represent non-circadian fluctuations. Among these genes, NCU09649 encodes a putative metallophosphoesterase that contains only one intron in its 5' UTR region (Figure 5A). We investigated the splicing of NCU09649 in the WT and dsprp5 strains under constant dark at DD12 through DD42. The RT-PCR results showed that both the unspliced and spliced species of NCU09649 oscillated in WT but not in dsprp5 (Figure 5B&C). Compared to the WT, the rhythmicity of the spliced transcripts of NCU09649 was significantly dampened in dsprp5. In addition, in the dsprp5 strain, the levels of spliced species were significantly decreased (Figure 5B, C), suggesting a role of PRP5 in the regulation of NCU09649 splicing.

In the Neurospora circadian clock, seven alternative splice variants of the core clock gene frq are observed (Diernfellner et al. 2005). At the protein level, these variants yield two FRQ isoforms, s-FRQ and l-FRQ, depending on exclusion or inclusion of frq I-6, respectively. The frq open reading frame (ORF) has three putative initiation codons (AUG), of which only the first and third function in the initiation of translation (Liu et al. 1998; Colot et al. 2005). The protein product that is translated from the first initiation codon is l-FRQ, whereas s-FRQ is translated from the third initiation codon and lacks 99 amino acid residues from its N-terminus. Splicing of frq I-6 removes the first initiation codon so that the frq mRNA lacking I-6 is translated into s-FRQ (Liu et al. 1998; Colot et al. 2005; Diernfellner et al. 2005; Neiss et al. 2008). We have previously reported that knockdown of prp5 represses the splicing of frq I-6, suggesting that alternative splicing in part explains the dysregulation of the circadian clock in the dsprp5 strain (Zhang et al. 2015).

Diernfellner et al. showed that splicing of frq I-6 displayed a rhythm under DD, suggesting that splicing of frq I-6 is under circadian control (Diernfellner et al. 2007). Here we further measured the levels of the spliced transcript variants in the WT and frq10 strain, which bears a
frame-shift mutation in the frq ORF and produces a truncated protein product with no circadian function (Aronson et al. 1994). RT-PCR using primers flanking frqI-6 was carried out to examine the expression of frq with spliced or unspliced I-6 under DD, in the WT and frq9 strains (Figure 5D). Considering that the oscillation at the transcriptional level might mask the analysis of splicing rhythmicity, we calculated the ratio of spliced isoforms vs. unspliced isoform. The results showed that the ratio of spliced/unspliced transcripts oscillated in WT which is consistent with the previous observation (Diernfellner et al. 2007). In contrast, both the unspliced and spliced species showed no overt rhythms in frq9 (Figure 5D).

**DISCUSSION**

In eukaryotes, the regulation of alternative splicing plays a critical role in regulating the normal rhythms of the circadian clock. The rhythmicity of spliced variants can be attributed to rhythmic transcription, rhythmic splicing or a combination of both (Koike et al. 2012; Partch et al. 2014; Lipton et al. 2015).

PRP5 is an RNA-dependent ATPase present in the commitment complex, which regulates pre-spliceosome formation and the release of spliced mRNA from the spliceosome. During splicing, Prp5p recruits U2 snRNP to pre-mRNA and hydrolyses ATP to stabilize the association of U2 in the pre-spliceosome in *Saccharomyces cerevisiae* (Kosowski et al. 2009). In this work, we revealed that PRP5 regulates the circadian rhythms of *Neurospora* may play a role in linking the circadian clock and downstream splicing events. Furthermore, some other spliceosomal factors, such as snRNA U4-1 and U5, have also been implicated in the regulation of circadian clock. Repression of PRP5 and other spliceosome components resulted in differential influences on frq premRNA splicing. For instance, knockdown of prp5 and U4-2 results in decreased frqI-6 splicing while knockdown of prmt5 results in an increase in frqI-6 splicing (Zhang et al. 2015). For NCU09649, knockdown of prp5 also led to decreased levels of spliced transcripts (Figure 3B), suggesting that PRP5 and U4-2 act to promote spicing, while PRMT5 represses splicing.

Both the expression patterns of prp5 RNA and PRP5 protein exhibited circadian rhythmicity (Figure 3E-H), moreover, we showed that the assembly of PRP5 in the spliceosome complex was governed by circadian clock (Figure 4). Taken together with its important role in pre-spliceosome formation (Kosowski et al. 2009), these findings suggest that circadian clock may regulate the composition and function of spliceosome and a set of splicing events as consequence.

FRQ is the core circadian regulator in *Neurospora*, and alternative splicing of frqI-6 exhibited overt circadian rhythms (Figure 5D), in agreement with the previous findings (Diernfellner et al. 2007; Zhang et al. 2015), these data confirm that the splicing of frqI-6 is rhythmically governed by the circadian clock.
As for NCU09649, the levels of both unspliced and spliced transcripts oscillated (Figure 5B, C), but the ratio of the spliced vs. unspliced species showed no overt rhythmicity, suggesting that both the transcription and splicing of NCU09649 are under control of the circadian clock. Repression of prp5 led to altered splicing patterns, suggesting that PRP5 plays an important role in mediating the pre-RNA splicing of NCU09649. Together, these findings suggest that PRP5 may bridge the circadian clock and alternative splicing through regulating the spliceosome function (Figure 5E).

The circadian period of the strain exclusively expressing l-FRQ is shorter compared to the WT strain which expresses l-FRQ and s-FRQ simultaneously while the strain exclusively expressing s-FRQ possesses a longer period (Liu et al. 1997). In this work, the heterokaryon knockout strains of U4-1 and U5 showed shorter circadian periods, which might be due to less splicing of frq I-6. Consistently, in absence of QA, dsprp5 displayed a shorter period (Figure 1G,H). The ratio of frq transcripts containing I-6 is significantly increased in dsprp5 strain (Zhang et al. 2015), however, the molecular rhythms dsprp5 was too dampened to calculate the period (Figure 2E). These data suggest that in addition to frq I-6 splicing, other unknown regulators, which are likely potential PRP5 targets, might be involved in determining the abnormal periodicity (Figure 5E). Decoupling between different regulatory layers might occur in the control of circadian clock, for instance, the fwd-1 null strain showed robust rhythms at the transcriptional level of frq but not the FRQ protein level (Larrondo et al. 2015). Though the conidiation rhythms and clock gene expression were affected in dsprp5 strain, at the molecular level, it still showed rhythmicities revealed by frq/FRQ expression and luciferase reporter assay (Figure 1 and Figure 2), suggesting that decoupling might occur between the flow from circadian oscillator to the output.

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