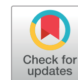

Circadian clock regulation of the glycogen synthase (*gsn*) gene by WCC is critical for rhythmic glycogen metabolism in *Neurospora crassa*

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Circadian clocks generate rhythms in cellular functions, including metabolism, to align biological processes with the 24-hour environment. Disruption of this alignment by shift work alters glucose homeostasis. Glucose homeostasis depends on signaling and allosteric control; however, the molecular mechanisms linking the clock to glucose homeostasis remain largely unknown. We investigated the molecular links between the clock and glycogen metabolism, a conserved glucose homeostatic process, in *Neurospora crassa*. We find that glycogen synthase (*gsn*) mRNA, glycogen phosphorylase (*gpn*) mRNA, and glycogen levels, accumulate with a daily rhythm controlled by the circadian clock. Because the synthase and phosphorylase are critical to homeostasis, their roles in generating glycogen rhythms were investigated. We demonstrate that while *gsn* was necessary for glycogen production, constitutive *gsn* expression resulted in high and arrhythmic glycogen levels, and deletion of *gpn* abolished *gsn* mRNA rhythms and rhythmic glycogen accumulation. Furthermore, we show that *gsn* promoter activity is rhythmic and is directly controlled by core clock component white collar complex (WCC). We also discovered that WCC-regulated transcription factors, VOS-1 and CSP-1, modulate the phase and amplitude of rhythmic *gsn* mRNA, and these changes are similarly reflected in glycogen oscillations. Together, these data indicate the importance of clock-regulated *gsn* transcription over signaling or allosteric control of glycogen rhythms, a mechanism that is potentially conserved in mammals and critical to metabolic homeostasis.

circadian rhythms | *Neurospora crassa* | glycogen metabolism | glycogen synthase | glycogen phosphorylase

Most organisms possess an endogenous circadian clock mechanism that, through the regulation of gene expression, generates self-sustained rhythms in biological processes. These clocks are reset each day to synchronize to 24-h environmental cycles of light–dark and temperature. In addition, clocks present in organs involved in metabolism, including the liver, pancreas, muscle, and adipose tissue, can be reset by feeding cues (1, 2), allowing the integration of nutritional signals with the clock to maintain metabolic homeostasis throughout the organism. Consequently, misalignment between feeding cycles and the endogenous clock, or through circadian disruption, leads to metabolic imbalance that promotes increased body weight, insulin resistance, as well as liver and cardiovascular disease (3–6). Despite the importance of the clock in metabolic homeostasis, the molecular mechanisms connecting the clock and nutritional signals to metabolic homeostasis are not fully understood. To gain insights into this mechanism, we investigated molecular links between the clock and glycogen metabolism in the model filamentous fungus, *Neurospora crassa*.

Glycogen, a branched polymer of glucose residues, is a major form of carbon and energy storage in evolutionarily diverse organisms and is utilized in times of nutritional deprivation (7, 8). For example, in the mammalian liver, glycogen can be broken down to yield glucose to maintain blood glucose levels during the daily cycle of fasting (9). Yeast cells that accumulate glycogen stores display a growth advantage over cells that cannot, indicating a key role for glycogen in overall fitness (10). Glycogen concentration is controlled by the activities of two opposing enzymes, glycogen synthase (GS) and glycogen phosphorylase (GP). GS, which utilizes UDP-glucose, catalyzes the addition of glucose residues via α1,4-linkages to the glycogen chain initiated by glycogenin, and branching enzyme introduces branch points via α1,6-linkages. GS activity is inhibited by phosphorylation, but this regulation can be overcome by the allosteric activator glucose 6-phosphate (8). GP, along with the debranching enzyme, breaks down glycogen to release glucose-1-phosphate from α1,4-linkages, and free glucose from α1,6-linkages (7). Similar to GS, GP is controlled by allosterism and reversible phosphorylation. In yeast cells, expression of the genes encoding GS, GP, and the branching and debranching enzymes are coordinately controlled by the Protein Kinase A (PKA) pathway (10), and *N. crassa* GSN was shown to be regulated by PKA (11).

Previous studies revealed that GS and GP activity cycles under control of the circadian clock in mouse liver, and that glycogen levels peak near the end of the active phase (12–14). Furthermore,

Significance

Circadian rhythms enable organisms to anticipate daily environmental cycles and control the timing of numerous biological processes, including metabolism, to optimize the health and survival of organisms. Glycogen metabolism is a conserved glucose homeostatic process; however, the molecular mechanisms linking the circadian clock and glycogen metabolism remain largely unknown. In this report, we demonstrate that circadian clock-dependent transcriptional regulation of glycogen synthase, *gsn*, regulates circadian oscillations of GSN protein and glycogen accumulation in the model filamentous fungus, *Neurospora crassa*.

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the core clock component and transcriptional activator CLOCK in mice directly binds to the promoter of hepatic *Gys2* encoding Glycogen Synthase 2 and drives its rhythmic expression (15). However, how the clock regulates the levels and activity of GS and/or GP, necessary for glucose homeostasis, remain largely unknown.

The well-studied circadian clock in *N. crassa* is composed of the FRQ/WCC (white collar complex) circadian oscillator, which forms a characteristic negative feedback loop that generates daily rhythms. In the FRQ/WCC oscillator, two PAS domain-containing GATA-type zinc finger transcription factors (TFs), White Collar 1 (WC-1) and White Collar 2 (WC-2) dimerize to form the White Collar Complex (WCC) (16–18). WCC functions as a positive element in the oscillator and activates transcription of the *frequency* (*frq*) gene (19–21). The negative component FRQ accumulates, enters the nucleus, interacts with FRQ-interacting RNA helicase (FRH) (22, 23) and CK1 (24), and inhibits the WCC (25–28). Progressive phosphorylation of FRQ relieves WCC inhibition, reinitiates the cycle, and leads to proteasome-dependent degradation of FRQ (29, 30). WC-1 is also a blue light photoreceptor (19, 31), and with its partner WC-2, functions to regulate light-responsive genes, as well as downstream clock-controlled genes (*ccgs*) (32–34). ChIP-seq in cells given a short light pulse to activate the WCC revealed that WCC binding occurs at the promoters of ∼200 genes, and TFs were enriched among these direct WCC targets, including CSP-1 and VOS-1 (34). *N. crassa* VOS-1 is the homolog of *Aspergillus nidulans* VosA involved in the control of development, metabolism, and stress responses (35, 36). CSP-1 functions primarily as a repressor to control the expression of ∼800 genes, including *wc-1* (37). Of the CSP-1 targets, ∼200 genes are involved in metabolism, and deletion of *csp-1* (Δ*csp-1*) results in the loss of circadian time-dependent membrane lipid synthesis (37). Furthermore, CSP-1 differentially regulates the expression of *wc-1* depending on glucose concentration to maintain the circadian period over a range of glucose concentrations, a process referred to as nutritional compensation (38, 39).

In this study, we show that glycogen accumulation, and *gsn* and *gpn* mRNA levels, are clock controlled. In addition, we provide several lines of evidence to support that rhythms in *gsn* mRNA levels are necessary for the rhythmic accumulation of glycogen. Rhythmic expression of *gsn* is accomplished by rhythmic binding of the WCC to the promoter of *gsn*. In addition, the WCC-controlled TF, VOS-1, cooperates with WCC and CSP-1 to modulate the amplitude and phase of the glycogen oscillation by regulating *gsn* rhythmicity.

Results

The *N. crassa* Circadian Clock Regulates Rhythmic Expression of Glycogen Metabolic Genes and Glycogen Abundance. To determine if glycogen levels are regulated by the circadian clock, WT and arrhythmic clock mutant (Δ*frq*) strains were cultured in constant darkness (DD), conditions in which the clock mechanism free runs with an endogenous ∼22.5-h period. Daily rhythms of glycogen abundance were observed in WT cells, with a peak during subjective night (DD32) (Fig. 1*A* and *SI Appendix*, Table S1). In contrast, glycogen levels were low and arrhythmic in Δ*frq* cells, compared with WT (Fig. 1 *A* and *B*), demonstrating circadian clock control of glycogen abundance. Consistent with rhythmic glycogen levels, both *gsn* and *gpn* mRNA levels cycled in WT (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S1*A* and Table S1), but not in Δ*frq* cells (Fig. 1 *C* and *D* and *SI Appendix*, Table S1), peaking at subjective dawn (DD12 and DD36). Similar results were obtained using strains containing the *gsn* or *gpn* promoters fused to the luciferase reporter. Both P*gsn-luc* and P*gpn-luc* levels cycled with a daily rhythm in WT, but not in Δ*frq* cells (*SI Appendix*, Fig. S1*B* and Table S1), demonstrating that *gsn* and *gpn* promoter activity, rather than mRNA turnover, are controlled by the circadian clock.

GSN and GPN have opposing activities in glycogen metabolism, synthesis versus breakdown, respectively. Therefore, it was somewhat surprising to find that *gsn* and *gpn* mRNA levels peaked

Fig. 1. The circadian clock regulates *gsn* and *gpn* mRNA and protein levels and rhythmic glycogen accumulation. (*A*) Plot of glycogen levels from WT (black line) and Δ*frq* cells (gray line) ($n \geq 3$, ±SEM). Rhythmicity was determined using *F* tests of fit of the data to a sine wave and is represented as a dotted line (WT black dotted line, $P < 0.001$). In Δ*frq* cells, rhythmicity was abolished as indicated by a better fit of the data to a line (dotted gray line). (*B*) The average glycogen content from all 12 time points in WT vs. the indicated strains ($n \geq 4$, ±SEM, Student's *t* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*C* and *D*) *gsn* and *gpn* RNA levels from WT and Δ*frq* cells harvested at the indicated times in DD (solid black lines). Rhythmicity was determined as described above in *A*. In WT cells, *gsn* and *gpn* data were best fit to a sine wave ($P < 0.01$; $n = 2$). In Δ*frq* cells ($n = 2$), rhythmicity was abolished as indicated by a better fit of the data to a line (dotted black lines). 28S rRNA was used as internal loading control. See *SI Appendix*, Fig. S1*A* for a representative Northern blot. (*E* and *F*) Representative Western blots of GSN-V5 and GPN-V5 from cells harvested at the indicated times in DD. Amido black staining of the membrane was used to normalize protein loading. The data are plotted on the bottom ($n = 3$, ±SEM), and fit to a sine wave (dotted line) as described above ($P < 0.002$). The shading in the plots, here and throughout the subsequent figures, represent subjective day (gray) and night (black), with the start of the subjective day representing circadian time (CT) 0, and the start of the subjective night representing CT12 as indicated in *A*. The peak phase of the rhythms (CT) are provided in *SI Appendix*, Table S1.

at the same time of day. GSN and GPN are the rate-limiting enzymes for glycogen accumulation and breakdown, respectively, suggesting that levels and/or activity of GSN and/or GPN may determine the rhythmic accumulation of glycogen. To begin to test this idea, we tagged GSN and GPN at the C terminus with a V5-epitope tag and measured GSN-V5 and GPN-V5 levels from cells harvested every 4 h in DD over 2 d using anti-V5 antibody. Total GSN and GPN levels exhibited circadian rhythms with a peak in the subjective night (∼DD32) (Fig. 1 *E* and *F*), which coincides with the peak of glycogen levels (Fig. 1*A*).

To determine if rhythmic glycogen abundance requires *gsn* or *gpn*, we assayed glycogen rhythms in **Δ***gsn* and **Δ***gpn* strains. The

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overall levels of glycogen are low in Δ*gpn* cells at all times of the day compared with WT cells, and while a low amplitude rhythm in glycogen levels in Δ*gpn* cells is observed in the data, the rhythm does not meet statistical significance (Figs. 1*B* and 2*A*). Therefore, we concluded that rhythmic accumulation of glycogen is disrupted in Δ*gpn* cells. As expected, no glycogen was detected in Δ*gsn* cells lacking glycogen synthase (Fig. 2*A*). In contrast, the *frq* promoter luciferase reporter transcriptional fusion construct (P*frq-luc*) displayed robust rhythmicity in Δ*gpn* cells, ruling out the possibility that the loss of glycogen accumulation rhythms in Δ*gpn* was the result of a defect in the core circadian clock mechanism (Fig. 2*B*). Furthermore, *gpn* mRNA rhythms were disrupted in Δ*gsn* cells, and *gsn* mRNA rhythms were abolished in Δ*gpn* cells, whereas the clock-controlled gene *ccg-1* mRNA (40) accumulated rhythmically in the mutant strains (Fig. 2*C*).

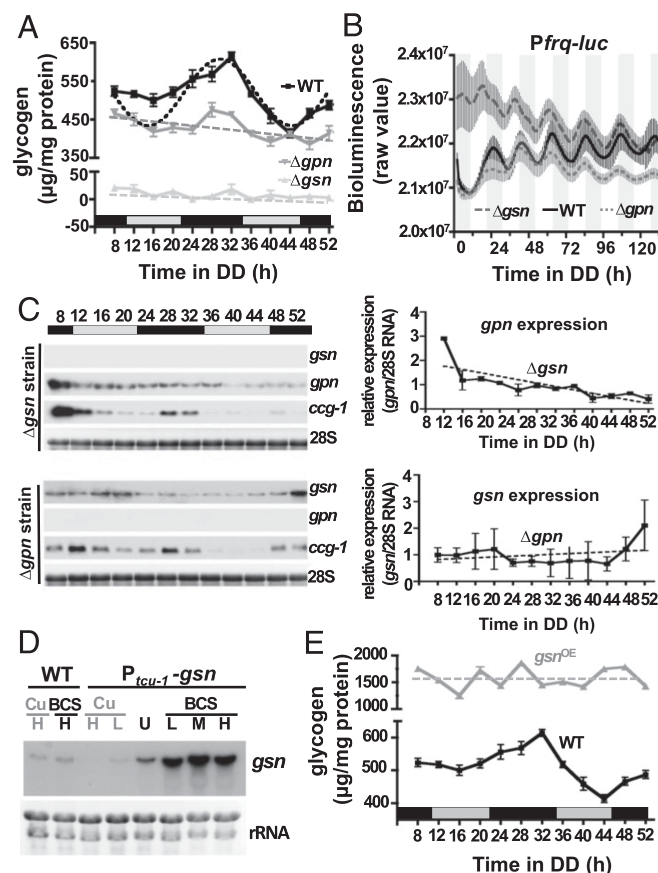

Fig. 2. Rhythms in *gsn* mRNA accumulation are required for rhythmic glycogen levels. (*A*) Plot of glycogen levels from WT (black line; replotted from Fig. 1*A*), Δ*gpn* (dark gray line), and Δ*gsn* cells (light gray line) ($n \geq 2$, ±SEM). Glycogen levels in Δ*gpn* and Δ*gsn* had a better fit of the data to a linear line. (*B*) Representative trace of P*frq-luc* in WT (black line), Δ*gpn* (dotted dark gray line), and Δ*gsn* (dotted light gray line) ($n \geq 3$, ±SEM). Bioluminescence data were analyzed by BioDare. Arbitrary units are shown. (*C*) Representative Northern blots of *gsn*, *gpn*, and clock-controlled gene *ccg-1* mRNA isolated from Δ*gsn* or Δ*gpn* cells harvested at the indicated times in DD. rRNA was used as a loading control. The data for *gsn* in Δ*gpn* cells, and *gpn* in Δ*gsn* cells, are plotted on the right (solid black lines, $n \geq 4$, ±SEM), with both having a better fit to a linear line. (*D*) Northern blot of *gsn* mRNA from WT and P_{tcu-1}-*gsn* cells treated with low [L; 25 µM Cu or bathocuproinedisulfonic acid (BCS)], medium (M; 100 µM Cu or BCS), high (H; 250 µM Cu or BCS) levels, or untreated (U), and harvested at DD24. rRNA served as a loading control. (*E*) Plot of glycogen accumulation from WT (black line) and P_{tcu-1}-*gsn* cells (gray line) treated with 250 µM BCS over the indicated times in DD to constitutively overexpress *gsn* mRNA (*gsn*^{OE}). Glycogen levels in *gsn*^{OE} were better fit to a linear line.

The loss of rhythmic *gsn* mRNA and glycogen levels in Δ*gpn* cells supported the hypothesis that cycling *gsn* mRNA is necessary for rhythmic glycogen accumulation. To test this hypothesis, we constructed a strain that overexpressed *gsn* from the *tcu-1* promoter (41). Constitutive overexpression of *gsn* resulted in disruption of the circadian rhythm of glycogen accumulation, and an approximately threefold increase in total glycogen levels compared with WT cells (Figs. 1*B* and 2 *D* and *E*). Further support for clock control of rhythmic gene expression being important for glycogen level rhythms is that while phosphorylated GSN accumulated rhythmically, the amount of phosphorylated GSN represented only a small fraction of total GSN (*SI Appendix*, Fig. S1*C*). Thus, under these growth conditions, signaling mechanisms that regulate GSN activity likely have only a minor role, if any, in regulating rhythmic glycogen accumulation. Taken together, these data support that circadian control of *gsn* is critical for rhythmic accumulation of glycogen. Therefore, we next focused on determining what controls rhythmic *gsn* expression, but also examined possible mechanisms of transcriptional regulation of *gpn*.

WCC Regulates Rhythmic Expression of *gsn*. WC-2 ChIP-seq from *N. crassa* cultures given a short light treatment to promote genome-wide WCC binding did not identify WC-2 binding sites near the *gsn* or *gpn* genes (34). However, based on the WCC-consensus binding site (19, 34, 42), we identified four putative WCC binding sites within 2 kb upstream of the translation start site of *gsn* (Fig. 3*A*). ChIP assays confirmed light-induced recruitment of WC-2 to the binding sites present in the *gsn* promoter, but as expected, not to *gpn*, which lacks WCC binding sites (Fig. 3*B*). Examination of WC-2 binding to the *gsn* promoter from cells grown in DD and harvested at different times of the day revealed that WC-2 is rhythmically recruited to the *gsn* promoter, with peak binding during the subjective day (DD14) (Fig. 3*C*). These data supported the idea that WCC directly regulates *gsn* rhythmic expression. We next examined if *gsn* mRNA and glycogen abundance rhythms were altered in Δ*wc-1* cells. As expected for loss of a core clock component, P*gsn-luc* and glycogen rhythms were abolished in Δ*wc-1* cells (Fig. 3 *D* and *E*), but overall glycogen levels in the mutant were similar to WT levels (Figs. 1*B* and 3*E*). Taken together, these data indicated that the core clock component WCC directly drives rhythmic expression of *gsn* necessary for rhythmic glycogen accumulation.

VOS-1 Influences Rhythmic *gsn* and *gpn* mRNA and Glycogen Levels. In addition to WCC binding sites, we identified potential VOS-1 binding sites in the *gsn* and *gpn* promoter regions based on the identification of sequences similar to the consensus *A. nidulans* VosA DNA binding site (5′-CTGGCCAAGGC-3′) (Fig. 3*A*) (43). Because *vos-1* is a direct target of the WCC (34), we first examined if the circadian clock controls rhythms in the expression of *vos-1*. Both P*vos-1–luc* and VOS-1–V5 showed robust circadian oscillations, with a peak in VOS-1–V5 during the subjective night (DD28) (Fig. 4 *A* and *B*). Furthermore, VOS-1 bound rhythmically to the *gsn* and *gpn* promoters, peaking in the subjective night (DD28) (Fig. 4 *C* and *D*), consistent with the nighttime peak levels of *vos-1* mRNA and protein, and preceding the peak in *gsn* and *gpn* mRNA levels (Fig. 1 *C* and *E*). In the Δ*vos-1* strain, both P*gsn-luc* and P*gpn-luc* were still rhythmic, but with a significantly reduced amplitude, and with an ∼4-h phase advance of the P*gsn-luc* rhythm compared with WT (Fig. 4 *E* and *F* and *SI Appendix*, Table S1). These data indicated that while VOS-1 is not necessary for rhythmicity of *gsn* and *gpn*, it contributes to the robustness of their rhythms. Furthermore, in Δ*vos-1* cells, glycogen accumulation was rhythmic, but with a lower amplitude and an ∼2-h phase advance (Fig. 4*G* and *SI Appendix*, Table S1), and the overall levels of glycogen were similar to WT levels (Fig. 1*B*).

CSP-1 Is Required for Rhythmic Expression of *gpn*, but Not *gsn*. CSP-1 is a direct target of the WCC (34), and previous ChIP-seq analyses indicated that CSP-1 physically binds to the *gpn* promoter

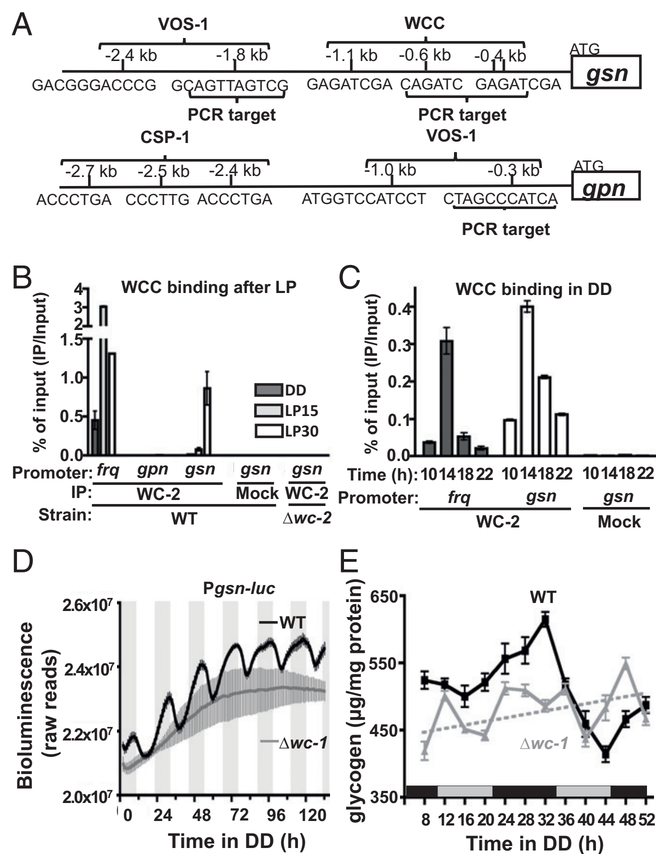

Fig. 3. The WCC directly controls *gsn* expression and promotes rhythmic glycogen accumulation. (*A*) Map of WCC, VOS-1, and/or CSP-1 binding sites in the promoter region of *gsn* and *gpn*. The regions amplified for ChIP-PCR for WCC and VOS-1 are indicated below (PCR target), and the primers are listed in *SI Appendix*, Table S3. (*B*) Plot of ChIP-qPCR data (% of input) for WC-2 binding (which complexes with WC-1 to form the WCC) to the indicated promoters from cells harvested at DD24 with or without a 15- or 30-min light treatment to induce WCC activity region ($n \geq 3$, ±SEM). WC-2 binding to the *frq* promoter served as a positive control. MockIP and Δ*wc-2* cells served as negative controls. (*C*) Plot of ChIP-qPCR data (% of input) for WC-2 binding to the indicated promoters from cells harvested at the indicated times in DD. MockIP served as the negative control. (*D*) Representative trace of bioluminescence signals from P*gsn-luc* in WT (black line) and Δ*wc-1* (gray line). Bioluminescence data were analyzed by BioDare (*SI Appendix*, Table S1). (*E*) Plot of glycogen levels from WT (black line; replotted from Fig. 1*A*), and Δ*wc-1* cells (gray line) ($n \geq 4$, ±SEM). Glycogen levels in Δ*wc-1* cells were better fit to a line (dotted gray line).

(37). To determine if CSP-1 regulates rhythmic *gpn* and/or *gsn* promoter activity, we assessed the expression of *gpn* and *gsn* in Δ*csp-1* cells. Rhythms in P*gpn-luc* were abolished (Fig. 5*A*), while P*gsn-luc* was rhythmic with a reduced amplitude (Fig. 5*B*) in Δ*csp-1* cells compared with WT cells. Importantly, rhythmic glycogen accumulation persisted in Δ*csp-1* cells with an ∼5-h phase advance (Fig. 5*C*). Although CSP-1 functions primarily as a transcriptional repressor (37), low levels of *gpn* expression in Δ*csp-1* cells compared with WT cells (Fig. 5*A*), suggested that CSP-1 may function as an activator of *gpn* expression. To test this further, we constructed strains with *csp-1* controlled by either the quinic acid (QA)-inducible *qa-2* promoter (44) or the *β-tubulin* promoter to constitutively induce the *csp-1* expression (45), and determined the levels of *csp-1* and *gpn*. The *β-tubulin* promoter drives constitutive overexpression of a target gene of interest (45). Increased expression of *csp-1* at 1 h of quinic acid induction led to a similar increase in *gpn* mRNA levels 1 h later, supporting that CSP-1 activates *gpn* transcription (Fig. 5*D*). Taken together, these data indicate that CSP-1 is required for rhythmic *gpn* transcription and modulates the phase and amplitude of glycogen accumulation rhythms through control of *gsn* mRNA expression. Importantly, these data support that the circadian control of *gsn* expression is the primary driver of rhythmic accumulation of glycogen, because the loss of *gpn* rhythms in Δ*csp-1* did not abolish rhythmic glycogen accumulation.

Discussion

Many metabolic functions are under control of the clock to ensure that they are produced at the appropriate time of day, such as stimulating catabolism during the active phase to support increased energy demands (32, 46, 47). The importance of clock control of metabolism is revealed by an increased incidence of metabolic disorders in mice and humans with a disrupted clock (3, 48). The clock component and nuclear receptor REV-ERBα has been shown to play a key role in connecting the clock to metabolism in mammals (49–51). *Rev-erbα* is expressed with a

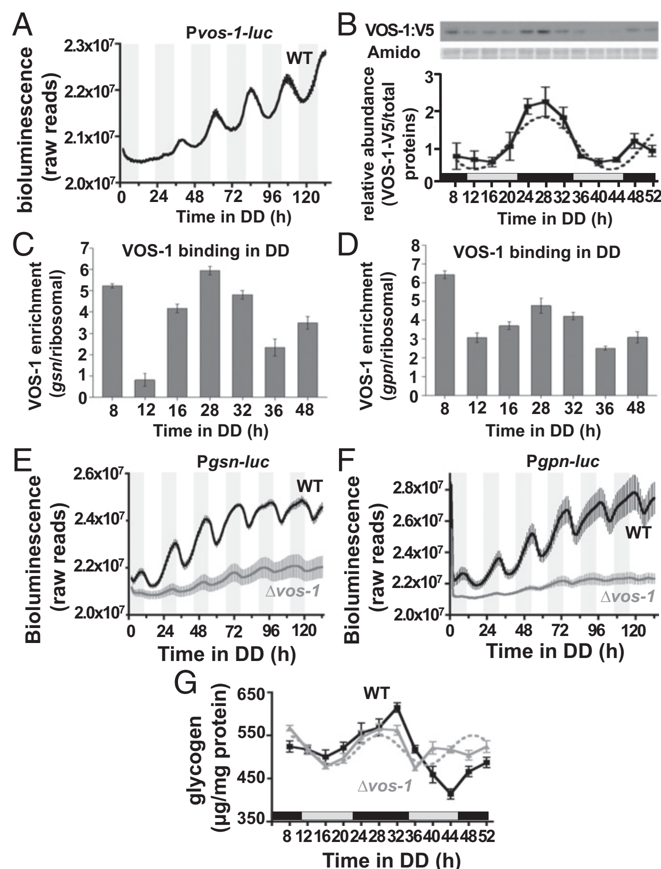

Fig. 4. VOS-1 binds rhythmically to the *gsn* promoter and is necessary for robust rhythms in *gsn* mRNA and glycogen accumulation. (*A*) Representative trace of bioluminescence signals from P*vos-1–luciferase* (P*vos-1–luc*) in WT cells grown in DD for the indicated times. Arbitrary units are shown. (*B*) Representative Western blot of VOS-1–V5 from cells harvested at the indicated times in DD. The data are plotted below ($n = 3$, ±SEM), and were fit to a sine wave ($P < 0.05$). Amido black staining of the membrane was used to normalize protein loading. (*C* and *D*) ChIP-qPCR of VOS-1 binding to the *gsn* and *gpn* promoter at the indicated time points in DD ($n = 2$, ±SEM). Nonspecific VOS-1 binding on the 60S rRNA was used for normalization of the signal. (*E* and *F*) Representative trace of bioluminescence signal from P*gsn-luc* and P*gpn-luc* in WT (black line) and Δ*vos-1* (gray line) cells ($n \geq 3$, ±SEM). Bioluminescence data were analyzed by BioDare (*SI Appendix*, Table S1). (*G*) Plot of glycogen levels from WT (black line; replotted from Fig. 1*A*) and Δ*vos-1* cells (gray line) ($n = 5$, ±SEM). Δ*vos-1* displays rhythmic glycogen accumulation ($P < 0.001$), but with a reduced amplitude and a phase advance compared with WT (*SI Appendix*, Table S1).

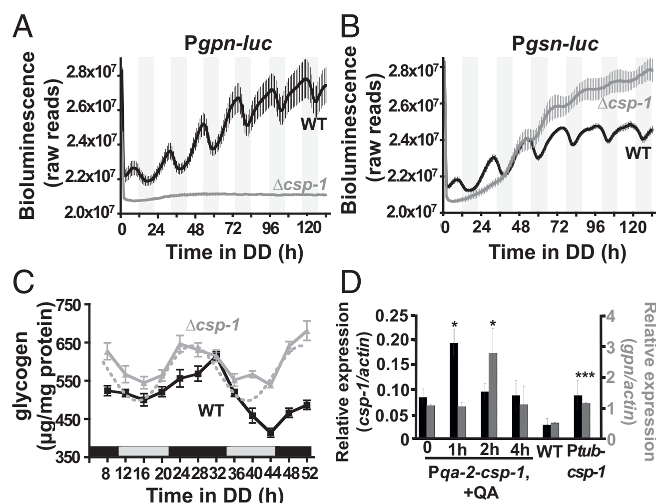

Fig. 5. CSP-1 regulates the rhythmic expression of *gpn*. (*A* and *B*) Representative trace of bioluminescence signals from P*gpn-luc* and P*gsn-luc* in WT and Δ*csp-1* cells (gray line) ($n \geq 3$, ±SEM) grown in DD for the indicated times. Arbitrary units are shown. (*C*) Plot of glycogen levels from WT (black line; replotted from Fig. 1*A*) and Δ*csp-1* cells (gray line) ($n = 4$, ±SEM). Δ*csp-1* displays rhythmic glycogen accumulation, but with a reduced amplitude and a phase advance compared with WT (*SI Appendix*, Table S1). (*D*) *gpn* mRNA from WT and *csp-1* overexpression cells (*Pqa-2–csp-1* and P*tub-csp-1*). *Pqa-2–csp-1* cells were harvested at 0, 60, 120, and 240 min after quinic acid treatment. Relative expression levels of *csp-1* (black) and *gpn* (gray) were quantified by RT-PCR with *actin* used for normalization ($n = 3$, ±SEM). Student's *t* test comparisons of levels to untreated (0) levels for P*qa-2–csp-1* $^{*}P < 0.05$, or to WT for P*tub–csp-1* $^{***}P < 0.001$.

circadian rhythm in several tissues, including the liver, adipose tissue, muscle, and pancreas, and in these tissues, modulates lipid, glucose, and bile acid metabolism (50, 52). Similarly, in *N. crassa*, the clock-controlled TF, CSP-1, connects the circadian clock to metabolism by regulating ~200 genes involved in metabolic pathways, including glucose metabolism (38, 47). However, the molecular mechanisms of circadian clock-controlled glucose homeostasis remain largely unknown. We utilized *N. crassa* as a model to uncover potentially conserved molecular mechanisms controlling rhythmic glycogen metabolism, a critical process in glucose homeostasis. We observed circadian oscillations of glycogen, *gsn* mRNA, *gpn* mRNA, and GSN and GPN protein levels. These data are consistent with previous animal studies demonstrating circadian rhythms of glucose metabolic parameters, including glycogen abundance, plasma glucose levels, and glucose tolerance (53). Moreover, hepatic glycogen synthase (*Gys2*) is a direct target of the mammalian core clock protein CLOCK, and both GYS2 and glycogen abundance show dampened circadian oscillations in *Clock* mutant mice (15).

We observed in-phase morning-specific mRNA levels of both *gsn* and *gpn* despite their opposing functions in glycogen metabolism. However, GSN and GPN function may not only depend on mRNA abundance, but also on their protein accumulation, enzymatic activities, and localization. In animals and fungi, GS and GP are regulated by allosterism and by reversible phosphorylation (7). Phosphorylated GS becomes inactive, whereas phosphorylation is required for the activation of GP. This results in a switch-like mechanism where one enzyme is active while the other one is inactive (7). While we have not yet investigated the impact of the clock on GPN phosphorylation in *N. crassa*, our data reveal the importance of transcription control of *gsn* and little, if any, role for phosphorylation of GSN in rhythmic glycogen accumulation (*SI Appendix*, Fig. S1*C*). In addition, in yeast and skeletal muscle cells, GS and GP display differences in their cellular localization that is dependent on glycogen concentration, with GS entering the nucleus when glycogen is depleted and GP remaining cytoplasmic (54–56). The nuclear localization of GS has been suggested to provide a warning signal that fuel levels are low, which then triggers transcription of genes necessary for increasing glycogen stores (56). These data suggest the possibility that loss of rhythmic *gsn* expression in Δ*gpn* cells, as well as loss of *gpn* rhythmicity in Δ*gsn* cells (Fig. 2*C*), may be due to changes in nuclear GSN-directed transcriptional control. As such, the coordinated regulation of *gsn* and *gpn* mRNA by the clock may provide a strategy to allow the organism to efficiently shift between glycogen synthesis and breakdown, depending on the time of day to maximize energy production in the active phase, or in response to nutritional stress. Consistent with this idea, glycogen synthase (*Gys1* and *Gys2*) and glycogen phosphorylase (*Pygl*) genes are robustly rhythmic in mouse liver, with similar peak expression levels during the early- to midsubjective night (57). Future experiments will be necessary to investigate potential clock and stress control of *N. crassa* GPN phosphorylation and activity, as well as the potential role for nuclear GSN in rhythmic *gpn* and *gsn* transcription.

Previous studies demonstrated that direct targets of CSP-1 peak in the evening, antiphase to direct WCC target genes that peak in the morning (37). However, our studies revealed that the cycling mRNAs of WCC target, *gsn*, and CSP-1 target, *gpn*, peak at the same time of day. To determine a possible mechanism for coordinated phase regulation of *gsn* and *gpn*, we revised our previous mathematical model (39) to identify molecular wirings and parameter space that would reproduce our experimental data (*SI Appendix*). Computer simulations suggest that the rhythmic expression of *gpn* is independently regulated by both monomeric CSP-1 and heterodimeric CSP-1/VOS-1 complex with stronger activation by CSP-1/VOS-1 complex to satisfy the in-phase relationship between *gsn* and *gpn* mRNA levels, and the loss of *gpn* rhythmicity in Δ*csp-1*. In other words, our model suggests that the phase and rhythmicity of *gpn* is determined by VOS-1 and CSP-1, respectively. On the other hand, the model suggests that *gsn* is independently regulated by WCC and VOS-1 with a stronger activation by VOS-1 to reproduce the reduced expression of *gsn* in Δ*vos-1* and the loss of rhythmicity of *gsn* in Δ*wc-1*. Our simulations suggest that WCC and CSP-1 regulate the rhythmic expression of *gsn* and *gpn*, respectively, and VOS-1 regulates the abundance and phase of *gsn* and *gpn*. These model-driven hypotheses will be experimentally validated in our future experiments. Furthermore, we plan to expand this model to investigate reciprocal regulation of GSN and GPN to determine the posttranslational modifications of GSN and GPN contributing to rhythmic accumulation and breakdown of glycogen.

Our data support that rhythmic *gsn* mRNA levels are necessary for rhythmic accumulation of glycogen. The main driver of rhythmic *gsn* expression appears to be through the rhythmic binding of WCC to the *gsn* promoter. First, conditions that alter *gsn* mRNA rhythms, amplitude, and/or phase (constitutive expression, deletion of *wc-1*, *vos-1*, or *csp-1*) similarly affect glycogen rhythms, whereas conditions that abolish *gpn* rhythms (deletion of *csp-1*) maintain rhythms in glycogen. Validation of this idea will require mutating the WCC binding sites in the promoter of *gsn* and assaying rhythms in *gsn* mRNA and glycogen levels. The role of CSP-1 and VOS-1, and possibly other TFs, in *gsn* regulation may be necessary for processing various inputs from the environment to adjust the timing of glycogen metabolism for maximum energy benefit. Interestingly, *csp-1* transcription was previously shown to be activated under high glucose (2%) conditions (37), identical to our growth conditions. It is therefore also of interest to determine if glycogen rhythms and phase are altered in different glucose conditions. The WCC is not only a circadian TF, but also functions as a blue-light photoreceptor (29), and *Aspergillus* VosA is involved in stress responsive pathways, fungal development, and carbohydrate metabolism (43). Thus, similar to WCC light responsiveness, VOS-1 likely responds to environmental signals, such as nutrient stress, to modulate the expression of *gsn*.

In conclusion, we demonstrate that *gsn* mRNA rhythms are necessary for the daily cycle of glycogen abundance in the simple

model organism *N. crassa*. The complex mechanism of *gsn* and *gpn* promoter regulation, which appears to be conserved in mammalian cells (14), as well as possible feedback regulation of GSN on *gpn*, and GPN on *gsn*, equips the fungus with the ability to anticipate daily environmental stress (clock control), while at the same time providing flexibility to deal with acute stress, including nutrient availability, to coordinate energy production and other physiological processes under normal and stressful conditions.

Materials and Methods

Strains and Culture Conditions. Strains for this study are described in *SI Appendix*, Table S2. Mutant strains were created as previously described (58). Primers for plasmid construction are listed in *SI Appendix*, Table S3. For circadian time course experiments, subjective day and night bars on the plots in the figures were determined based on the period of the rhythm (*SI Appendix*, Table S1) as previously described (40).

Other Methods. Culture conditions, RNA extraction, Northern blotting, protein extraction, Western blot assays, bioluminescence assay, ChIP-qPCR, glycogen quantification, and data analysis are described in *SI Appendix*, *Materials and Methods*.

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1. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. *Science* 291:490–493.
2. Damiola F, et al. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14:2950–2961.
3. Turek FW, et al. (2005) Obesity and metabolic syndrome in circadian clock mutant mice. *Science* 308:1043–1045.
4. Kennaway DJ, Owens JA, Voultsios A, Boden MJ, Varcoe TJ (2007) Metabolic homeostasis in mice with disrupted clock gene expression in peripheral tissues. *Am J Physiol Regul Integr Comp Physiol* 293:R1528–R1537.
5. Rudic RD, et al. (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2:e377.
6. Froy O (2010) Metabolism and circadian rhythms–Implications for obesity. *Endocr Rev* 31:1–24.
7. Roach PJ, Depaoli-Roach AA, Hurley TD, Tagliabracci VS (2012) Glycogen and its metabolism: Some new developments and old themes. *Biochem J* 441:763–787.
8. Adeva-Andany MM, González-Lucán M, Donapetry-García C, Fernández-Fernández C, Ameneiros-Rodríguez E (2016) Glycogen metabolism in humans. *BBA Clin* 5:85–100.
9. Wasserman DH (2009) Four grams of glucose. *Am J Physiol Endocrinol Metab* 296: E11–E21.
10. Anderson C, Tatchell K (2001) Hyperactive glycogen synthase mutants of Saccharomyces cerevisiae suppress the glc7-1 protein phosphatase mutant. *J Bacteriol* 183:821–829.
11. Freitas FZ, de Paula RM, Barbosa LC, Terenzi HF, Bertolini MC (2010) cAMP signaling pathway controls glycogen metabolism in Neurospora crassa by regulating the glycogen synthase gene expression and phosphorylation. *Fungal Genet Biol* 47:43–52.
12. Ishikawa K, Shimazu T (1976) Daily rhythms of glycogen synthetase and phosphorylase activities in rat liver: Influence of food and light. *Life Sci* 19:1873–1878.
13. Ishikawa K, Shimazu T (1980) Circadian rhythm of liver glycogen metabolism in rats: Effects of hypothalamic lesions. *Am J Physiol* 238:E21–E25.
14. Roesler WJ, Khandelwal RL (1985) Diurnal variations in the activities of the glycogen metabolizing enzymes in mouse liver. *Int J Biochem* 17:81–85.
15. Doi R, Oishi K, Ishida N (2010) CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2. *J Biol Chem* 285:22114–22121.
16. Ballario P, et al. (1996) White collar-1, a central regulator of blue light responses in Neurospora, is a zinc finger protein. *EMBO J* 15:1650–1657.
17. Denault DL, Loros JJ, Dunlap JC (2001) WC-2 mediates WC-1-FRQ interaction within the PAS protein-linked circadian feedback loop of Neurospora. *EMBO J* 20:109–117.
18. Cheng P, Yang Y, Gardner KH, Liu Y (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of Neurospora. *Mol Cell Biol* 22:517–524.
19. Froehlich AC, Liu Y, Loros JJ, Dunlap JC (2002) White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. *Science* 297:815–819.
20. Vitalini MW, et al. (2010) *Cellular and Molecular Biology of Filamentous Fungi* (ASM Press, Washington, DC), pp 442–466.
21. Lee K, Dunlap JC, Loros JJ (2003) Roles for WHITE COLLAR-1 in circadian and general photoperception in Neurospora crassa. *Genetics* 163:103–114.
22. Cheng P, He Q, He Q, Wang L, Liu Y (2005) Regulation of the Neurospora circadian clock by an RNA helicase. *Genes Dev* 19:234–241.
23. Shi M, Collett M, Loros JJ, Dunlap JC (2010) FRQ-interacting RNA helicase mediates negative and positive feedback in the Neurospora circadian clock. *Genetics* 184:351–361.
24. Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC (2009) Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the Neurospora circadian clock. *Mol Cell* 34:354–363.
25. He Q, Liu Y (2005) Degradation of the Neurospora circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. *Biochem Soc Trans* 33:953–956.
26. Schafmeier T, et al. (2005) Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. *Cell* 122:235–246.
27. Schafmeier T, et al. (2008) Circadian activity and abundance rhythms of the Neurospora clock transcription factor WCC associated with rapid nucleo-cytoplasmic shuttling. *Genes Dev* 22:3397–3402.
28. Hong CI, Ruoff P, Loros JJ, Dunlap JC (2008) Closing the circadian negative feedback loop: FRQ-dependent clearance of WC-1 from the nucleus. *Genes Dev* 22:3196–3204.
29. He Q, et al. (2003) FWD1-mediated degradation of FREQUENCY in Neurospora establishes a conserved mechanism for circadian clock regulation. *EMBO J* 22:4421–4430.
30. Larrondo LF, Olivares-Yañez C, Baker CL, Loros JJ, Dunlap JC (2015) Circadian rhythms. Decoupling circadian clock protein turnover from circadian period determination. *Science* 347:1257277.
31. Cheng P, Yang Y, Wang L, He Q, Liu Y (2003) WHITE COLLAR-1, a multifunctional neurospora protein involved in the circadian feedback loops, light sensing, and transcription repression of wc-2. *J Biol Chem* 278:3801–3808.
32. Hurley JM, et al. (2014) Analysis of clock-regulated genes in Neurospora reveals widespread posttranscriptional control of metabolic potential. *Proc Natl Acad Sci USA* 111:16995–17002.
33. Crosthwaite SK, Dunlap JC, Loros JJ (1997) Neurospora wc-1 and wc-2: Transcription, photoresponses, and the origins of circadian rhythmicity. *Science* 276:763–769.
34. Smith KM, et al. (2010) Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for neurospora white collar complex. *Eukaryot Cell* 9:1549–1556.
35. Ni M, Yu JH (2007) A novel regulator couples sporogenesis and trehalose biogenesis in Aspergillus nidulans. *PLoS One* 2:e970.
36. Krijgsheld P, et al. (2013) Development in Aspergillus. *Stud Mycol* 74:1–29.
37. Sancar G, et al. (2011) A global circadian repressor controls antiphasic expression of metabolic genes in Neurospora. *Mol Cell* 44:687–697.
38. Sancar G, Sancar C, Brunner M (2012) Metabolic compensation of the Neurospora clock by a glucose-dependent feedback of the circadian repressor CSP1 on the core oscillator. *Genes Dev* 26:2435–2442.
39. Dovzhenok AA, Baek M, Lim S, Hong CI (2015) Mathematical modeling and validation of glucose compensation of the neurospora circadian clock. *Biophys J* 108:1830–1839.
40. Loros JJ, Denome SA, Dunlap JC (1989) Molecular cloning of genes under control of the circadian clock in Neurospora. *Science* 243:385–388.
41. Lamb TM, Vickery J, Bell-Pedersen D (2013) Regulation of gene expression in Neurospora crassa with a copper responsive promoter. *G3 (Bethesda)* 3:2273–2280.
42. Chen CH, Ringelberg CS, Gross RH, Dunlap JC, Loros JJ (2009) Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in Neurospora. *EMBO J* 28:1029–1042.
43. Ahmed YL, et al. (2013) The velvet family of fungal regulators contains a DNA-binding domain structurally similar to NF-κB. *PLoS Biol* 11:e1001750.
44. Giles NH, et al. (1985) Gene organization and regulation in the qa (quinic acid) gene cluster of Neurospora crassa. *Microbiol Rev* 49:338–358.
45. Matsu-Ura T, Baek M, Kwon J, Hong C (2015) Efficient gene editing in *Neurospora crassa* with CRISPR technology. *Fungal Biol Biotechnol* 2:4.
46. Green CB, Takahashi JS, Bass J (2008) The meter of metabolism. *Cell* 134:728–742.
47. Sancar C, Sancar G, Ha N, Cesbron F, Brunner M (2015) Dawn- and dusk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in Neurospora. *BMC Biol* 13:17.
48. Scheer FA, Hilton MF, Mantzoros CS, Shea SA (2009) Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci USA* 106:4453–4458.
49. Duez H, Staels B (2008) The nuclear receptors Rev-erbs and RORs integrate circadian rhythms and metabolism. *Diab Vasc Dis Res* 5:82–88.
50. Le Martelot G, et al. (2009) REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* 7:e1000181.
51. Feng D, et al. (2011) A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* 331:1315–1319.
52. Preitner N, et al. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110:251–260.
53. Lamia KA, Storch KF, Weitz CJ (2008) Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci USA* 105:15172–15177.
54. Cid E, Cifuentes D, Baqué S, Ferrer JC, Guinovart JJ (2005) Determinants of the nucleocytoplasmic shuttling of muscle glycogen synthase. *FEBS J* 272:3197–3213.
55. Ferrer JC, Baqué S, Guinovart JJ (1997) Muscle glycogen synthase translocates from the cell nucleus to the cystosol in response to glucose. *FEBS Lett* 415:249–252.
56. Wilson WA, et al. (2010) Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiol Rev* 34:952–985.
57. Hughes ME, et al. (2009) Harmonics of circadian gene transcription in mammals. *PLoS Genet* 5:e1000442.
58. Hong CI, et al. (2014) Circadian rhythms synchronize mitosis in Neurospora crassa. *Proc Natl Acad Sci USA* 111:1397–1402.
59. Colot HV, et al. (2006) A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. *Proc Natl Acad Sci USA* 103: 10352–10357.