Metabolic compensation of the *Neurospora* clock by a glucose-dependent feedback of the circadian repressor CSP1 on the core oscillator

Gencer Sancar, Cigdem Sancar, and Michael Brunner

Biochemistry Center, University of Heidelberg, D-69120 Heidelberg, Germany

Conidial separation 1 (CSP1) is a global transcription repressor. It is expressed under control of the white collar complex (WCC), the core transcription factor of the circadian clock of *Neurospora*. Here we report that the length of the circadian period decreases with increasing glucose concentrations in *csp1* mutant strains, while the period is compensated for changes in glucose concentration in wild-type strains. Glucose stimulated CSP1 expression. Overexpression of CSP1 caused period lengthening and, eventually, complete dampening of the clock rhythm. We show that CSP1 inhibits expression of the WHITE COLLAR 1 (WC1) subunit of the WCC by repressing the *wc1* promoter. Glucose-dependent repression of *wc1* transcription by CSP1 compensated for the enhanced translation of WC1 at high glucose levels, resulting in glucose-independent expression of the WCC and, hence, metabolic compensation that maintained a constant circadian period. Thus, the negative feedback of CSP1 on WC1 expression constitutes a molecular pathway that coordinates energy metabolism and the circadian clock.

**Keywords:** circadian clock; metabolism; glucose compensation; feedback loop; *Neurospora*

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Circadian clocks are self-sustained molecular oscillators that are based on interconnected transcriptional and post-transcriptional feedback loops. They coordinate the rhythmic expression of a large number of genes in anticipation of environmental perturbations associated with the day–night cycle of the earth (Dibner et al. 2010; Diernfellner and Schafmeier 2011; Hogenesch and Ueda 2011; Lowrey and Takahashi 2011). A major function of circadian clocks is to regulate metabolic processes to establish cellular homeostasis in a rhythmic environment (Gatfield and Schibler 2008; Duez and Staels 2009; Asher and Schibler 2011; Bray and Young 2011; Huang et al. 2011; Sassone-Corsi 2012; Stratmann and Schibler 2012).

In mammals, the core circadian transcription factor CLOCK/BMAL1 activates expression of the *Period (Per)* and *Cryptochrome (Cry)* genes, and the PER and CRY proteins rhythmically repress CLOCK/BMAL1 in a negative feedback loop [Takahashi et al. 2008]. In a second loop, CLOCK/BMAL1 activates the *Rev-erb* and *Ror* nuclear receptors genes [Preitner et al. 2002], which encode repressors and activators, respectively, that control rhythmic expression of *Bmal1* and probably also the *Clock, Per,* and *Cry* genes [Preitner et al. 2002; Liu et al. 2007; Bugge et al. 2012; Cho et al. 2012]. Although rhythmic expression of BMAL1 is not required for the function of the clock, hepatic double deletion of the genes for the partially redundant repressors, *Rev-erb*-α and *Rev-erb*-β, causes arrhythmicity in mouse livers [Bass 2012; Cho et al. 2012; Stratmann and Schibler 2012]. The *Rev-erb* nuclear receptors control the expression of many genes involved in the metabolism of lipids and glucose and thus allow the clock to rhythmically regulate cellular metabolism. When Rev-erb function is compromised in the liver, animals develop metabolic syndrome and obesity [Gatfield and Schibler 2008; Duez and Staels 2009; Bass 2012; Bugge et al. 2012; Cho et al. 2012; Solt et al. 2012; Stratmann and Schibler 2012].

Metabolism, in turn, feeds back on the circadian clock via NAD⁺, a substrate of the histone deacetylase SIRT1, which is recruited by CLOCK/BMAL1 to circadian genes [Nakahata et al. 2008; Bellet et al. 2011]. Additional signaling pathways likely exist, and the REV-ERBs are candidates for the cross-talk between metabolism and circadian clocks [Solt et al. 2012; Stratmann and Schibler 2012].

In *Neurospora*, the WHITE COLLAR 1 (WC1) and WC2 subunits form the heterodimeric white collar complex (WCC), which is the core transcription factor of the circadian clock [Crosthwaite et al. 1997; Dunlap and...
Loros 2004; Heintzen and Liu 2007). The WCC activates morning-specific expression of the circadian clock gene *frequency* (*frq*). WC1 is a photoreceptor (Froehlich et al. 2002, He et al. 2002). In light–dark cycles, the WCC dimerizes (Malzahn et al. 2010) and activates expression of the photoreceptor gene *vivid* (*vvd*) (Schwerdtfeger and Linden 2003; Zoltowski et al. 2007). FRQ and VVD inhibit the WCC in distinct negative feedback loops: FRQ forms a complex with CK1α and FRH (Gorl et al. 2001; Cheng et al. 2005) and inhibits the WCC by supporting its phosphorylation (Schafmeier et al. 2005). VVD inhibits the light-activated WCC (Chen et al. 2010) by disrupting WCC dimers (Malzahn et al. 2010). Many morning-specific genes are directly activated by the WCC (Chen et al. 2009; Smith et al. 2010).

We recently showed that the WCC-controlled gene *conidial separation 1* (*csp1*) encodes a global circadian repressor that modulates expression of ~800 genes in *Neurospora* (Sancar et al. 2011). Many of these genes are rhythmically expressed with an evening-specific phase. Target genes of CSP1 are predominantly involved in metabolism of lipids and glucose in particular.

Here we report that glucose metabolism, in turn, has the potential to feed back on the core FRQ/WCC molecular oscillator via CSP1. CSP1 is a glucose-dependent repressor of *wc-1* transcription. This negative feedback loop compensates the core oscillator against glucose and other carbon sources and thus coordinates cellular metabolism with the circadian clock.

### Results

CSP1 is a circadian repressor with some similarities to the yeast transcription repressors NRG1 and NRG2 (Sancar et al. 2011). Since NRG1 and NRG2 are regulated by glucose (Kuchin et al. 2002, Berkey et al. 2004; Vyas et al. 2005), we asked whether CSP1 modulates the *Neurospora* circadian clock in response to glucose levels. To assess the period length of the clock, we analyzed the circadian conidiation rhythm of a *csp1* mutant and a corresponding control strain (wild-type [WT] *bd*) in race tubes containing 0%, 0.1%, 0.3%, and 0.5% glucose (Fig. 1A). We found that the period length of the *csp1* mutant decreased with increasing concentrations of glucose (0% glucose: 22.20 ± 0.18 h; 0.5% glucose: 19.90 ± 0.10 h) but that the period length of the *bd* control strain was essentially independent of glucose concentration (0% glucose: 22.45 ± 0.15 h; 0.5% glucose: 22.15 ± 0.05 h) [Fig. 1B]. Similar results were obtained when fructose and sucrose were used as carbon sources [Supplemental Fig. S1A,B].

In addition, we generated a Δ*csp1* *bd* strain that expresses a luciferase reporter under the control of the *frq* promoter and followed its clock-controlled bioluminescence rhythm in constant darkness (Fig. 1C). At a low glucose level (0.05%), the period length of Δ*csp1* *frq-luc* was 20.56 ± 0.11 h (*n* = 15), while it was 18.96 ± 0.10 h (*n* = 12) at a high glucose level (0.3%). The period length of the corresponding *bd* *frq-luc* control strain (Gooch et al. 2008) was nearly unaffected by glucose (low glucose: 20.58 ± 0.11 h, high glucose: 20.35 ± 0.03 h). These results suggest that CSP1 is required to compensate the circadian clock for changes in glucose concentration.

To determine how CSP1 levels affect the circadian clock, we generated a *frq-luc* reporter strain that expresses CSP1-R1Flag-His, a stable functional version of CSP1 (Sancar et al. 2011), under the control of the quinic acid (QA)-inducible *qa2* promoter. In the absence of QA, the *qa2-csp1-R1Flag-His* strain displayed a robust conidiation rhythm on race tubes with a period of 22.18 ± 0.07 h. At 60 μM QA, the period was ~2.5 h longer (24.52 ± 0.45 h), and at 6 mM QA, the overt conidiation rhythm was lost [Fig. 2A, top panel]. The conidiation rhythm of a corre-
its expression in response to glucose levels in a strain expressing csp1flag-His under the control of the endogenous csp1 promoter (Sancar et al. 2011). Levels of csp1flag-His RNA and CSP1Flag-His protein were higher in 2% than in 0.1% glucose [Fig. 3A,B]. Moreover, addition of 2% glucose to csp1Flag-His grown in low glucose resulted in induction of csp1Flag-His RNA and protein [Fig. 3C,D]. Even expression of the stable CSP1-R1Flag-His version under the control of the endogenous csp1 promoter was elevated at high glucose [Supplemental Fig. S2C]. The data indicate that glucose regulates expression of csp1 on the level of transcription.

Similar results were obtained when the glucose-dependent expression of nontagged CSP1 was analyzed in wild-type bd and wild-type strains [Supplemental Fig. S2A,B]. Notably, glucose-dependent expression of CSP1 was also observed in a Δwc2 strain [Supplemental Fig. S2D], indicating that glucose-induced csp1 transcription is not dependent on the WCC. In addition to the glucose, expression of CSP1Flag-His was induced by adding fructose and sucrose to the medium [Supplemental Fig. S2E,F]. This suggests that the availability of a carbon source is critical for CSP1 expression.

Analysis of our published CSP1 chromatin immunoprecipitation [ChIP] combined with deep sequencing [ChIP-seq] [Sancar et al. 2011] data suggests that CSP1 binds to the wc1 promoter (Fig. 4A). ChIP of CSP1 from strains that express either functional CSP1 [csp1Flag-His] or a DNA-binding mutant of CSP1 [Znmut-CSP1Flag-His] confirmed the binding of CSP1 to the wc1 promoter [Supplemental Fig. S3A]. To assess the functional relevance of CSP1 binding to the wc1 promoter, we measured wc1 RNA and WC1 protein expression in wild-type, Δcsp1, csp1Flag-His and csp1-R1Flag-His strains [Fig. 4B,C]. Wild type and csp1Flag-His expressed similar levels of wc1 RNA and WC1 protein,
entire \( wc1 \) promoter for the promoter of the nuclear pore complex gene \( nup98-96 \), which is not regulated by CSP1 (Sancar et al. 2011). Expression of WC1 was slightly less under the control of the \( nup98-96 \) than in a control strain, yet WC1 was functional and supported light-induced expression of target genes (Supplemental Fig. 4). The period length of the \( nup-wc1 \) strain decreased with increasing glucose concentration [Fig. 5] and displayed glucose dependence similar to the \( csp1 \) mutant strain. These data suggest that regulation of \( wc1 \) transcription by CSP1 is a major pathway for glucose compensation of the clock.

Expression levels of WC1 determine the period length of the circadian clock (Cheng et al. 2001). We therefore analyzed how glucose-dependent regulation of \( wc1 \) by CSP1 affects \( wc1 \) RNA and WC1 protein. At 0.1% glucose, when CSP1 is expressed at low levels, the levels of \( wc1 \) RNA were slightly higher (\(-1.4\)-fold) in \( \Delta csp1 \) than in wild type. At 2% glucose, \( wc1 \) RNA was less abundant in both strains relative to the ribosomal RNA that was used for normalization. However, \( wc1 \) RNA was \(-2.5\)-fold more abundant in \( \Delta csp1 \) than in wild type, reflecting the glucose-dependent increase of CSP1 expression in wild type [Fig. 6A]. Comparing the low- and high-glucose conditions suggests that CSP1 is a rather moderate transcription repressor under the conditions analyzed. We then analyzed WC1 protein [Fig. 6B]. At 0.1% glucose, when CSP1 expression is low, WC1 levels were similar in wild type and \( \Delta csp1 \). At 2% glucose, when CSP1 expression is high, WC1 levels in wild type were similar to those demonstrating that the epitope tagging did not compromise the function of CSP1. Levels of \( wc1 \) RNA and WC1 protein were elevated in \( csp1-R1Flag-His \) transformants of \( D \) used for normalization. (\( an \) e g a t i v ef e e d b a c kl o o pb yr e p r e s i n gt h e results suggest that CSP1 inhibits WC1 expression in a negative feedback loop by repressing the \( wc1 \) promoter. CSP1 modulates the expression of \(-800 \) genes (Sancar et al. 2011). To determine whether binding of CSP1 to the \( wc1 \) promoter is sufficient for glucose compensation of the circadian clock, it would be ideal to eliminate the CSP1-binding sites. However, the \( wc1 \) promoter region contains 69 potential CSP1-binding sites and CSP1 binds over an extended region, which makes a mutagenesis approach unfeasible [Fig. 4A]. We therefore exchanged the

![Figure 4](https://example.com/figure4)

**Figure 4.** CSP1 regulates \( wc1 \) expression by binding to the \( wc1 \) promoter. (A) ChIP-seq analysis of CSP1-binding sites of the \( wc1 \) promoter. Sequence reads are plotted above the genomic region of the \( wc1 \) locus. The arrows show the location of the WCC-binding sites [Smith et al. 2010]. The bar indicates the region used to design the primers used for ChIP-PCR. (B) RNA levels of \( wc1 \) in wild type [WT], \( \Delta csp1 \), \( csp1Flag-His \), and \( csp1-R1Flag-His \) were determined by qRT–PCR (\( \pm \)SEM, \( n = 4 \)). 28s rRNA was used for normalization. (C) Western blots showing WC1 and CSP1 levels of two independent experiments in wild type, \( \Delta csp1 \), \( csp1Flag-His \), and \( csp1-R1Flag-His \). Tubulin [TUB] was used as a loading control.

![Figure 5](https://example.com/figure5)

**Figure 5.** Expression of \( wc1 \) by a CSP1-independent promoter results in loss of glucose compensation of the circadian clock. (A) The \( nup-98-96 \) promoter was cloned in front of the \( wc1 \) ORF and transformed into \( \Delta wc1 \) \( bd \). Representative race tube assay of the \( nup-wc1 \) \( bd \) at different glucose concentrations. (B) Graph showing the free-running period length of the \( nup-wc1 \) \( bd \). The period lengths were \( 22.70 \pm 0.08 \) h [\( n = 9 \)] at 0% glucose, \( 22.44 \pm 0.03 \) h [\( n = 9 \)] at 0.1% glucose, \( 21.56 \pm 0.14 \) h [\( n = 9 \)] at 0.3% glucose, and \( 21.32 \pm 0.12 \) h [\( n = 9 \)] at 0.5% glucose. The period lengths are shown \( \pm \)SEM. [*] \( P < 0.05 \), [**] \( P < 10^{-5} \).
at 0.1% glucose, while expression levels of WC1 were elevated about twofold in \( \Delta \text{csp1} \) strains. The data suggest that CSP1 represses \( \text{wc1} \) transcription in a glucose-dependent manner, which apparently results in glucose-compensated expression of WC1.

The availability of glucose (or another carbon source) as an energy source affects metabolism in a rather general manner (Schneper et al. 2004; Ferenci 2007; Boer et al. 2010). We therefore measured the rate of protein synthesis as a function of glucose. Incorporation of \(^{35}\text{S}-\text{Met} \) was substantially higher at 2% than at 0.1% glucose in both wild type and \( \Delta \text{csp1} \), suggesting a reduced translation rate in low glucose. The observations suggest that the CSP1-dependent repression of \( \text{wc1} \) RNA at high glucose levels counterbalances the enhanced general translation efficiency. Thus, the fine-tuned modulation of \( \text{wc-1} \) transcription by CSP1 compensates the expression levels of WC1 against changes in glucose concentration.

**Discussion**

We show here that the *Neurospora* repressor CSP1 regulates transcription of \( \text{wc1} \) in response to glucose as well as fructose and sucrose. WC1 is the limiting subunit of the WCC, the core transcription activator of the circadian clock, and expression levels of the WCC determine the period length of the clock (Cheng et al. 2001). We show that glucose-dependent repression of \( \text{wc-1} \) transcription by CSP1 compensates for enhanced translation of WC1 at high levels of glucose, resulting in glucose-independent expression of the WCC (Fig. 7). Hence, CSP1 transduces information about the metabolic state of the cell to the core circadian oscillator.
and compensates the circadian clock against glucose-related changes of biochemical rates.

Metabolism enhances biochemical reaction rates, such as transcription and translation, in a rather general manner (Schneider et al. 2004; Ferenci 2007; Boer et al. 2010). Even moderate changes of expression rates could unbalance physiological processes and disturb cellular homeostasis. CSP1 is a global repressor that regulates ~800 genes in Neurospora (Sancar et al. 2011). It seems to compensate for the enhanced translation efficiency at elevated glucose (i.e., production of more polypeptides per RNA) on a global scale by reducing the rate of transcription of a large number of genes.

CSP1 is a short-lived protein ($t_{1/2} \approx 30$ min) that is rapidly inactivated by phosphorylation and then degraded (Sancar et al. 2011). Its transcription is controlled in a light- and clock-dependent manner by the WCC and an unidentified transcription factor. Since the WCC is a morning-specific activator, the target genes of CSP1 are expressed in the (subjective) morning. Hence, genes that are transcribed under the control of a noncircadian activator and rhythmically repressed by CSP1 tend to peak in the (subjective) evening.

One of these genes is wc1, and we show here that its transcription is repressed by CSP1 in a glucose-dependent manner. The transcription of wc1 and csp1 is regulated in a similar fashion (Kaldi et al. 2006; Sancar et al. 2011), which is crucial for glucose compensation of the clock. Basic transcription of both genes is independent of the WCC, indicating that unidentified transcription factors control their expression. In addition, the WCC activates transcription of csp1 and wc1 in a light-dependent manner. Regulation of csp1 and wc1 by a constitutive and a rhythmic activator and by a rhythmic glucose-dependent repressor predicts that the circadian expression profile of these genes (i.e., amplitude and phase) should depend on the metabolic state (e.g., glucose concentration). Overexpression versus deletion of csp1 indicates that CSP1 is a strong transcription repressor that has the potential to regulate wc-1 expression over a broad range. Yet under supposedly physiological conditions (0%–2% glucose), the dynamic range of glucose-dependent regulation of wc1 transcription in wild-type strains is rather narrow (~2.5 times). This is due to a negative feedback loop of Csp1 on its own gene, which limits the WCC-dependent amplitude of the csp1 RNA and CSP1 protein rhythm. Although the range of wc1 regulation is rather narrow, it covers the physiologically relevant conditions. We show that overexpression of Csp1 above physiological levels represses wc1 to an extent that compromises the circadian clock so that the cells become arrhythmic.

Regulation of wc1 transcription by CSP1 displays a striking similarity to that of the mammalian Bmal1 gene by REV-ERB-α and its partially redundant homolog, REV-ERB-β (Preitner et al. 2002; Liu et al. 2008; Cho et al. 2012). REV-ERB-α/β and Csp1 are global repressors that regulate a large number of genes involved in metabolism and link the circadian clock to energy homeostasis (Sancar et al. 2011; Bugge et al. 2012; Cho et al. 2012; Solt et al. 2012). REV-ERB-α affects in particular metabolism of lipids and glucose in a circadian fashion (Raspe et al. 2002; Anzulovich et al. 2006; Yin et al. 2007; Duez et al. 2008, Le Martelot et al. 2009; Cho et al. 2012). Similarly, CSP1 controls rhythmic expression of genes involved in lipid and glucose metabolism (Sancar et al. 2011).

The circadian clocks of Neurospora and mammals are evolutionarily distant, and CSP1 and REV-ERB-α/β are not related by sequence. Yet both components have the potential to sense, directly or indirectly, metabolic signals and adjust the phase (or free-running period) of their circadian oscillators in response to the metabolic state of the organism. Thus, tight coupling of circadian oscillators with metabolism seems to be a central function of circadian clocks to coordinate metabolic processes and establish homeostasis in a rhythmic environment.

Materials and methods

Neurospora strains and culture conditions

Strains indicated with bd carry the ras-1<sup>bd</sup> mutation (Belden et al. 2007) facilitating the detection of circadian comitant rhythms on race tubes. Fungal Genetics Stock Center (FGSC) #2489 was used as the wild-type strain. The csp1 mutant strain (FGSC #2555) carries a Cys-to-Tyr exchange in the DNA-binding domain, hence, it is inactive [Lambrechts et al. 2009]. The bd, his-3 strain was used for the transformation of frq-luciferase reporter constructs. The strain Δcsp1 [FGSC #11348] was generously crossed to his-3 by Deborah Bell-Pedersen’s group for transformation of the frq-luciferase reporter [Gooch et al. 2008]. Δwc1 bd his-3 (FGSC #9001) was used to generate nup-wc1 bd qa2-csp1-R1Flag-His<sup>D</sup> strains. Standard growth medium contained 2% glucose, 0.5% L-arginine, 1× Vogel’s medium, and 10 ng/ml biotin. In the experiments where different carbon sources and amounts were used, strains were grown in petri plates with 20 mL of standard medium with the indicated carbons source until mycelial mats formed. Mycelial pads (1 cm) were cut out and grown for 1 d in 100-mL flasks with 50 mL of Vogel’s medium with the indicated carbon [120 rpm at 25°C]. No additional carbon source was added to race tubes with QA.

Protein analysis

Neurospora protein extraction was performed as described (Schafmeier et al. 2006). Protein concentration was estimated by measuring absorption at 280 nm [NanoDrop, PeqLab]. Western blotting was performed as described [Gorl et al. 2001]. Nitrocellulose filters were stained with Ponceau S to control uniform loading of the gels. Enhanced chemiluminescence signals were detected with X-ray films. For denaturing extracts, peqGOLD TriFAST reagent [PeqLab] was used following the manufacturer’s protocol. To assess the translation rate at low glucose (0.1%) and high glucose (2%), mycelial discs were labeled with [35S]-Met (3.3 Ci/mL, 3.3 pM) for 15 min in 30 mL of Vogel’s medium. Labeling was terminated by adding 3.3 nM nonradioactive methionine.

RNA analysis

RNA was prepared with peqGOLD TriFAST [PeqLab]. The reverse transcription was done with the QuantiTect reverse
transcription kit (Qiagen) following the manufacturer’s instructions. Transcript levels were analyzed by quantitative real-time PCR in 96-well plates with the StepOnePlus real-time PCR system [Applied Biosystems]. TaqMan Gene Expression Master Mix [Applied Biosystems], TaqMan, and UPL probes [Roche] were used. Primers and probes are listed in Supplemental Table S1.

Generation of knock-in cassettes and Neurospora transformations

The yeast in vivo recombination system (Colot et al. 2006) was used to generate qa2-csp1-R1Flag-His. The 5’ region of csp1, the qa promoter, and a previously generated csp1-R1Flag-His knock-in cassette fragment [Sancar et al. 2011] were amplified with the primers listed in Supplemental Table S1. Transformation of Neurospora was performed as described (Colot et al. 2006).

Tandem ChIP

Nuclear extracts of csp1FlagHis and Znmut csp1FlagHis were subjected to tandem ChIP with Ni-NTA enrichment, followed by anti-Flag immunoprecipitation as described (Sancar et al. 2011). Primers and probes used for the ChIP-PCR are listed in Supplemental Table 1.

Real-time luciferase measurements

Luciferase reporter assays were carried out as described [Sancar et al. 2011]. We used 0.3% glucose as the carbon source where high-glucose conditions were used.

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Gencer Sancar, Cigdem Sancar and Michael Brunner

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