Modulation of Circadian Gene Expression and Metabolic Compensation by the RCO-1 Corepressor of *Neurospora crassa*

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ABSTRACT *Neurospora crassa* is a model organism for the study of circadian clocks, molecular machineries that confer ~24-hr rhythms to different processes at the cellular and organismal levels. The FREQUENCY (FRQ) protein is a central component of the *Neurospora* core clock, a transcription/translation negative feedback loop that controls genome-wide rhythmic gene expression. A genetic screen aimed at determining new components involved in the latter process identified *regulation of conidiation 1* (*rco-1*), the ortholog of the *Saccharomyces cerevisiae* Tup1 corepressor, as affecting period length. By employing bioluminescent transcriptional and translational fusion reporters, we evaluated *frq* and FRQ expression levels in the *rco-1* mutant background observing that, in contrast to prior reports, *frq* and FRQ expression are robustly rhythmic in the absence of RCO-1, although both amplitude and period length of the core clock are affected. Moreover, we detected a defect in metabolic compensation, such that high-glucose concentrations in the medium result in a significant decrease in period when RCO-1 is absent. Proteins physically interacting with RCO-1 were identified through co-immunoprecipitation and mass spectrometry; these include several components involved in chromatin remodeling and transcription, some of which, when absent, lead to a slight change in period. In the aggregate, these results indicate a dual role for RCO-1: although it is not essential for core-clock function, it regulates proper period and amplitude of core-clock dynamics and is also required for the rhythmic regulation of several *clock-controlled* genes.

KEYWORDS circadian clocks; corepressor; *Neurospora crassa*; core-clock mechanism; frequency

THE circadian oscillator of *Neurospora crassa* is based on a negative transcriptional/translational feedback loop (TTFL) where the expression of the *frequency* (*frq*) gene is rhythmically inhibited by its gene product, FRQ (Aronson *et al.* 1994). *frq* transcription is controlled by the White Collar Complex (WCC) (Crosthwaite *et al.* 1997), formed by the White Collar 1 (WC-1) and White Collar 2 (WC-2) GATA-type transcription factors (TFs) that recruit the activating SWI/SNF complex (Wang *et al.* 2014). As FRQ is produced,

it dimerizes and interacts with FRQ Interacting RNA Helicase (FRH) and casein kinase 1 (CK1), leading to the inhibition of WCC and correlated with phosphorylation events that involve several kinases, including CK1, thereby closing the TTFL. FRQ itself is progressively phosphorylated, which affects its interaction with other partners and localization, leading finally to its degradation (reviewed in Baker *et al.* 2012; Montenegro-Montero and Larrondo 2013; Montenegro-Montero *et al.* 2015). These phosphorylations (Baker *et al.* 2009), and not the degradation of FRQ itself, is what is key in determining circadian period (Larrondo *et al.* 2015).

Up to 40% of the transcriptional units in *Neurospora* are subject to circadian regulation (Zhu *et al.* 2001; Correa *et al.* 2003; Nowrousian *et al.* 2003; Dong *et al.* 2008; Hurley *et al.* 2014). Among the phenotypic traits that are under circadian control is asexual reproduction (conidiation), which has been used for years as an easy-to-score readout of the clock,

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allowing robust evaluation of circadian rhythmicity. Thus, when *Neurospora* is grown under constant dark conditions: constant dark (DD) in horizontal hollow-glass tubes known as race tubes, the generation of a conidial band is observed every 22.5 hr (Pittendrigh *et al.* 1959; Sargent *et al.* 1966), revealing the period dictated by the circadian oscillator. Beginning nearly 50 years ago, genetic screens for rhythm mutants using race tubes yielded a number of mutations in genes encoding clock components, in particular *frq*, and in genes encoding ancillary factors modulating core-clock dynamics (Feldman and Hoyle 1973; Gardner and Feldman 1980; Loros *et al.* 1986). The molecular analyses of these and other mutations have contributed to the detailed understanding of conserved core-clock mechanisms across phyla (Shigeyoshi *et al.* 1997). However, in the race tube assay there are many steps of regulation separating the circadian oscillator from the observed final rhythm in asexual development and, therefore, mutations in any of the genes connecting the oscillator with rhythmic banding may yield a strain that is overtly arrhythmic while still possessing a functional clock. Additionally, while in general molecular outputs from the clock were not expected to feed back to influence the oscillator itself (Bell-Pedersen *et al.* 1992), counterexamples to this have emerged as diverse outputs have been shown to influence the state of the oscillator (Pregueiro *et al.* 2006; O'Neill *et al.* 2008; Sancar *et al.* 2012; Hong *et al.* 2013). In particular in higher organisms, recent findings have shown that the regulation of metabolism by the circadian clock and its components is reciprocal, and that core elements of the circadian clock are able to sense the alterations in cell metabolism (Brewer *et al.* 2005; Liu *et al.* 2007; Kalsbeek *et al.* 2008; Nakahata *et al.* 2008, 2009; Bass and Takahashi 2010; Grimaldi *et al.* 2010; Cho *et al.* 2012; Jeyaraj *et al.* 2012). Given this and the observation that large proportions of the genomes of model systems appear to be clock controlled (e.g., Hurley *et al.* 2014), it follows that there must be means to insulate the clock from aspects of metabolism, in particular those aspects that can be affected by the environment; otherwise, the clock would lose its utility in the same way that a clock whose period is strongly influenced by ambient temperature would lose adaptive significance. The fact that circadian systems needed such compensation mechanisms, explicitly temperature and nutritional compensation, has been long appreciated (e.g., Hastings and Sweeney 1957; Pittendrigh and Caldarola 1973), but only recently have genetic determinants of such compensation been identified. In such mutants the clock maintains its rhythm but displays altered periodicity when grown, for instance, under different glucose concentrations. Genes influencing nutritional/metabolic compensation include the transcriptional repressor CSP-1 (Sancar *et al.* 2012) whose loss leads to a loss of metabolic compensation evidenced as period shortening at high-glucose levels, and the RNA helicase PRD-1 whose loss leads to the reverse effect, period lengthening under conditions of high nutritional carbon (Emerson *et al.* 2015).

In this study, by employing a candidate reverse genetics approach and a luciferase-based platform, we show that the transcriptional corepressor RCO-1 is involved in the regulation of rhythmic gene expression downstream from the central oscillator. RCO-1 was first identified in *Neurospora* as a regulator of conidiation, since mutants of this protein exhibited augmented expression of conidiation (*con*) genes during mycelial growth and a phenotypic impairment of this process (Yamashiro *et al.* 1996). Later on, RCO-1 was also implicated in the regulation of light responses (Olmedo *et al.* 2010). Although it has been recently reported that RCO-1 regulates *frq* expression and is therefore essential for clock function (Zhou *et al.* 2013; Liu *et al.* 2015), bioluminescence-based analyses conducted on solid media have revealed that although conidiation is impaired in a $\Delta rco-1$ strain, the central clock remains functional albeit with a slower period. RCO-1 acts as a corepressor and primarily controls the rhythmic expression of numerous genes associated with clock-controlled processes. Although several TFs were identified as specific integrators of RCO-1 activity, including HSF-2, a PHD TF and the metabolic-related TFs CSP-1 and CRE-1, these interactors do not appear to play a key part in regulating these circadian roles. Thus, our results indicate that RCO-1 is a protein that not only controls the expression of different biological processes in *Neurospora*, but that also impinges core-clock dynamics modulating proper period length and metabolic compensation of the circadian clock.

Materials and Methods

Strains and culture conditions

Fungal Genetics Stock Center (FGSC) 2489 (*wt*, A) and FGSC 11371 ($\Delta rco-1$, A) strains were used in this study. For race-tube and luciferase assays, these strains were crossed with a *his-3::frq_{c-box}-luc*, *ras-1^{bd}* reporter strain. Cultures were performed in solid media (Vogel's 1 $\times$, 0.03% glucose, 0.05% arginine) supplemented with quinic acid (QA) (0.01 M) unless indicated otherwise. For monitoring *in vivo* FRQ levels, a *frq^{luc}* strain (Larrondo *et al.* 2012) was crossed with FGSC 11371 ($\Delta rco-1$, A). Strains containing translational fusions with LUC, V5, or HA were generated through knockin strategies as previously described (Larrondo *et al.* 2009) and backcrossed to FGSC 2489 (*wt*, A) to obtain homokaryotic strains. Published $\Delta rco-1$ strains (referred to as RCO^{ZZ}, as in Zhou *et al.* (2013) were kindly provided by the authors. RCO^{ZZ}, *his-3* was crossed to *frq_{c-box}-luc* or *frq^{luc}* reporter strains, and *luc⁺*, RCO^{ZZ} progeny were analyzed. In all cases, the absence of the *rco-1* sequence was confirmed by a multiplex PCR.

Time course experiments

To assess messenger RNA (mRNA) and protein levels of genes of interest, time-course experiments were performed in constant conditions (25° and DD). Unless indicated otherwise, time courses were conducted on solid media conditions as

previously described (Larrondo *et al.* 2015). Briefly, the strains were pregrown in Petri dishes with 25 ml of liquid medium containing Vogel's salts, 2% sucrose, 0.5% arginine, and 50 ng/ml biotin for 48 hr in constant light: constant light (LL); in some cases, Tween-80 at 0.2% was used as a wetting agent (Olmedo *et al.* 2010). From there, tissue pads of 5 mm were cut and inoculated on plates with solid media using cellophane-covered Petri dishes with 30 ml of maltose media (1× Vogel's salts, 0.5% maltose, 2% agar) (Schneider *et al.* 2009) or low nitrogen- CCD (LN-CCD) media (1× Vogel's salts, 0.03% glucose, 0.05% arginine, 50 ng/ml biotin, 1.5% agar), supplemented with QA 0.01 M. For each time course, 13 plates were inoculated and incubated in LL for 4 hr, after which plates were transferred individually every 4 hr to DD conditions. After 48 hr, cultures were harvested under a safe red light, ground in liquid nitrogen, and RNA or protein extracted.

RNA extraction and RT-qPCR analysis

RNA was extracted with TRIzol as previously described (Kramer 2007) and then analyzed by quantitative RT-PCR. One microgram of total RNA was treated with 1 unit DNase (Promega, Madison, WI) at 37° for 1 hr. After inactivation of DNase by incubation at 65° for 15 min, the reverse transcription reaction was carried out according to the manufacturer's protocol (SuperScript III, Invitrogen, Carlsbad, CA). Analysis of complementary DNA (cDNA) was performed using the SYBR Green-based method.

Luciferase reporter assays

Unless specified otherwise in the figure legends, all *Neurospora* strains were inoculated in 96-well plates and grown at 25° in 12 hr light, 12 hr darkness (LD) cycle for 72 hr before being transferred DD for luciferase analyses. The luciferase reporter assay analyses were performed as reported (Larrondo *et al.* 2015). Briefly, 96 well plates were inoculated with LN-CCD media (1× Vogel's salts, 0.03% glucose, 0.05% arginine, 50 ng/ml biotin, 1.5% agar), supplemented with QA 0.01 M and 12.5 μM luciferin. Luciferase signals were acquired with a PIXIS 1024B camera (Princeton Instruments) and quantified with a customized macro developed for the ImageJ software; luminescent signals were accumulated for 5 min, three times per hour over the time course in DD. For metabolic compensation assays, glucose concentration was changed as indicate in the text.

Protein extraction, co-immunoprecipitation, and Western blotting

Protein lysates and Western blot analyses were performed as described (Hurley *et al.* 2012) with slight modifications. Protein lysates were prepared with DTT, PMSF, and Halt protease inhibitor cocktail (Thermo Scientific). For Western blot analysis, between 20 and 100 μg of total protein was loaded per lane. Anti-V5 antibody (Invitrogen) was diluted 1:5000. Anti-HA antibody (Sigma, St. Louis, MO) was diluted 1:1000. SuperSignal West Pico ECL (Pierce

Chemical, Rockford, IL) was used for signal development. For co-immunoprecipitation (Co-IP) assays, 2 mg of total protein was incubated with 50 μl of anti-V5 agarose beads (Sigma) for 2 hr at 4° and washed four times with cold protein extraction buffer (PEB) and resuspended in 50 μl of 2× loading buffer.

Mass-spectrometry analysis

Liquid medium containing Vogel's salts, 2% glucose, and 50 ng/ml biotin was used. Disks of mycelia were cut from mats of mycelia, transferred to 500 ml of fresh medium in 1000-ml flasks, and shaken at 125 rpm under LL at 25° for 48 hr. Approximately 80 mg of protein was combined with 50 μl Dynabeads α-V5 conjugated (5 μg antibody/mg Dynabeads) and incubated for 90 min. Beads were washed four times with 1 ml cold PEB [50 mM HEPES/KOH (pH 7.4), 137 mM NaCl, 10% glycerol, 0.4% NP-40, 5 mM EDTA containing 0.5 PMSF], and proteins were eluted by adding 50 μl Inge's 2× sample buffer minus DTT. Purified proteins were reduced by adding DTT to 5 mM and incubated at 70° for 20 min. Alkylation of cysteines was carried out by adding iodoacetamide to 13 mM final concentration. Samples were then loaded directly onto a SDS-PAGE gel or Trichloroacetic acid (TCA) precipitated (in the latter case, the antibody α-V5 was cross-linked to the Dynabeads). To control for nonspecific interactions, lysates prepared from the wild-type (WT) strain were carried through the purification protocol in parallel. The list of proteins identified by tandem mass spectrometry (MS) in the WT samples was subtracted from the list of proteins identified in RCO-1^{V5} in both protocols. An arbitrary cutoff of 20 or 10 peptides (identified for each protein) was chosen, and a list of interactors was generated (Supplemental Material, Table S1).

Determination of FRQ levels through the luciferase assay system

In order to evaluate FRQ levels, strains carrying a translational fusion between FRQ and LUC were used (Larrondo *et al.* 2012). The strains were grown in plates with 25 ml of liquid medium containing 1× Vogel's salts, 2% sucrose, 0.5% arginine, and 50 ng/ml biotin for 48 hr; in some case Tween-80 at 0.2% was used as a wetting agent. Tissue pads of 5 mm were cut and inoculated in plates with LN-CCD media supplemented with QA (0.01 M) (Olmedo *et al.* 2010). Cultures were incubated at 25° in LL condition for 48 hr; after this time, the tissue was harvested, dried, and snap frozen with liquid nitrogen. Mycelia were ground and processed according to the manufacturer's instructions (Promega, luciferase assays system E1500). Luminescence detection was performed using a Cytation 3 plate reader (BioTek): briefly, into a 96 Microwell white polystyrene plate (NUNC no. 136101) 20 μl of cell lysate was dispensed, after which 100 μl of luciferase assay reagent was added and the samples were mixed for 10 sec. In one well only the luciferase assay reagent was dispensed, and this well was used as a background control. The light produced during 10 sec was measured. Total

Figure 1 The absence of SUB-1 or RCO-1 affects the rhythmic expression of a *con-10^{LUC}* circadian output reporter. A luciferase reporter (*con-10^{LUC}*) was used as a proxy for circadian output regulation, and the impact of eliminating selected TFs was evaluated. The absence of SUB-1 ($\Delta sub-1$) leads to decreased amplitude and a phase change of CON-10^{LUC} expression, while in $\Delta rco-1$ expression of CON-10^{LUC} appears high and arrhythmic. The upper row depicts graphs containing raw luminescence data (A), while in the graphs in the lower row LUC levels were normalized using Spectrum resampling (B) (Costa *et al.* 2013).

protein concentration was determined by Bradford assay (Bradford 1976). The results were plotted, indicating the luminescence (Arbitrary Units)/total protein, and at least five biological and two technical replicates were measured.

Results

A luciferase-based reverse genetics approach identifies new components involved in the regulation of rhythmic gene expression downstream from the central oscillator

As a way of identifying new transcriptional regulators involved in the transmission of temporal information downstream of the FRQ-WCC oscillator (FWO), we utilized a luciferase-based approach in which the ORF of *con-10* (Lauter and Yanofsky 1993), a robustly rhythmic clock-controlled gene (*cgc*), was fused to an optimized luciferase reporter sequence (Gooch *et al.* 2008; Larrondo *et al.* 2012). Because the CON-10^{LUC} reporter is a translational fusion (at the endogenous *con-10* locus), KOs of transcriptional regulators of interest (TFs) (Colot *et al.* 2006) can be crossed to it without risk of mutation by RIPping (repeat-induced point mutation) (Galagan and Selker 2004) the *con-10* sequence as it would happen with ectopic transcriptional reporters. In order to test the feasibility of such a *cgc* reporter for future genetic screens, we began with a proof-of-principle candidate strategy by crossing TF KO strains that we suspected could modulate *con-10* expression. Among the first five tested KO strains, we detected different degrees of changes in CON-10^{LUC} dynamic levels (Table S2), with $\Delta sub-1$

and $\Delta rco-1$ displaying the most dramatically impaired circadian patterns (Figure 1). While SUB-1 is a GATA type TF known to be involved in late light responses (Chen *et al.* 2009), RCO-1 is part of a corepressor transcriptional complex that has also been reported to modulate light responses in *Neurospora* (Olmedo *et al.* 2010; Ruger-Herreros *et al.* 2014). Analysis of the oscillator in the absence of SUB-1 confirmed that, as previously reported (Chen *et al.* 2009) the oscillator is functional in that strain (data not show), and therefore this report will only focus on RCO-1.

RCO-1 is not essential for core-clock function although it influences circadian parameters

In *Neurospora*, rhythmic conidiation is a clear output of the circadian clock that can be observed under DD conditions in strains carrying the *ras-1^{bd}* mutation (Belden *et al.* 2007). Since the $\Delta rco-1$ mutant has impaired asexual development (Yamashiro *et al.* 1996), we were unable to evaluate rhythmic conidiation in race tubes. Moreover, it has been reported that in $\Delta rco-1$ strains, phenotypic and molecular circadian output, as well as core-clock function are lost (Zhou *et al.* 2013). We confirmed, as shown in Figure 2A, that the absence of RCO-1 leads to reduced growth, loss of conidia formation, and absence of rhythmic banding. We then utilized an *in vivo* bioluminescence transcriptional reporter of the clock (*frq_{c-box}-luc*) (Gooch *et al.* 2014a; Larrondo *et al.* 2015) to evaluate the state of the central oscillator in the *rco-1* mutant strain. This luciferase core clock reporter revealed the presence of robust rhythms, and moreover a significant increase in period length (~4 hr) (Figure 2B). In

Figure 2 The absence of RCO-1 abrogates rhythms in conidiation and leads to a functional circadian oscillator with altered period and amplitude. (A) Race tube assays of WT (*rco-1⁺*, *ras-1^{bd}*) and $\Delta rco-1$ ($\Delta rco-1$, *ras-1^{bd}*) strains in DD confirms the absence of overt circadian rhythms in conidiation in $\Delta rco-1$. (B) WT (*rco-1⁺*, *ras-1^{bd}*) and $\Delta rco-1$, *ras-1^{bd}* strains carrying the transcriptional reporter *frq_{C-box}-luc* were analyzed, confirming the presence of a functional core circadian oscillator in $\Delta rco-1$, which exhibits a long period and decreased amplitude. (C) The absence of RCO-1 was analyzed in combination with the *frq⁷* allele, observing a further increase in period consistent with a FWO-based rhythm. The luminescence data were analyzed using Spectrum resampling (Costa *et al.* 2013). Period $\pm$ SEM, WT: 20.89 ± 0.5 ; $\Delta rco-1$: 25.88 ± 0.36 ; *frq⁷* WT: 28.66 ± 0.16 ; *frq⁷* $\Delta rco-1$: 33.74 ± 0.26 . (D) Real-time qPCR analyses show rhythms in *frq* mRNA levels in time courses conducted in solid media conditions. Three biological replicates were measured and *frq* expression was normalized using two reference genes: *tbp* (NCU04770) and *suc* (NCU08336). For plotting, DD4 of WT strain was set to 1, and other time points were normalized accordingly. To evaluate *frq* rhythmicity, data were analyzed with ARSER (Yang and Su 2010), revealing that its expression was rhythmic ($P < 0.05$) in both strains. (E) Representative Western blots of samples derived from time courses as described in D, reveal oscillating levels of FRQ in WT and $\Delta rco-1$ strains. A densitometric analysis (right) was conducted for three biological replicates of each strain. Standard error is shown. For plotting, the LL time point was set to 1 and the other points were normalized accordingly.

order to further confirm that these oscillations depend on the negative element FRQ and do not reflect a FRQ-Less Oscillator (FLO) (Brody *et al.* 2010), we employed the *frq⁷* allele, which lengthens the period of the canonical core circadian clock but

does not affect FLO-based phenomena. As shown in Figure 2C, in a *frq⁷* background, $\Delta rco-1$ displays an even longer period for the reporter gene, consistent with the rhythms in $\Delta rco-1$ depending on a FRQ-based TTFL.

Figure 3 *rco-1* transcription and translation are rhythmic although its mRNA and protein levels do not appear to oscillate. (A) Reporter construct containing the *rco-1* promoter region (3000 bp) fused to *luciferase* was integrated at the *his-3* locus and the luminescence data were measured as previously described. (B) A translational fusion between RCO-1 and LUC was engineered at the endogenous *rco-1* locus by homologous recombination, and the resulting strain was analyzed under the same experimental conditions as A. (C) *rco-1* and *frq* mRNA levels were measured over a time course experiment conducted under solid media conditions, utilizing *tbp* (NCU04770) and *suc* (NCU08336) as reference genes. (D) Quantification of FRQ and RCO-1 protein levels from time courses as described in C. As V5-tagged strains of FRQ or RCO-1 were employed to facilitate detection, Western blots were performed with α -V5 antibody, followed by densitometric analyses (levels at DD4 was set as 1 for normalization), confirming rhythmic expression of FRQ and constant nonrhythmic levels of RCO-1. The figure is representative of three biological replicates. (E) Cycloheximide (CHX) was used to determine RCO-1^{V5} stability. Samples were grown in liquid culture media (LCM) 0.03% glucose for 48 hr after which CHX 10 μ g/ml was added. Cultures were transferred to DD and harvested every 2 hr. Western blot with α V5 (left) and the corresponding densitometric analysis (right) reveal that RCO-1 exhibits high stability. The data correspond to three biological replicates. FRQ^{V5} was used as a control of an unstable protein.

Table 1 Putative targets of RCO-1 involved in clock regulate or related process

Gene number	Gene description	Biological process
NCU00629	6-phosphofructokinase	C-Carbon metabolism
NCU01227	Probable succinyl-CoA ligase	
NCU01528	Glyceraldehyde-3-phosphate dehydrogenase-1	
NCU02139	Annexin ANXC4	
NCU02713	Conidial separation-1	
NCU03317	Glucosyltransferase	
NCU03415	Aldehyde dehydrogenase	
NCU04851	Conserved hypothetical protein	
NCU04866	All development altered - 6	
NCU05855	O-methyltransferase	
NCU07027	Glycogen phosphorylase	
NCU07442	Betaine aldehyde dehydrogenase	
NCU09873	Phosphoenolpyruvate carboxykinase	
NCU10007	Malate synthase	
NCU11201	Phosphoglycerate mutase	
NCU06660	Plasma membrane proteolipid 3	Response to light stimulus
NCU07649	Integral membrane protein	
NCU05964	Developmental regulator VosA	
NCU07846	Conserved hypothetical protein	
NCU07441	Conserved hypothetical protein	
NCU09883	Conserved hypothetical protein	
NCU09043	Caleosin domain containing protein	
NCU03863	Conserved hypothetical protein	
NCU04120	Calmodulin A	
NCU04414	Conserved hypothetical protein	
NCU04990	Protein serine/threonine kinase	
NCU05599	40S ribosomal protein S28	
NCU05770	Peroxidase/catalase 2	Response to stress
NCU05789	Secreted glucosidase	
NCU06340	TPR repeat protein	
NCU07024	Osmotic sensitive-2	
NCU07232	Heat shock protein 30	
NCU07277	Anchored cell wall protein-8	
NCU10051	Flavohemoglobin	
NCU00212	Histone h4	
NCU01634	Histone H4-1	
NCU02621	C2H2 transcription factor	
NCU02713	Conidial separation-1	
NCU03552	Conserved hypothetical protein	Transcriptional control
NCU04851	Conserved hypothetical protein	
NCU04866	All development altered - 6	
NCU06095	Grainy-head homolog	
NCU06266	Histone-lysine N-methyltransferase	
NCU08055	b-ZIP transcription factor IDI4	
NCU08159	Conserved hypothetical protein	
NCU08594	Conserved hypothetical protein	
NCU09033	C6 transcription factor	
NCU04414	Conserved hypothetical protein	
NCU07846	Conserved hypothetical protein	

These results demonstrate the existence of circadian rhythms in $\Delta rco-1$, unambiguously revealed *in vivo* by employing the abovementioned clock reporter. Intriguingly, Zhou *et al.* (2013) reported elevated and arrhythmic FRQ levels as well as nonoscillatory *frq* expression in the absence of RCO-1 by Western and Northern blots, respectively. To gain further insights into those published results, circadian time course experiments on solid media were performed and both FRQ and *frq* mRNA levels were analyzed. As shown in Figure 2, D and E, rhythmic expression of FRQ and *frq* was detected in both WT *N. crassa* and in the $\Delta rco-1$ strain

obtained from the FGSC KO collection (Colot *et al.* 2006). Also, to obtain additional proof of these circadian rhythms, we analyzed the mutant strain using a destabilized luciferase reporter (Cesbron *et al.* 2013) containing the same clock promoter (*c-box*) at a different locus (*csr*) (Bardiya and Shiu 2007), further confirming that *frq* expression is rhythmic in the absence of RCO-1 (Figure S1). Importantly, in addition to the $\Delta rco-1$ strains obtained from the FGSC, we also analyzed the mutant *rco-1* strain developed by Zhou *et al.* (2013), herein referred to as RCO^{ZZ} to differentiate it from the one generated by the *Neurospora* KO consortium (Colot

Figure 4 RCO-1 regulates the expression of several genes involved in a variety of processes. (A) The expression of several putative RCO-1 target genes was evaluated by RT-qPCR in WT and $\Delta rco-1$ strains under LL conditions. The plots indicate the results for a set of genes from each biological process described in Table 1 and also for some genes previously described as targets of RCO-1. Almost all analyzed genes present higher expression levels in the $\Delta rco-1$ mutant. The data corresponded to three biological replicates. A Mann-Whitney test was performed; significance is indicated with an asterisk (* $P < 0.05$). (B) Heat map representation of gene expression (measured through RT-qPCR) in absence of RCO-1 in three independent biological replicates (Nb); only 58% of the evaluated genes changed their expression levels in absence of the corepressor.

et al. 2006). Thus, RCO^{ZZ} was crossed with our clock reporter strains and evaluated under our experimental conditions, revealing rhythmicity of both clock reporters as analyzed by bioluminescent traces (Figure S2) and further confirming that RCO-1 is not essential for core-clock function. We confirmed the observations by Zhou *et al.* (2013) regarding higher *frq* expression as well as WC-1-independent expression of this gene in the absence of RCO-1 (Figure S3). In the aggregate, although RCO-1 is not an essential clock component, the absence of RCO-1 affects key circadian parameters such as period and amplitude, suggesting that it directly or indirectly affects core-clock components.

***rco-1* transcription and translation are rhythmic although its mRNA and protein levels do not appear to oscillate**

To evaluate the role of RCO-1 in determining the period length of the clock, we first assessed if *rco-1* itself is a *cgc*. For this purpose, we generated a transcriptional (Figure 3A) and translational (Figure 3B) luciferase-based reporter by fusing *luc* (Gooch *et al.* 2008) to the *rco-1* promoter or ORF, respectively, and observed rhythmic expression for both transcriptional and translational reporters (Figure 3, A and B). In contrast, steady-state mRNA and protein levels (Figure 3, C and D) showed nonoscillatory expression, a pattern seen for other clock-controlled genes in *N. crassa* (Hurley *et al.* 2014). Transcriptional reporters of two negative control genes were evaluated on the same experimental conditions, showing no obvious circadian rhythmicity (Figure S4). This reinforces the idea that the oscillations seen for *rco-1* have a *bona fide* circadian component and are not just part of a global oscillatory phenomenon. Importantly, evidence of rhythmic transcription but flat mRNA is not new (Wuarin and Schibler 1990; Millar and Kay 1991), and lately it has

received increasing attention as genomewide studies have shown it to be a widespread phenomenon (Menet *et al.* 2012; Montenegro-Montero and Larrondo 2015). In addition, highly stable proteins may fail to display oscillatory behavior even if their transcription is rhythmic (Millar *et al.* 1992). To confirm that the latter could partially explain overall RCO-1 levels, we evaluated its stability, finding that indeed RCO-1 is very stable (Figure 3E), indicating that although both transcription and translation of RCO-1 have a circadian component to them, total levels remain constant. Recently, the existence of rhythms in Mg^{++} levels in several systems, including *Neurospora*, was reported (Feeney *et al.* 2016). In the case of mammalian cells, it was shown that they could impact translation through the mechanistic Target Of Rapamycin (mTOR) pathway. Therefore, in the future it will be important to understand the contribution of Mg^{++} oscillations in the post-transcriptional regulation of *Neurospora* *cgc*s.

***RCO-1* regulates the circadian expression of several genes that belong to clock-controlled processes**

Because RCO-1 is a corepressor, it is expected to regulate the expression of a number of genes. Published chromatin immunoprecipitation sequencing (ChIP-seq) data generated using a tagged version of RCO-1 (RCO-1^{FLAG-HIS}) revealed >600 putative RCO-1 targets after a light pulse of 30 min (Sancar *et al.* 2011). Comparing these reported ChIP targets with lists of putative *cgc*s from microarrays, bioluminescence reporter analyses, and RNA sequencing (RNA-seq) (Correa *et al.* 2003; Nowrousian *et al.* 2003; Hurley *et al.* 2014), we found that 32% of the RCO-1 targets correspond to potential *cgc*s. Among the identified genes, carbon metabolism and stress and light responses were recognized as biological categories along with several genes involved in transcriptional control

Figure 5 RCO-1 is involved in proper circadian control of several clock-controlled genes. Transcriptional luciferase reporters were used to evaluate the circadian expression of RCO-1 target genes in WT and $\Delta rco-1$ strains, revealing the loss of proper circadian control in $\Delta rco-1$. Bioluminescence was measured as previously described.

(Table 1). Consistent with its role as corepressor, RCO-1 negatively regulates the expression of several of these genes (Figure 4). However, some genes showed decreased expression in the absence of RCO-1, suggesting that this protein could be necessary for the recruitment of activators, or alternatively, that it represses a repressor of these genes. The effect of RCO-1 on *ccg* expression was confirmed using reporter constructs consisting of a codon-optimized luciferase gene (*luc*) (Gooch *et al.* 2008) under the control of the promoters of *ccg-13*, *gapdh* (*ccg-7*), *desaturase* (NCU09497), or *csp-1* (Figure 5) observing that, except for *gapdh*, rhythmicity was completely abolished (see also Figure S5) in a $\Delta rco-1$ strain. Indeed, *gapdh* rhythmicity was limited to only the first 2 days during which a defect in period and amplitude was also observed, suggesting that regulators in addition to RCO-1 impact this gene. RCO-1 clearly has a role in the control of output pathways, although rhythmicity may be only partially altered for some *ccgs*.

RCO-1 interacts with several transcription factors

Like its homolog Tup1 in yeast, RCO-1 is part of a corepressor complex with RCM-1 (Olmedo *et al.* 2010; Sancar *et al.* 2011). In *Saccharomyces cerevisiae*, Tup1 participates in the control of the expression of ~7% of the genome (DeRisi *et al.* 1997; Green and Johnson 2004) through its association with different TFs (Treitel and Carlson 1995; Park *et al.* 1999; Proft and Serrano 1999; Znaidi *et al.* 2004; Crisp *et al.* 2006; Hanlon *et al.* 2011). To better identify the biological processes that RCO-1 controls in *Neurospora*, we searched for

TFs that interact with this corepressor using Co-IP in a RCO-1^{V5} tagged strain followed by MS analyses (Table 2). Applying a strict cutoff, considering interactors with coverage of ≥ 20 peptides, yielded a list of 40 candidates including four TFs and three proteins with roles in chromatin remodeling, several interactions of which (HSF-2, CRE-1, and HDA-1) were confirmed biochemically (Figure S6). To determine whether the loss of regulation of any of these TFs or chromatin modifiers could be the cause of the long period in $\Delta rco-1$ strains, we examined the bioluminescent core-clock reporter (*frq_{C-box}-luc*) in individual KO of the TFs *hsf-2* (NCU08480), NCU05242, NCU05411 and a *phd* TF (NCU01238) as well as the chromatin regulator *hda-1*; however, we detected a modest period length shortening only in the absence of the PHD TF NCU01238 (Figure S7).

Metabolic compensation of the clock is impaired in absence of RCO-1

As described above, a metabolic compensation mechanism allows *Neurospora* to keep circadian period relatively constant despite variations in availability of carbon (such as glucose) or other nutrients (Sancar *et al.* 2012; Emerson *et al.* 2015). Consequently, when the fungus is unable to compensate for the excess of this nutrient, period may change as reported in the absence of the RNA helicase PRD-1 or the TF CSP-1 (Sancar *et al.* 2011, 2012). Based on RCO-1's function as a corepressor, and its ability to interact with CSP-1, we hypothesized that its absence should also produce a defect in metabolic compensation. First, we confirmed the reported

Table 2 Transcriptional regulators that interact with RCO-1 as inferred from mass spectrometry experimental data

Gene number	Gene description
NCU08480	Heat shock factor-2
NCU01238	PDH transcription factor
NCU05242	C2H2 transcription factor
NCU03875	Chromatin remodeling factor 4-1
NCU05411	Pathway-specific nitrogen regulator
NCU07556	Chromatin remodeling factor 8-1
NCU06394	Chromatin remodeling complex subunit

See Table S2 for extended data.

period decrease for $\Delta csp-1$ as a function of increasing glucose concentrations in race tubes (Figure S8) (Sancar *et al.* 2012), although this assay could not be performed for the $\Delta rco-1$ strain due to its lack of overt rhythms in conidiation. Therefore, we evaluated circadian dynamics in low- or high-glucose conditions (LN-CCD + 0% or 0.5% glucose, respectively), of WT and $\Delta rco-1$ strains utilizing the *frq_{c-box}-luc* reporter (Figure 6). Under low-glucose conditions, $\Delta rco-1$ exhibited a lengthened period compared to WT (as described earlier in the text, Figure 2), while a clear decrease in its period was observed in high-glucose medium, a change not observable in the WT strain. Surprisingly, under these experimental conditions (96-well plates), we failed to clearly observe the metabolic compensation defect previously described by Sancar *et al.* (2012) for $\Delta csp-1$ (Figure S9). Nevertheless, the effect of RCO-1 on circadian metabolic compensation is comparable to that played by CSP-1, which was expected; however, based on the fact that we can detect it under these assay conditions makes it likely that the role of RCO-1 in response to glucose is not only the direct consequence of its interaction with CSP-1, but by other TFs associated with RCO-1, or due to downstream effectors that somehow feedback to control core-clock mechanisms. Importantly, under the experimental conditions utilized to identify RCO-1 interactors, we failed to identify CSP-1 among RCO-1's most abundant partners (cutoff of 20 peptides), although we did observe it when the cutoff was less strict (10 peptides). *In toto*, these results reinforce the idea that although RCO-1 is not a core component of *Neurospora* circadian oscillator, it may play an ancillary role in the maintenance of proper period length and metabolic compensation of the clock, and is likely to do so via multiple interacting partners.

Discussion

In the search for transcriptional regulators involved in modulating circadian expression in *N. crassa*, we identified the corepressor RCO-1. Although much can be inferred regarding its molecular biology based on precedents from yeast, little is known about RCO-1's role in the biology of *Neurospora*. Studies performed in the late 1990s (Yamashiro *et al.* 1996; Lee and Ebbole 1998) indicate that this protein represses genes expressed during the process of conidiation (*con* genes); later

on, a relationship between photoadaptation and this corepressor was established (Olmedo *et al.* 2010; Ruger-Herreros *et al.* 2014), revealing that RCO-1 is necessary for proper photoadaptation of a small subset of genes.

Because RCO-1 is part of a corepressor complex (Olmedo *et al.* 2010; Sancar *et al.* 2011), and its yeast homolog Tup1 interacts with several proteins controlling different biological processes (Hanlon *et al.* 2011), we expected RCO-1 to regulate the expression of a large subset of genes in *Neurospora*. Indeed a $\Delta rco-1$ strain possesses a severe phenotype: slow growth and impairment in sexual and asexual reproduction, indicating a pivotal role of this protein in development and metabolism. Using new methods based on the *in vivo* measurement of luciferase activity in *Neurospora* (Gooch *et al.* 2008; Larrondo *et al.* 2012; Larrondo *et al.* 2015; Montenegro-Montero *et al.* 2015), we were able to evaluate circadian gene expression in the absence of this protein. Our first findings on *con-10^{LUC}* (the gene that we are utilizing as bait to identify circadian regulators) revealed that RCO-1 not only represses *con-10^{LUC}* expression but that it was also necessary for its rhythmic expression. This phenomenon could be explained in two ways: RCO-1 participates in the control of circadian output pathways or it impairs core-clock function (and therefore output as well). To evaluate the latter proposal, the expression of a transcriptional reporter of the core clock (*frq_{c-box}-luc*) (Gooch *et al.* 2014b; Larrondo *et al.* 2015; Montenegro-Montero *et al.* 2015) was analyzed along with overall levels of *frq* expression. Consistent with an earlier report (Zhou *et al.* 2013) we found WC-1-independent and elevated expression of *frq*/FRQ in strains lacking RCO-1; however, in independent analyses conducted in two separate laboratories we were unable to reproduce the arrhythmicity previously reported for $\Delta rco-1$ mutants (Zhou *et al.* 2013; Liu *et al.* 2015), using either stock strains from the *Neurospora* knockout project or the strain developed and utilized in the prior report (Zhou *et al.* 2013). Rather, the central oscillator remains functional in the absence of RCO-1 but displays, interestingly, a longer period in comparison with a WT strain. This behavior was also observed using a destabilized luciferase (*frq_{c-box}-luc^{PEST}*) (Cesbron *et al.* 2013) at a different locus in *Neurospora* or with a *frq^{luc}* translational reporter (Larrondo *et al.* 2012), and was also observed in a long period *frq⁷* mutant background. It is important to notice that in those published reports (Zhou *et al.* 2013; Liu *et al.* 2015), assays were performed under growth conditions that differ from ours: while they grew *Neurospora* on high-glucose liquid media, we mainly utilized solid media and low-glucose levels. Since RCO-1 interacts with several TFs that mediate glucose and metabolic responses (Figure S6 and (Sancar *et al.* 2011)) it is plausible that under such conditions (*i.e.*, high glucose and liquid) the absence of RCO-1 could lead to a stronger circadian phenotype, including low amplitude rhythms that may escape their detection methods. In addition, in liquid medium, biomass is mainly composed of submerged mycelia, a situation that could magnify the misregulation produced by the lack of RCO-1. Actually, such differences are quite

Figure 6 RCO-1 is required for proper metabolic compensation of the clock. WT and $\Delta rco-1$ strains carrying the transcriptional reporter *frq_{c-box}-luc* were inoculated in 96-well plates containing LN-CDD QA 0.01 M media with 0% glucose (low glucose, LG) or 0.5% glucose (high glucose, HG), and kept under LD 12:12 conditions for 72 hr prior to CCD analysis. Bioluminescence was measured as previously described. The absence of RCO-1 leads to reduced period in high-glucose conditions, indicating a loss of metabolic compensation of the clock. Period $\pm$ SEM. WT LG: 21.08 $\pm$ 0.23, HG: 20.94 $\pm$ 0.04; $\Delta rco-1$ LG: 24.52 $\pm$ 0.07, HG: 22.41 $\pm$ 0.095.

informative as they reveal that a dynamic network of transcription factors actively participates in *Neurospora*'s ability to respond to daily changes in the environment (including growth conditions) as well as in the maintenance of the basic circadian machinery that can also influence this network.

It is likely that in $\Delta rco-1$, the period defect arises from the effects of regulatory factors other than CSP-1 partnering with this corepressor. For example, eliminating the *phd* TF NCU01238 (Figure S7) provides a subtle defect in period, leading to a faster clock. Recently, studies in *Arabidopsis* have indicated that members of the Groucho/Tup1 corepressor family TOPLESS/TOPLESS-RELATED interact with histone deacetylases (Wang *et al.* 2013), and that depletion of the gene family results in a lengthening of the circadian period, a phenotype similar to the one observed here. Although we showed that the absence of *hda-1* by itself does not generate a change of period (Figure S7), in *Neurospora* there are four histone deacetylase genes and it is conceivable that one of these other genes may compensate for the lack of *hda-1*. It also remains possible that other RCO-1-interacting chromatin modifiers, such as the histone methyltransferase SET-2, could mediate this period effect. Indeed, defects in H3K36 methylation caused by the absence of SET-2 have been reported to trigger WC-1-independent *frq* transcription (Zhou

et al. 2013). Nevertheless, although RCO-1 is known to interact with RCM-1 and SET-2 at the *frq* promoter impacting its expression (Zhou *et al.* 2013; Liu *et al.* 2015), the TF(s) and *cis*-elements that mediate the recruitment of the RCO-1/RCM-1 complex to this locus still remain unknown.

Our results suggest that RCO-1 plays a major circadian role as a transcriptional regulator controlling clock output. To identify which circadian biological processes are regulated by this corepressor, we examined *in silico* putative RCO-1 targets (defined by published ChIP-seq data) in response to a light pulse (Sancar *et al.* 2011), cross-referencing them with a list of circadianly expressed genes (Hurley *et al.* 2014). Thus, we found that 32% of the putative RCO-1 target genes appear to be clock regulated and belong to different biological categories including metabolism, stress, and light response as well as transcriptional control. That an important percentage of RCO-1 targets genes correspond to *ccgs* strongly indicates RCO-1 itself, and the phenomenon of transcriptional repression, as central to circadian regulation and in particular to the circadian modulation of metabolism. In this way we can begin to understand how RCO-1 impacts metabolic/nutritional compensation.

The importance of nutritional parameters as environmental cues that can regulate circadian period and expression has been described in different organisms (Kohsaka *et al.* 2007; Nakahata *et al.* 2008; Xu *et al.* 2011; Hatori *et al.* 2012). A partner of RCO-1, CSP-1, has been reported as a major transcriptional regulator of metabolic genes in *Neurospora*, playing a key role in nutritional compensation of the clock, such that a decrease in period length is observed in high glucose in $\Delta csp-1$ strains (Sancar *et al.* 2011, 2012). Our results indicate that RCO-1 plays an equal or even greater role in this process, not only through its interaction with CSP-1, but also through its interactions with other TFs and/or chromatin regulators that separately influence expression of genes impacting metabolism. Importantly, although we recapitulated the metabolic compensation defect of $\Delta csp-1$ on race tubes, we were unable to detect a significant defect under the growth conditions utilized for 96-well luciferase analyses. Similarly, studies on a *prd-1* mutant, highlighted the difference in phenotypes observed in race tubes or 96-well assays that, for *prd-1*, were mainly due to changes in sugar levels and an underlying defect in metabolic compensation. Although we tried eliminating media composition as a variable, we could not observe a significant metabolic compensation defect for $\Delta csp-1$, which contrasts with the clear phenotype registered for $\Delta rco-1$. These observations strengthen the previous arguments regarding the participation of other transcriptional regulators that may interact with this corepressor and could therefore fully explain the phenotypes of $\Delta rco-1$.

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Literature Cited

- Aronson, B. D., K. A. Johnson, J. J. Loros, and J. C. Dunlap, 1994 Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. *Science* 263: 1578–1584.
- Baker, C. L., A. N. Kettenbach, J. J. Loros, S. A. Gerber, and J. C. Dunlap, 2009 Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol. Cell* 34: 354–363.
- Baker, C. L., J. J. Loros, and J. C. Dunlap, 2012 The circadian clock of *Neurospora crassa*. *FEMS Microbiol. Rev.* 36: 95–110.
- Bardiya, N., and P. K. Shiu, 2007 Cyclosporin A-resistance based gene placement system for *Neurospora crassa*. *Fungal Genet. Biol.* 44: 307–314.
- Bass, J., and J. S. Takahashi, 2010 Circadian integration of metabolism and energetics. *Science* 330: 1349–1354.
- Belden, W. J., L. F. Larrondo, A. C. Froehlich, M. Shi, C. H. Chen *et al.*, 2007 The band mutation in *Neurospora crassa* is a dominant allele of *ras-1* implicating RAS signaling in circadian output. *Genes Dev.* 21: 1494–1505.
- Bell-Pedersen, D., J. C. Dunlap, and J. J. Loros, 1992 The *Neurospora* circadian clock-controlled gene, *cgc-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes Dev.* 6: 2382–2394.
- Bradford, M. M., 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
- Brewer, M., D. Lange, R. Baler, and A. Anzulovich, 2005 SREBP-1 as a transcriptional integrator of circadian and nutritional cues in the liver. *J. Biol. Rhythms* 20: 195–205.
- Brody, S., K. Oelhafen, K. Schneider, S. Perrino, A. Goetz *et al.*, 2010 Circadian rhythms in *Neurospora crassa*: downstream effectors. *Fungal Genet. Biol.* 47: 159–168.
- Cesbron, F., M. Brunner, and A. C. Diernfellner, 2013 Light-dependent and circadian transcription dynamics in vivo recorded with a destabilized luciferase reporter in *Neurospora*. *PLoS One* 8: e83660.
- Chen, C. H., C. S. Ringelberg, R. H. Gross, J. C. Dunlap, and J. J. Loros, 2009 Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in *Neurospora*. *EMBO J.* 28: 1029–1042.
- Cho, H., X. Zhao, M. Hatori, R. T. Yu, G. D. Barish *et al.*, 2012 Regulation of circadian behaviour and metabolism by REV-ERB- α and REV-ERB- β . *Nature* 485: 123–127.
- Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew *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.
- Correa, A., Z. A. Lewis, A. V. Greene, I. J. March, R. H. Gomer *et al.*, 2003 Multiple oscillators regulate circadian gene expression in *Neurospora*. *Proc. Natl. Acad. Sci. USA* 100: 13597–13602.
- Costa, M. J., B. Finkenstadt, V. Roche, F. Levi, P. D. Gould *et al.*, 2013 Inference on periodicity of circadian time series. *Biostatistics* 14: 792–806.
- Crisp, R. J., E. M. Adkins, E. Kimmel, and J. Kaplan, 2006 Recruitment of Tup1p and Cti6p regulates heme-deficient expression of Aft1p target genes. *EMBO J.* 25: 512–521.
- Crosthwaite, S. K., J. C. Dunlap, and J. J. Loros, 1997 *Neurospora* *wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. *Science* 276: 763–769.
- DeRisi, J. L., V. R. Iyer, and P. O. Brown, 1997 Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278: 680–686.
- Dong, W., X. Tang, Y. Yu, R. Nilsen, R. Kim *et al.*, 2008 Systems biology of the clock in *Neurospora crassa*. *PLoS One* 3: e3105.
- Emerson, J. M., B. M. Bartholomai, C. S. Ringelberg, S. E. Baker, J. J. Loros *et al.*, 2015 *period-1* encodes an ATP-dependent RNA helicase that influences nutritional compensation of the *Neurospora* circadian clock. *Proc. Natl. Acad. Sci. USA* 112: 15707–15712.
- Feeney, K. A., L. L. Hansen, M. Putker, C. Olivares-Yanez, J. Day *et al.*, 2016 Daily magnesium fluxes regulate cellular time-keeping and energy balance. *Nature* 532: 375–379.
- Feldman, J. F., and M. N. Hoyle, 1973 Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics* 75: 605–613.
- Galagan, J. E., and E. U. Selker, 2004 RIP: the evolutionary cost of genome defense. *Trends Genet.* 20: 417–423.
- Gardner, G. F., and J. F. Feldman, 1980 The *frq* locus in *Neurospora crassa*: a key element in circadian clock organization. *Genetics* 96: 877–886.
- Gooch, V. D., A. Mehra, L. F. Larrondo, J. Fox, M. Touroutoudis *et al.*, 2008 Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. *Eukaryot. Cell* 7: 28–37.
- Gooch, V. D., A. E. Johnson, B. J. Bourne, B. T. Nix, J. A. Maas *et al.*, 2014a A kinetic study of the effects of light on circadian rhythmicity of the *frq* promoter of *Neurospora crassa*. *J. Biol. Rhythms* 29: 38–48.
- Gooch, V. D., A. E. Johnson, L. F. Larrondo, J. J. Loros, and J. C. Dunlap, 2014b Bright to dim oscillatory response of the *Neurospora* circadian oscillator. *J. Biol. Rhythms* 29: 49–59.
- Green, S. R., and A. D. Johnson, 2004 Promoter-dependent roles for the *Srb10* cyclin-dependent kinase and the *Hda1* deacetylase in Tup1-mediated repression in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 15: 4191–4202.
- Grimaldi, B., M. M. Bellet, S. Katada, G. Astarita, J. Hirayama *et al.*, 2010 PER2 controls lipid metabolism by direct regulation of PPARGamma. *Cell Metab.* 12: 509–520.
- Hanlon, S. E., J. M. Rizzo, D. C. Tatomer, J. D. Lieb, and M. J. Buck, 2011 The stress response factors Yap6, Cin5, Phd1, and Skn7 direct targeting of the conserved co-repressor Tup1-Ssn6 in *S. cerevisiae*. *PLoS One* 6: e19060.
- Hastings, J. W., and B. M. Sweeney, 1957 On the mechanism of temperature independence in a biological clock. *Proc. Natl. Acad. Sci. USA* 43: 804–811.
- Hatori, M., C. Vollmers, A. Zarrinpar, L. DiTacchio, E. A. Bushong *et al.*, 2012 Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab.* 15: 848–860.
- Hong, S., S. A. Kim, M. L. Guerinet, and C. R. McClung, 2013 Reciprocal interaction of the circadian clock with the iron homeostasis network in *Arabidopsis*. *Plant Physiol.* 161: 893–903.
- Hurley, J. M., C. H. Chen, J. J. Loros, and J. C. Dunlap, 2012 Light-inducible system for tunable protein expression in *Neurospora crassa*. *G3 (Bethesda)* 2: 1207–1212.
- Hurley, J. M., A. Dasgupta, J. M. Emerson, X. Zhou, C. S. Ringelberg *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.
- Jeyaraj, D., F. A. Scheer, J. A. Ripperger, S. M. Haldar, Y. Lu *et al.*, 2012 Klf15 orchestrates circadian nitrogen homeostasis. *Cell Metab.* 15: 311–323.

- Kalsbeek, A., E. Poppen, I. Schallij, C. Van Heijningen, J. van der Vliet *et al.*, 2008 Circadian control of the daily plasma glucose rhythm: an interplay of GABA and glutamate. *PLoS One* 3: e3194.
- Kohsaka, A., A. D. Laposky, K. M. Ramsey, C. Estrada, C. Joshi *et al.*, 2007 High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab.* 6: 414–421.
- Kramer, C., 2007 Isolation of total RNA from *Neurospora mycelium*. *Methods Mol. Biol.* 362: 291–303.
- Larrondo, L. F., H. V. Colot, C. L. Baker, J. J. Loros, and J. C. Dunlap, 2009 Fungal functional genomics: tunable knockout-knock-in expression and tagging strategies. *Eukaryot. Cell* 8: 800–804.
- Larrondo, L. F., J. J. Loros, and J. C. Dunlap, 2012 High-resolution spatiotemporal analysis of gene expression in real time: in vivo analysis of circadian rhythms in *Neurospora crassa* using a FREQUENCY-luciferase translational reporter. *Fungal Genet. Biol.* 49: 681–683.
- Larrondo, L. F., C. Olivares-Yanez, C. L. Baker, J. J. Loros, and J. C. Dunlap, 2015 Decoupling circadian clock protein turnover from circadian period determination. *Science* 347: 1257277.
- Lauter, F. R., and C. Yanofsky, 1993 Day/night and circadian rhythm control of con gene expression in *Neurospora*. *Proc. Natl. Acad. Sci. USA* 90: 8249–8253.
- Lee, K., and D. J. Ebbole, 1998 Tissue-specific repression of starvation and stress responses of the *Neurospora crassa* con-10 gene is mediated by RCO1. *Fungal Genet. Biol.* 23: 269–278.
- Liu, C., S. Li, T. Liu, J. Borjigin, and J. D. Lin, 2007 Transcriptional coactivator PGC-1 α integrates the mammalian clock and energy metabolism. *Nature* 447: 477–481.
- Liu, X., H. Li, Q. Liu, Y. Niu, Q. Hu *et al.*, 2015 Role for Protein Kinase A in the *Neurospora* Circadian Clock by Regulating White Collar-Independent frequency transcription through phosphorylation of RCM-1. *Mol. Cell. Biol.* 35: 2088–2102.
- Loros, J. J., A. Richman, and J. F. Feldman, 1986 A recessive circadian clock mutation at the FRQ locus of *neurospora crassa*. *Genetics* 114: 1095–1110.
- Menet, J. S., J. Rodriguez, K. C. Abruzzi, and M. Rosbash, 2012 Nascent-Seq reveals novel features of mouse circadian transcriptional regulation. *eLife* 1: e00011.
- Millar, A. J., and S. A. Kay, 1991 Circadian control of cab gene transcription and mRNA accumulation in *Arabidopsis*. *Plant Cell* 3: 541–550.
- Millar, A. J., S. R. Short, N. H. Chua, and S. A. Kay, 1992 A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell* 4: 1075–1087.
- Montenegro-Montero, A., and L. F. Larrondo, 2013 The *Neurospora* circadian system: from genes to proteins and back in less than 24 hours, *Neurospora. Genomics and Molecular Biology*, edited by D. Kasbekar, and K. McCluskey. Caister Academic Press, Norfolk, UK.
- Montenegro-Montero, A., and L. F. Larrondo, 2015 In the driver's seat: the case for transcriptional regulation and coupling as relevant determinants of the circadian transcriptome and proteome in eukaryotes. *J. Biol. Rhythms* 31: 37–47.
- Montenegro-Montero, A., P. Canessa, and L. F. Larrondo, 2015 Around the fungal clock: recent advances in the molecular study of circadian clocks in *Neurospora* and other fungi. *Adv. Genet.* 92: 107–184.
- Nakahata, Y., M. Kaluzova, B. Grimaldi, S. Sahar, J. Hirayama *et al.*, 2008 The NAD⁺-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* 134: 329–340.
- Nakahata, Y., S. Sahar, G. Astarita, M. Kaluzova, and P. Sassone-Corsi, 2009 Circadian control of the NAD⁺ salvage pathway by CLOCK-SIRT1. *Science* 324: 654–657.
- Nowrousian, M., G. E. Duffield, J. J. Loros, and J. C. Dunlap, 2003 The frequency gene is required for temperature-dependent regulation of many clock-controlled genes in *Neurospora crassa*. *Genetics* 164: 923–933.
- O'Neill, J. S., E. S. Maywood, J. E. Chesham, J. S. Takahashi, and M. H. Hastings, 2008 cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science* 320: 949–953.
- Olmedo, M., L. Navarro-Sampedro, C. Ruger-Herreros, S. R. Kim, B. K. Jeong *et al.*, 2010 A role in the regulation of transcription by light for RCO-1 and RCM-1, the *Neurospora* homologs of the yeast Tup1-Ssn6 repressor. *Fungal Genet. Biol.* 47: 939–952.
- Park, S. H., S. S. Koh, J. H. Chun, H. J. Hwang, and H. S. Kang, 1999 Nrg1 is a transcriptional repressor for glucose repression of STA1 gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 19: 2044–2050.
- Pittendrigh, C. S., B. V. G. Bruce, N. S. Rosenzweig, and M. L. Rubin, 1959 A biological clock in *neurospora*. *Nature* 184: 169–170.
- Pittendrigh, C. S., and P. C. Caldarola, 1973 General homeostasis of the frequency of circadian oscillations. *Proc. Natl. Acad. Sci. USA* 70: 2697–2701.
- Pregueiro, A. M., Q. Liu, C. L. Baker, J. C. Dunlap, and J. J. Loros, 2006 The *Neurospora* checkpoint kinase 2: a regulatory link between the circadian and cell cycles. *Science* 313: 644–649.
- Proft, M., and R. Serrano, 1999 Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confers HOG-dependent osmotic regulation. *Mol. Cell. Biol.* 19: 537–546.
- Ruger-Herreros, C., M. Gil-Sanchez Mdel, G. Sancar, M. Brunner, and L. M. Corrochano, 2014 Alteration of light-dependent gene regulation by the absence of the RCO-1/RCM-1 repressor complex in the fungus *Neurospora crassa*. *PLoS One* 9: e95069.
- Sancar, G., C. Sancar, B. Brugger, N. Ha, T. Sachsenheimer *et al.*, 2011 A global circadian repressor controls antiphasic expression of metabolic genes in *Neurospora*. *Mol. Cell* 44: 687–697.
- Sancar, G., C. Sancar, and M. Brunner, 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.
- Sargent, M. L., W. R. Briggs, and D. O. Woodward, 1966 Circadian nature of a rhythm expressed by an invertase-less strain of *Neurospora crassa*. *Plant Physiol.* 41: 1343–1349.
- Schneider, K., S. Perrino, K. Oelhafen, S. Li, A. Zatzepin *et al.*, 2009 Rhythmic conidiation in constant light in vivid mutants of *Neurospora crassa*. *Genetics* 181: 917–931.
- Shigeyoshi, Y., K. Taguchi, S. Yamamoto, S. Takekida, L. Yan *et al.*, 1997 Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* 91: 1043–1053.
- Treitel, M. A., and M. Carlson, 1995 Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl. Acad. Sci. USA* 92: 3132–3136.
- Wang, B., A. N. Kettenbach, S. A. Gerber, J. J. Loros, and J. C. Dunlap, 2014 *Neurospora* WC-1 recruits SWI/SNF to remodel frequency and initiate a circadian cycle. *PLoS Genet.* 10: e1004599.
- Wang, L., J. Kim, and D. E. Somers, 2013 Transcriptional co-repressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. *Proc. Natl. Acad. Sci. USA* 110: 761–766.
- Wuarin, J., and U. Schibler, 1990 Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm. *Cell* 63: 1257–1266.
- Xu, K., J. R. DiAngelo, M. E. Hughes, J. B. Hogenesch, and A. Sehgal, 2011 The circadian clock interacts with metabolic

- physiology to influence reproductive fitness. *Cell Metab.* 13: 639–654.
- Yamashiro, C. T., D. J. Ebbole, B. U. Lee, R. E. Brown, C. Bourland *et al.*, 1996 Characterization of *rco-1* of *Neurospora crassa*, a pleiotropic gene affecting growth and development that encodes a homolog of Tup1 of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 16: 6218–6228.
- Yang, R., and Z. Su, 2010 Analyzing circadian expression data by harmonic regression based on autoregressive spectral estimation. *Bioinformatics* 26: i168–i174.
- Zhou, Z., X. Liu, Q. Hu, N. Zhang, G. Sun *et al.*, 2013 Suppression of WC-independent frequency transcription by RCO-1 is essential for *Neurospora* circadian clock. *Proc. Natl. Acad. Sci. USA* 110: E4867–E4874.
- Zhu, H., M. Nowrousian, D. Kupfer, H. V. Colot, G. Berrocal-Tito *et al.*, 2001 Analysis of expressed sequence tags from two starvation, time-of-day-specific libraries of *Neurospora crassa* reveals novel clock-controlled genes. *Genetics* 157: 1057–1065.
- Znaidi, S., B. Pelletier, Y. Mukai, and S. Labbe, 2004 The *Schizosaccharomyces pombe* corepressor Tup11 interacts with the iron-responsive transcription factor Fep1. *J. Biol. Chem.* 279: 9462–9474.

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Modulation of Circadian Gene Expression and Metabolic Compensation by the RCO-1 Corepressor of *Neurospora crassa*

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Fig S1. Expression of destabilized luciferase reporter at the *csr-1* locus.

A transcriptional reporter, *frq_{c-box}-luc^{PEST}*, was integrated at the *csr-1* locus in *wt* and $\Delta rco-1$ strains, and transformants were selected based on cyclosporine resistance. Bioluminescence measurements indicate rhythmic expression of LUC levels in both strains, confirming an increased period in $\Delta rco-1$, as also seen in Figure 2. Period $\pm$ SEM: WT: 21.0 ± 0.12 , $\Delta rco-1$: 23.3 ± 0.11 .

A)

B)

Figure S2. The central circadian oscillator is also functional in $\Delta rco-1$ strains obtained from other sources.

The $\Delta rco-1$, *his-3* strain generated by Zhuo et al., and named therein *RCO^{zz}* (Zhou et al, 2013), was crossed with our clock reporters and evaluated under the same conditions described for Figure 2B. Both transcriptional (A, *frq_{c-box}-luc*) and translational (B, *FRQ^{LUC}*) reporters confirm that the circadian clock remains functional in the absence of RCO-1, albeit period and amplitude are affected, as was also observed for the $\Delta rco-1$ obtained from the FGSC and analyzed in this work (Figure 2B).

A)

B)

Figure S3. *frq* mRNA and FRQ protein levels are elevated in the absence of RCO-1.

(A) *frq* mRNA levels were evaluated in WT and $\Delta rco-1$ strains in cultures on solid medium (LNN-CCD + QA) in LL conditions. *tbp* (NCU04770) and *act* (NCU04173) were used as reference genes. The Mann Whitney test was performed, and significance is indicated with an asterisk * ($p < 0.05$).

(B) FRQ^{LUC} protein levels were evaluated from solid medium cultures (LNN-CCD + QA) kept in LL conditions, by quantifying LUC levels which were normalized by protein content. FRQ levels were determined in WT, $\Delta rco-1$, $\Delta wc-1$ and $\Delta wc-1/\Delta rco-1$ strains, appearing elevated in the $\Delta rco-1$ compared to WT, and in $\Delta wc-1/\Delta rco-1$ compared to $\Delta wc-1$.

NCU07666_{prom}-luc

NCU07039_{prom}-luc

Figure S4. Examples of transcriptional reporters not exhibiting rhythmic expression. Transcriptional luciferase reporters of the promoters of *NCU07666* and *NCU07039* were generated by selecting 1.5 Kb of each promoter and transforming them into *Neurospora*. Luciferase levels were evaluated under the same conditions described for Figure 2B. No circadian expression was obvious by eye or software analysis for any of these reporters.

A)

B)

Figure SS. Rhythmicity of *ccg-13* and *csp-1* mRNA levels is lost in the absence of RCO-1.

WT and $\Delta rco-1$ strains were grown in LCM 2% glucose for 48 and 72 hours respectively. After this time, tissue pads of 5mm were cut and inoculated in maltose media (Vogel's 1X, maltose 0,5%, agar 2%). Time course experiments were performed as previously described (Larrondo et al, 2015) and 3 biological replicates were measured. WT and $\Delta rco-1$ strains are shown in the left and right panels, respectively. Measurement of *ccg-13* (A) and *csp-1* (B) expression by RT-qPCR was normalized using two reference genes: *tbp* (NCU04770), *suc* (NCU08336). To evaluate rhythmicity, data were analyzed with ARSER (Yang and Su 2010) finding that the expression of both genes was rhythmic ($p < 0.05$) in the WT strain, and this rhythmicity was lost in a $\Delta rco-1$ strain.

Figure S6. RCO-1 interacts with several transcriptional regulators *in vivo*.

Co-IP analyses revealed the interaction of RCO-1 with the TFs HSF-2, CRE-1 and with the putative histone deacetylase HDA-1. RCO-1 was C-terminally tagged with a V5 or HA epitopes while HSF-2 and CRE-1 were tagged with V5 and HDA-1 with HA. Immunoprecipitations were performed with an α-V5 antibody, and western blotting was conducted with α-V5 and α-HA.

Figure S7. Status of the core-oscillator in the absence of regulatory proteins that interact with RCO-1.

WT, $\Delta NCU01238$, $\Delta NCU05411$, $\Delta NCU05242$, $\Delta NCU08480$ and $\Delta hda-1$ strains carrying the transcriptional reporter *frq_{c-box}-luc* were analyzed under a CCD camera in DD and the bioluminescence traces were plotted. Only in the absence of NCU01238 (encoding for a PHD TF) is a change of period observed. Period $\pm$ SEM: WT: 20.6 ± 0.10 , $\Delta NCU01238$: 19.7 ± 0.19 .

Figure S8. Glucose dependence of period length in race tube assays in the $\Delta csp-1$ strain.

(A) Representative race tube assays of the WT ($csp-1^+; ras-1^{bd}$) and $\Delta csp-1; ras-1^{bd}$ strains at different glucose concentrations. (B) Graph showing the period length of the strains analyzed in A. The period of the $\Delta csp-1$ strain decreases as glucose concentration increases, similar to what has been previously described (Sancar et al. 2012).

Figure S9. $\Delta csp-1$ does not exhibit a metabolic compensation defect when analyzed in 96-well plates and LNN-CCD media.

The $\Delta csp-1$ strain containing a core-clock-luciferase reporter was analyzed under the same conditions under which $\Delta rco-1$ or *prd-1* exhibited metabolic compensation defects. While changes in period upon varying glucose concentration were observed for both $\Delta rco-1$ and *prd-1* (Emerson et al. 2015), $\Delta csp-1$ behaved like WT under those experimental conditions. Period $\pm$ SEM: WT 0% Glucose: 21.08 ± 0.125 , 0.3% Glucose: 21.45 ± 0.125 , 0.5% Glucose: 20.9 ± 0.045 ; $\Delta csp-1$: 0% Glucose: 20.83 ± 0.092 , 0.3% Glucose: 20.85 ± 0.088 , 0.5% Glucose: 20.43 ± 0.54 ; $\Delta rco-1$: 0% Glucose: 23.52 ± 0.075 , 0.3% Glucose: 22.69 ± 0.069 , 0.5% Glucose: 22.41 ± 0.09 .

NCU Number	Description	Number of peptides	Number of novel peptide	Coverage
NCU06052	Transcriptional repressor rco-1	399	43	0.38
NCU08480	Heat shock factor-2	90	31	0.50
NCU06842	Putative uncharacterized protein	78	26	0.27
NCU01634	Histone H4	60	14	0.59
NCU03126	Predicted protein	56	12	0.29
NCU01238	PDHD transcription factor	45	36	0.23
NCU05242	C2H2 transcription factor	40	17	0.17
NCU02695	Predicted protein	40	19	0.37
NCU10042	Enolase	37	19	0.37
NCU07914	Phosphoglycerate	35	19	0.49
NCU01528	Glyceraldehyde 3-phosphate-dehydrogenase	35	15	0.47
NCU09345	Nmt1 protein	35	13	0.32
NCU02136	Transaldolase	33	17	0.47
NCU03982	78 kDa glucose-regulated protein homolog	31	25	0.39
NCU06512	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	31	21	0.29
NCU03875	Chromatin remodeling factor 4-1	31	30	0.29
NCU05411	Pathway-specific nitrogen regulator	30	21	0.19
NCU09285	Putative uncharacterized protein	30	18	0.43
NCU04899	Malate dehydrogenase	30	17	0.49
NCU11000	Predicted protein	29	18	0.24
NCU01754	Alcohol dehydrogenase	28	15	0.39
NCU06338	DNA topoisomerase 2	28	20	0.13
NCU02435	Histone H2B	26	10	0.58
NCU02003	Elongation factor 1-alpha	26	15	0.37
NCU02806	14-3-3 protein 7	26	14	0.44
NCU09602	Heat shock 70 kDa protein	25	16	0.29
NCU06110	Thiamine thiazole synthase	25	6	0.16
NCU07700	Elongation factor 2	24	15	0.21
NCU07556	Chromatin remodeling factor 8-1	24	23	0.14
NCU04232	Predicted protein	23	12	0.26
NCU05488	Putative uncharacterized protein	23	14	0.58
NCU01680	Plasma membrane ATPase	22	13	0.15
NCU08550	Putative uncharacterized protein	22	10	0.25
NCU09477	ADP, ATP carrier protein	21	12	0.28
NCU06550	Probable pyridoxine biosynthesis protein pdx-1	21	11	0.32
NCU00489	Cytoplasmic ribosomal protein subunit S3	21	15	0.54
NCU06394	Chromatin remodeling complex subunit	21	21	0.28
NCU01635	Histone H3	20	10	0.35
NCU04173	Actin	20	12	0.40
NCU04035	Protein hir-1	20	17	0.19
NCU02957	Putative uncharacterized protein	20	11	0.32

Table S2. Data obtained by tandem MS. This list corresponded to the proteins identified in the samples coming from gel extraction protocol. A threshold of 20 peptides was used to select the proteins.

Gene annotation	
CSP-1	Defect in oscillation
HSF-2	Higher level of expression/Slight defect in oscillation
VAD-4	Low amplitude
SUB-1	Change of phase and amplitude
RCO-1	High level of expression, arrhythmicity

Table S1. KO of transcriptions factors that exhibit altered CON-10^{LUC} circadian expression.