Published in final edited form as:

Science. 2015 January 30; 347(6221): 1257277. doi:10.1126/science.1257277.

Decoupling circadian clock protein turnover from circadian period determination

Luis F. Larrondo^{1,2,*}, Consuelo Olivares-Yañez¹, Christopher L. Baker^{2,†}, Jennifer J. Loros^{2,3}, and Jay C. Dunlap^{2,*}

¹Millennium Nucleus for Fungal Integrative and Synthetic Biology, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

²Department of Genetics, Geisel School of Medicine at Dartmouth, Hanover, NH 03755, USA

³Department of Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, NH 03755, USA

Abstract

The mechanistic basis of eukaryotic circadian oscillators in model systems as diverse as *Neurospora*, *Drosophila*, and mammalian cells is thought to be a transcription-and-translation–based negative feedback loop, wherein progressive and controlled phosphorylation of one or more negative elements ultimately elicits their own proteasome-mediated degradation, thereby releasing negative feedback and determining circadian period length. The *Neurospora crassa* circadian negative element FREQUENCY (FRQ) exemplifies such proteins; it is progressively phosphorylated at more than 100 sites, and strains bearing alleles of *frq* with anomalous phosphorylation display abnormal stability of FRQ that is well correlated with altered periods or apparent arrhythmicity. Unexpectedly, we unveiled normal circadian oscillations that reflect the allelic state of *frq* but that persist in the absence of typical degradation of FRQ. This manifest uncoupling of negative element turnover from circadian period length determination is not consistent with the consensus eukaryotic circadian model.

Circadian clocks provide individuals with the ability to anticipate daily changes associated with the transition of day to night (1, 2). In organisms as diverse as humans, mice, fungi, insects, cyanobacteria, and plants, the molecular components of the circadian clocks have been identified in molecular detail (1, 3–6). In eukaryotes, the circadian oscillator underlying these subcellular clocks is generally viewed as comprising transcription and translation-based negative feedback loops (TTFLs) with interconnected feedback loops (1). Although posttranslational oscillators have been described (7, 8), their generalizability is

Copyright 2015 by the American Association for the Advancement of Science; all rights reserved.

*Corresponding author. jay.c.dunlap@dartmouth.edu (J.C.D.); llarrondo@bio.puc.cl (L.F.L.).

†Present address: Jackson Laboratory, Bar Harbor, ME, USA.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/347/6221/1257277/suppl/DC1

Figs. S1 to S7

Tables S1 and S2

Movies S1 and S2

still being tested. Thus, in the circadian paradigm for fungi and animals, (Per-Arnt-Sim) domain-containing transcription factors drive the expression of genes [e.g., *frequency* (*frq*), *period* (*per*)] whose products lead to the inhibition of their own transcription; only after degradation of these products can reinitiation of the cycle begin, and period length is thus obligately coupled to turnover kinetics of clock proteins (9–13). In the case of *Neurospora crassa*, transcription of the *frq* gene encoding the negative element FRQ is controlled by the White-Collar complex (WCC, the positive element), a heterodimer of PAS-containing GATA transcription factors White Collar-1 (WC-1) and White Collar-2 (WC-2). Beginning shortly after its synthesis, FREQUENCY (FRQ) is progressively phosphorylated, finally reaching a multi-phosphorylated (hyperphosphorylated) state that leads to its proteasome-mediated degradation (14, 15). Such changes, including timely turnover, are thought to underlie the daily rhythms in negative element abundance and phosphorylation that characterize eukaryotic circadian oscillators (13, 16).

More specifically, data supporting the importance of phosphorylation dynamics to clock protein turnover and period determination come from animals (10) and *Neurospora* (14, 17, 18) in which precise spatiotemporal arrangement of phosphorylation events dictates period and controls negative element stability. A key aspect in FRQ degradation is the association of its hyperphosphorylated isoforms with FWD-1, the substrate-recruiting subunit of an SCF (SKP/Cullin/ F-box)–type ubiquitin ligase that facilitates FRQ ubiquitination and presentation to the proteasome (17–21). FWD-1 is the ortholog of mammalian β -TrCP (22) and *Drosophila* Slimb (23, 24), which have similar roles in those circadian systems. That FRQ stability is a determinant of period length has been supported by the fact that strains bearing long-period *frq* alleles display a more stable FRQ, whereas this protein exhibits decreased half-life in strains with shorter periods (9, 14, 17, 18). Consistent with this, mutant strains in which ubiquitin-dependent proteasomal degradation of FRQ is severely altered show neither overt rhythms nor molecular circadian rhythmicity in FRQ abundance, as detected by Western blotting (15, 19, 25, 26). Also consistent with this model, mutations of genes encoding *Neurospora* COP9 signalosome components lead to destabilization of FWD-1, impairment of FRQ degradation, and apparent loss of both overt and molecular rhythmicity (25–27).

Results

Circadian rhythms in the absence of proper FRQ degradation

Overt circadianly regulated developmental rhythms (daily spore production) are reported to be lost in *fwd-1* mutants (19). Codon-optimized luciferase developed for *Neurospora* can report in vivo, and with high precision, *frq* expression dynamics or changes in abundance of FRQ, by the use of transcriptional (an ectopic construct in which the *frq* promoter controls luciferase expression) or translational (luciferase fused to the C terminus of the FRQ protein, *frq^{luc}*) reporters (6, 28–30). We therefore crossed *Neurospora frq^{luc}* to a *fwd-1* strain to assess whether, under free-running conditions, FRQ protein displayed residual regulation. We confirmed the absence of overt developmental rhythms (Fig. 1A). But, to our surprise, dynamic analysis of bioluminescence revealed oscillations in LUC activity—a direct measurement of FRQ-LUC synthesis given the short in vivo half-life of LUC (28)—in

fwd-1 cells (Fig. 1, B to D, and movie S1), this despite the presence of high amounts of hyperphosphorylated FRQ and attenuated turnover (fig. S1 and Fig. 2A). This suggests that complete or even substantial daily turnover of the negative element in the circadian clock, here FRQ, was not required to relieve the negative arm of the feedback loop in order to reinitiate new cycles of FRQ synthesis. We explicitly tested this possibility, confirming biochemically that, as described (19), abundance of FRQ in liquid cultures does not display detectable cycling in strains lacking FWD-1 (Fig. 2, A and B). Nevertheless, if synthesis of FRQ-LUC was dynamically tracked in vivo by measuring bioluminescence in 96-well plates, rhythms were evident in the *fwd-1* strain (Fig. 2C). To further validate that the *fwd-1* strain behaved as reported (19), we confirmed its genotype by polymerase chain reaction (PCR) and examined FRQ abundance by Western blot and by luciferase activity after blocking protein synthesis (fig. S1). In the absence of FWD-1, FRQ degradation was reduced and amounts appear elevated, consistent with previous work (19, 31). Our results, therefore, are incompatible with the current TTFL paradigm as applied to *Neurospora* and, taken at face value, compel a revised model in which FRQ stability as measured by its degradation rate is not a period determinant.

FRQ-LUC oscillations correspond to bona fide circadian rhythms

To confirm that rhythms in abundance of FRQ-LUC rely on a functional FRQ-WCC circadian oscillator, we tested this reporter in a strain carrying a point mutation in FRQ-interacting RNA helicase (*frh*) that leads to a clock-null phenotype (6, 32). In this strain, LUC levels were erratic, with no hints of rhythms, validating a central role for FRH (32, 33) in the FRQ-WCC oscillator (fig. S2). In addition, when examined in a WCC-deficient strain, amounts of FRQ-LUC were extremely low and arrhythmic (fig. S2). Taken together, this revalidates the *frq^{luc}* system (30) as a reliable reporter of the *Neurospora* circadian oscillator.

FRQ-LUC oscillations in strains lacking the COP9 signalosome

To confirm that these results were not restricted to only FWD-1 loss-of-function strains, we examined rhythms in strains bearing mutations in one of several components of the COP9 signalosome (CSN), which is required to deneddylate and thereby stabilize the class of SCF E3 ubiquitin ligases for which proteins like FWD-1 facilitate substrate recognition (34). For instance, loss of CSN-2 reduces CSN activity, causing a sharp decrease in amounts of FWD-1, and therefore stabilizing FRQ (25). This strain shows no rhythms in bulk FRQ amounts, and overt circadian rhythms are replaced by unstable developmental rhythms having periods between 40 and 60 hours (25). In our hands, analysis of rhythmic spore production (race-tube assay) (Fig. 1) of the *csn-2* strain confirmed the loss of overt circadian rhythms and the presence of unstable long-period cycles in conidiation, whereas real-time quantification of FRQ synthesis using the *frq^{luc}* reporter confirmed the coexistence of both circadian and developmental rhythms of differing period lengths (fig. S3, and movie S2). Thus, in *csn-2* cells, although stability of FRQ is increased (25), circadian rhythms in FRQ expression are still readily detectable; overt circadian developmental rhythms, however, are masked by long-period developmental cycles, a scenario similar to that in the *Neurospora chol-1* mutant (29). In light of recent reports that other CSN components influence FRQ stability and compromise both molecular and overt circadian rhythms (26,

27), we also examined those strains for cryptic circadian rhythmicity with the *frq^{luc}* reporter. In agreement with what we had observed for *csn-2*, we confirmed circadian rhythms in *csn-1*, *csn-4*, and *csn-5* strains (fig. S4), providing additional evidence of circadian rhythms despite altered FRQ stability.

Synthesis of new FRQ in the presence of hyperphosphorylated FRQ

In general, *Neurospora* circadian protein expression analyses have used samples from liquid cultures (35). Because the luciferase assays reported herein were conducted on agar plates, we confirmed that media conditions (solid versus liquid) are not producing major differences in FRQ stability. Comparisons of FRQ abundance in wild-type (WT) and *fwd-1* strains grown on solid media confirmed that, as observed for liquid cultures, degradation of FRQ was impaired in the absence of FWD-1 (fig. S5A). Time courses under these conditions showed clear oscillations of FRQ abundance in WT cells and revealed that although FRQ is more abundant and stable in *fwd-1*, it is nonetheless possible to distinguish its turnover and new synthesis (fig. S5, B and C), something not easily observed in samples coming from liquid cultures. In the aggregate, these results confirm that new FRQ can be produced in a timely manner even in the presence of hyperphosphorylated FRQ and that although FRQ degradation is impaired, molecular rhythms can still persist.

frq expression in *fwd-1* reflects the allelic state of the oscillator

To further corroborate the existence of a functional circadian oscillator in strains devoid of *fwd-1* (*fwd-1*), we employed a reporter in which only the *frq* CLOCK-Box of the *frq* promoter (36) drives *luc* expression (*frq_C-box-luc*) (Fig. 3). Rhythms were visualized in these strains, indicating that the WCC is active and therefore can still drive transcription from circadian cis elements even in the presence of high amounts of FRQ.

That these rhythms depend on the circadian oscillator in the normal manner was evidenced by the characteristic systematic alteration of period length seen in strains bearing various *frq* alleles, whether or not they were devoid of FWD-1. Rhythmicity persisted in the absence of proper FRQ turnover in *fwd-1*, displayed a long period in the presence of long-period *frq* alleles such as *frq⁷* or *frq^{S548A}*, and was short in the presence of short-period alleles such as *frq¹* or *frq^{S900A}* (Fig. 3 and table S2). However, periodicity was influenced by FWD-1, although the variation appeared to show no obvious pattern: Loss of FWD-1 altered the period of strains with less stable FRQ—as *frq⁺* or *frq^{S900A}*—as well as of strains with more stable FRQ, as *frq⁷* or *frq^{S548A}* (Fig. 3, B and C). Loss of FWD-1 even made an arrhythmic strain rhythmic (*frq^{5xS→D}*, having clustered S to D mutations at phosphorylated residues S538, S540, S541, S545, and S548 within the PEST1 region (17) (Fig. 3C).

Half-life of FRQ is a correlative measurement, and not a determinant factor, of period length

The above-mentioned results emphasized the unforeseen nature of these circadian rhythms: The period alterations in strains such as *frq¹*, *frq⁷*, or *frq^{S900A}* have been explained by changes in FRQ stability, and indeed FRQ half-life strongly correlates with period in these strains (9) (Fig. 4) in a manner similar to the correlation of locomotor rhythm period with PER half-life in *Drosophila* (13). That is, although in a WT *Neurospora* strain with a period

of about 22 hours FRQ had a half-life of close to 2.8 hours, in *frq⁷* cells (period ~29 hours), FRQ half-life was about 3.8 hours, whereas in *frq¹* cells (period ~16 hours) half-life was ~2 hours (9), yielding a strong correlation (Fig. 4D). However, examination of *fwd-1* strains confirmed that, in agreement with previous reports (19), FRQ half-life is increased (Fig. 4, A and B). Current models would predict that these increased half-lives would yield circadian periods of 39 hours or more (Fig. 4C). Instead, for all six *frq* alleles examined, we observed that period lengths in the absence of *fwd-1* remained close to those reported for turnover-proficient strains. Similar apparent inconsistencies between observed period length and stability can be found in the literature for other clustered FRQ phosphosite mutants that appear to cripple FRQ degradation without undue effects on clock-signaling phosphorylations (18, 37, 38), as well as the presence of altered FRQ profiles in overtly rhythmic strains (18). Although period was not unaffected by loss of FWD-1, a finding consistent with possible side effects of its loss on other cellular proteins, the decisive finding is that period length is not correlated with FRQ half-life: Clock protein stability does not determine clock period length and, in this canonical TTFL, the circadian negative element does not have to be completely or even substantially removed to release repression by the negative arm of the circadian feedback loop.

Inhibition of kinase activity lengthens period independently of FRQ stability

We and others were initially led to hypotheses positing an essential role for protein turnover in the clock, both from the observed rhythms in negative element abundance—among the first and most solid generalizations to arise in the comparative molecular clocks literature (1–3, 10, 11)—and also from the clear cycles in phosphorylation of negative elements that precede turnover (14). Thus, given the inescapable conclusion from our data that total clearance of the negative element is not an essential step in the TTFL, we were led to reexamine phosphorylation. We used 6-dimethylaminopurine (6-DMAP), a broad-spectrum kinase inhibitor that blocks clock protein phosphorylation, and examined its effects in both WT (*fwd-1⁺*) strains and in *fwd-1* strains deficient in clock protein turnover. Consistent with previous data (14), we observed that 6-DMAP lengthened period in a dose-dependent manner and did so independently of changing the inherent stability of FRQ as influenced by FWD-1 (fig. S6, A and B). Inhibition of kinase activity also had similar effects in the long-period *frq⁷* mutant that has a more stable FRQ protein (fig. S6), but the pertinent question in light of the clock model is whether this period lengthening is accompanied by changes in FRQ stability. We examined FRQ stability in two concentrations of inhibitor that lengthen period (150 μ M and 250 μ M 6-DMAP) in three genetic backgrounds and found that the period lengthening was not accompanied by stabilization of FRQ, as previous models would predict (fig. S7, A to D). We reconfirmed published data (14) showing that higher doses (5 mM of 6-DMAP) can stabilize FRQ, but note that the stabilization effect was reduced in *fwd-1* strains in which FRQ has already been stabilized by loss of its primary connection to the proteasome (fig. S7, E and F). Overall, these data confirm the importance of daily phosphorylation to circadian cycling and period length but support a model in which the half-life of a circadian negative element is a correlative measurement, and not a determinant factor, of period length.

Discussion

Reevaluating the relation between FRQ half-life and period determination

Our data lead us to several seemingly inescapable conclusions. One is that the long-standing relation between clock protein phosphorylation, clock protein stability, and period determination ought to be revisited. The data indicate that it is the qualities of FRQ rather than its quantity that are crucial and that the strong correlations between clock protein phosphorylation and half-life and between half-life and period length are, in fact, just correlations that do not imply in all cases cause and effect. Equally important is the related conclusion that clock proteins such as FRQ acting as negative elements do not need to be mostly eliminated by turnover before the next cycle of their synthesis can start. Thus, although degradation is the final outcome of FRQ posttranslational modifications, phosphorylation may be the key mechanism determining clock speed and the point at which the next cycle of *frq* synthesis is initiated. Figure 5 presents a cartoon context summarizing both the obligate and the associated events in the circadian cycle as an aid in viewing these distinctions. Our data indicate two general classes of conceptual phosphorylation events on a clock protein, clock-signaling phosphorylations (CSP) and termination-signaling phosphorylations (TSP). In this model, clock speed is dictated by the rate at which a sequential series of CSP—and the associated secondary, tertiary, and quaternary structural as well as intracellular localization changes resulting from these CSP—take place in and among the clock protein complex of FRQ/FRH/CK1/WC-1/ WC-2 (17). Under normal circumstances, these sequential CSP events will lead inexorably to TSP and to degradation of the negative element (e.g., FRQ) that may ordinarily be extremely rapid (unless strains have impaired protein turnover). However, once CSPs are completed, the rate of clock protein turnover per se is not a factor in determining circadian period length. This allows many predictions, among them that mutations causing a long period may delay CSP (pre-TSP) either directly by affecting phosphorylation sites or by eliciting structural changes that delay some key phosphorylation events. In this context, longer half-life of the clock protein does not cause a longer period by delaying the subsequent onset of new clock protein synthesis but rather is a consequence of a delayed cascade of phosphorylation events (including, necessarily, TSPs).

Predictions derived from the proposed model

This model provides novel perspectives and makes several predictions. For instance, clearance of the TTFL negative element (here FRQ) is not required before reinitiation of its synthesis (Fig. 2); changes in phosphorylation, turnover, and period length do not need to be correlated (Fig. 4); and a broad-spectrum kinase inhibitor can lengthen period without changing negative element stability (figs. S7 and S8). We predicted that CSP and TSP would be distinct and identifiable; however, this was problematic. For instance, TSP (*sensu stricto*) were predicted to alter stability but not period and might include the “recreational phosphorylation” sites in FRQ (17), especially those residues phosphorylated late in the cycle whose mutation has no effect on period. However, these have not been seen, perhaps because phosphodegrons act as charged domains rather than as just one or two charged amino acids. The distinction between CSP and TSP may, however, explain the anomalous

frq^{5xS→D} mutant (Fig. 3C) whose arrhythmicity is suppressed by *fwd-1 17 fwd 17 39 40* ability to repress, later phosphorylations can no longer affect clock function.

It is premature to broadly generalize these findings to all TTFLs; however, data from animal clocks are not inconsistent with these ideas. For instance, phosphorylation of *Drosophila* PER (dPER) by Doubletime kinase (DBT) at Ser47 (S47), a key step in controlling the speed of that clock, elicits phosphorylation of nearby residues to generate a high-affinity atypical SLIMB-binding site that leads to PER turnover (12). Ablation of SLIMB is correlated with constitutively high amounts of negative elements and behavioral arrhythmicity (23); however, a dPER S47A mutant showing reduced affinity for SLIMB and a long period length displayed persistent multiply phosphorylated dPER at all times of day, including times coincident with cyclical synthesis of new PER; this result is most straightforwardly interpreted as reinitiation of new PER synthesis in the presence of inactive old PER (12). The existence of such a class of PER invisible to the feedback loop is a prediction from the revised model, and identification of DBT-driven phosphorylation of S47 as a period-determining step (CSP in our terminology) does not contradict the revised model presented here. Although in most cases in *Drosophila*, alterations in period have been attributed to changes in PER degradation rates, the effect of some mutants such as DBT^S cannot be simply explained by effects on PER stability (13). In mammals, epistasis among mutations that impact the stability of PER and CRY proteins is unexpectedly absent (41). Consistent with data from *Neurospora*, including those reported here, inhibition of kinases (casein kinase I, among others) in animals reliably lengthens circadian period in whole organisms or in cell lines by up to 6 hours (10, 16–18, 22, 37, 42, 43); although these data are usually interpreted in terms of phosphorylation promoting F-box protein substrate recognition leading to turnover, the effects of reduction of these F-box protein levels have been less consistent. Knockdown of β -TRCP1 lengthened rhythms in NIH3T3 cells by only ~1 hour (42), and the circadian period of β -TrCP1 homozygote knockout mice is identical to that of WT animals (43); in contrast, knockdown of β -TRCP2 in U2OS cells leads to more dramatic period lengthening that is further enhanced when RNA interference is directed to both β -TRCPs (44). Mutation or knockdown of FBXL3 leads to increased CRY1 amounts and marked period lengthening (44–46), an effect that can be minimized by suppressing FBXL21 expression (47, 48); both results suggest that CRY1 levels and stability mediate the period effect. However, changes in CRY1 stability observed by eliminating FBXL21 alone (an F-Box protein that stabilizes CRYs) or in combination with FBXL3 (47), or by mutating phosphosites within CRY1 (49), do not clearly correlate with the observed changes in period. Plausible explanations for some of these data may exist, among them possible paralog compensation, cellular compartment-specific effects, or effects of F-box protein ablation on proteins other than PERs and CRYs; however, another possibility is that in these strains it is the phosphorylation status per se of the clock proteins that is crucial, rather than the derivative effects of phosphorylation on their stability or the timing of their ultimate turnover, each of which can be uncoupled from period determination. It may be that in these systems, the heretofore obligate coupling between clock protein stability and period determination, as well as the requirement of negative element turnover to reinitiate the circadian feedback loop, warrant a closer look.

Materials and methods

Strains

Strains used in this study are listed in table S1. Knockouts for *csn* components, *fwd-1* or *wc-1*, generated by the *Neurospora* functional ge-nomics program (50) as well as *frq*⁷ and *frq*¹ strains, were obtained from the Fungal Genetics Stock Center (Kansas City, Missouri, USA) (51). Strains containing the *frq*^{V5H6} (herein indistinctively mentioned as *frq*^{V5} or *frq*^{KI}) or other mutant *frq* alleles based on this knock-in construct have already been described (17), as well as the *frh*^{R806H} mutant (32).

Crosses between reporter strains and strains of interest were conducted as described in www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm and www.fgsc.net/methods/stanford.html. For race-tube analyses, progeny bearing the *ras-1*^{bd} allele [as determined by PCR (52)] were selected. Importantly, we did not observe differences between *ras-1*^{wt} or *ras-1*^{bd} in luciferase analyses. The reporter strains used in these studies correspond to (i) A *frq*^{luc} translational fusion in which the *luc* sequence has been fused in frame to the second-to-last codon of the *frq* gene, as already reported (30), and (ii) a reporter in which the Clock-box of the *frq* promoter (53) drives the expression of *luc* (28). Briefly, this construct, called *frq*_{c-box}-*luc*, contains a modified promoter region present in plasmid pAF 45 (36), where the pLRE (proximal light regulatory element) has been mutated, whereas the dLRE (Clock-box element) has been left intact (36). This reporter plasmid, which contains a resected *frq* promoter, was transformed into a *Neurospora* histidine auxotrophic strain (no. 87-74, *ras-1*^{bd} *his-3*), thereby targeting the transgene to *his-3*, and then backcrossed to strain 74A. Progeny from this cross were selected, genotyped, and used as a parental in the crosses described in this study. Genotypes of the different strains used herein were confirmed by PCR (see below).

Culture conditions

Race-tube studies were conducted as previously described (14). Liquid culture media (LCM 2%) consisted of 2% glucose, 0.5% arginine, 50 ng/ml biotin, and 1x Vogel's salts. Circadian cultures and FRQ degradation experiments were performed as already reported (14, 54). Race tubes that were examined for bioluminescence were supplemented with a sterile luciferin solution to a final concentration of 25 μM. Solid LCM 2% media refers to LCM 2% supplemented with 1.5% agar.

For in vivo monitoring of bioluminescence in multiple strains, 96-well plates were used. Liquid nitrogen-cooled charge-coupled device (LN-CCD) media (0.03% glucose, 0.05% arginine, 50 ng/ml biotin, 1.5% agar, and 25 uM luciferin) supplemented with quinic acid (QA) (to a final concentration of 0.01 M) were used as solid media for the 96-well plate assays throughout the studies. Although quinic acid does not affect the *Neurospora* circadian clock (55), we found it to dramatically increase the amplitude of the luciferase reporter signals when performing assays in 96-well plates, probably due to an increase of a me-tabolizable carbon source. Routinely, three wells were inoculated for each strain, using a freshly prepared spore suspension. Plates were covered with a sterile film permeable to O₂ and CO₂ (USA Scientific) and incubated at 25°C under constant light in a Percival

incubator, before transferring to DD conditions to be examined for dynamic lucif-erase expression. 6-DMAP (Sigma) was added to liquid or solid media in the concentrations indicated.

Protein analyses from solid media cultures

Analyses were performed from samples grown on Petri plates containing agar media, as described above (LN-CCD + QA). These plates were overlaid with sterile wet cellophane, on top of which, at the center of the plate, 5 μ l of a 10^5 -spore suspension were inoculated. Plates were kept in an upside-up position at 25° C LL (constant light) for 4 hours, after which they were transferred to DD (constant darkness) conditions to then be harvested after a conventional staggered culture protocol schedule (35). For FRQ stability analyses, plates were grown for 36 hours in 25°C LL, previous to the transfers until completing 12 hours in DD. To harvest the biomass, a microscope slide was used to collect the fungal tissue growing on top of the cellophane and frozen immediately in an Eppendorf tube in liquid nitrogen. Samples were then processed for protein extraction.

Luciferase-based analyses

Bioluminescence analyses were conducted using either a VersArray 1300B or a 1024B PIXIS CCD system from Princeton Instruments. Both cameras were housed within Percival incubators, under constant dark conditions and constant temperature (25°C). The cameras are controlled with the WinView software (Princeton Instruments), and acquisition times were set to 10 min with a 20-min delay between images (two frames per hour). Data from the image series were extracted with a customized macro developed for ImageJ (<http://rsbweb.nih.gov/ij>) and customized with an Excel interface. Data were plotted as raw bioluminescence or normalized by dividing each frame of an individual region of interest (ROI) by the average luminescence registered in that particular ROI over time. Period determination was assessed with the BRASS software (Biological Rhythms Analysis Software System, P.E. Brown, Warwick University).

Protein preparation and Western blots

Protein extractions, analyses, and Western blots were conducted as previously described (17), using either FRQ antisera or α -V5 antibody (Invitrogen, Carlsbad, CA) at a 1:250 or 1:5000 dilution respectively.

DNA analyses

Neurospora genomic DNA was prepared from small liquid cultures using the Puregene DNA isolation kit (Gentra Systems). Genotyping was performed using sequence-specific primers for the amplification of *fwd-1* [Forward (Fwd), ATGGCC-GGCTTTCCTCCACGA; Reverse (Rev), ATCCTCAT-CAGCGGGCAACA; product size, 711 base pairs (bp)], *csn-2* (Fwd, ATGTCCGACGACGATTTTCATG; Rev, GTAGTGTGAGGGAGTAGAAT; product size, 470 bp), *csn-1* (Fwd, ATGGACGACCGCAAATTAGC; Rev, CGCCGGAGCTGGCTTCGACA; product size, 980 bp), *csn-4* (fwd, ATGGTCTCCTCGGAAGTAAG; Rev, CATCTTCTCCAGGATGCCGA; product size, 858 bp), and *csn-5* (Fwd, GCTCGGCGAGAGCAA-CCGTT; Rev,

GAGGGACGCTCTGTCCATCA; product size, 713 bp), and the amplification of the *mus-51* locus was used as amplification control in the multiplexing PCR reactions (Fwd, TGGAGGAA-GGATCAGGATGAG; Rev, CTGGTTGGCGCAGAA-GAGCA; product size, 522 bp). The presence of the *ras-1^{bd}* mutation was evaluated as previously reported (52).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institute of General Medical Sciences of the National Institutes of Health to J.C.D. (R01 GM34985 and P01 GM68087) and J.J.L. (R01 GM083336), Millennium Nucleus for Fungal Integrative and Synthetic Biology (NC120043), and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1090513 and 1131030) to L.F.L. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank I. Edery and A. Kramer for comments on the manuscript, V. Caballero for technical assistance, and the Fungal Genetics Stock Center at the University of Missouri for strains.

References

1. Dunlap JC. Molecular bases for circadian clocks. *Cell*. 1999; 96:271–290.10.1016/S0092-8674(00)80566-8 [PubMed: 9988221]
2. Rosbash M. The implications of multiple circadian clock origins. *PLOS Biol*. 2009; 7:e62.10.1371/journal [PubMed: 19296723]
3. Bell-Pedersen D, et al. Circadian rhythms from multiple oscillators: Lessons from diverse organisms. *Nat Rev Genet*. 2005; 6:544–556.10.1038/nrg1633 [PubMed: 15951747]
4. Montenegro-Montero, A.; Larrondo, LF. *Neurospora: Genomics and Molecular Biology*. McCluskey, K.; Kasbekar, DP., editors. Caister Academic Press; Norfolk: 2013. p. 243–271.
5. Duong HA, Robles MS, Knutti D, Weitz CJ. A molecular mechanism for circadian clock negative feedback. *Science*. 2011; 332:1436–1439.10.1126/science.1196766 [PubMed: 21680841]
6. Hurley JM, Larrondo LF, Loros JJ, Dunlap JC. Conserved RNA helicase FRH acts nonenzymatically to support the intrinsically disordered *Neurospora* clock protein FRQ. *Mol Cell*. 2013; 52:832–843.10.1016/j.molcel.2013.11.005 [PubMed: 24316221]
7. O'Neill JS, Reddy AB. Circadian clocks in human red blood cells. *Nature*. 2011; 469:498–503.10.1038/nature09702 [PubMed: 21270888]
8. O'Neill JS, et al. Circadian rhythms persist without transcription in a eukaryote. *Nature*. 2011; 469:554–558.10.1038/nature09654 [PubMed: 21270895]
9. Ruoff P, Loros JJ, Dunlap JC. The relationship between FRQ-protein stability and temperature compensation in the *Neurospora* circadian clock. *Proc Natl Acad Sci USA*. 2005; 102:17681–17686.10.1073/pnas.0505137102 [PubMed: 16314576]
10. Brown SA, Kowalska E, Dallmann R. (Re)inventing the circadian feedback loop. *Dev Cell*. 2012; 22:477–487.10.1016/j.devcel.2012.02.007 [PubMed: 22421040]
11. Blau J. PERspective on PER phosphorylation. *Genes Dev*. 2008; 22:1737–1740.10.1101/gad.1696408 [PubMed: 18593875]
12. Chiu JC, Vanselow JT, Kramer A, Edery I. The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes Dev*. 2008; 22:1758–1772.10.1101/gad.1682708 [PubMed: 18593878]
13. Syed S, Saez L, Young MW. Kinetics of doubletime kinase-dependent degradation of the *Drosophila* period protein. *J Biol Chem*. 2011; 286:27654–27662.10.1074/jbc.M111.243618 [PubMed: 21659538]

14. Liu Y, Loros J, Dunlap JC. Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. *Proc Natl Acad Sci USA*. 2000; 97:234–239.10.1073/pnas.97.1.234 [PubMed: 10618401]
15. He Q, Liu Y. Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. *Biochem Soc Trans*. 2005; 33:953–956.10.1042/BST20050953 [PubMed: 16246019]
16. Reischl S, Kramer A. Kinases and phosphatases in the mammalian circadian clock. *FEBS Lett*. 2011; 585:1393–1399.10.1016/j.febslet.2011.02.038 [PubMed: 21376720]
17. Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol Cell*. 2009; 34:354–363.10.1016/j.molcel.2009.04.023 [PubMed: 19450533]
18. Tang CT, et al. Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. *Proc Natl Acad Sci USA*. 2009; 106:10722–10727.10.1073/pnas.0904898106 [PubMed: 19506251]
19. He Q, et al. FWD1-mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. *EMBO J*. 2003; 22:4421–4430.10.1093/emboj/cdg425 [PubMed: 12941694]
20. Heintzen C, Liu Y. The *Neurospora crassa* circadian clock. *Adv Genet*. 2007; 58:25–66.10.1016/S0065-2660(06)58002-2 [PubMed: 17452245]
21. Querfurth C, et al. Circadian conformational change of the *Neurospora* clock protein FREQUENCY triggered by clustered hyperphosphorylation of a basic domain. *Mol Cell*. 2011; 43:713–722.10.1016/j.molcel.2011.06.033 [PubMed: 21884974]
22. Eide EJ, et al. Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol Cell Biol*. 2005; 25:2795–2807.10.1128/MCB.25.7.2795-2807.2005 [PubMed: 15767683]
23. Grima B, et al. The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature*. 2002; 420:178–182.10.1038/nature01122 [PubMed: 12432393]
24. Ko HW, Jiang J, Edery I. Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature*. 2002; 420:673–678.10.1038/nature01272 [PubMed: 12442174]
25. He Q, Cheng P, He Q, Liu Y. The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex. *Genes Dev*. 2005; 19:1518–1531.10.1101/gad.1322205 [PubMed: 15961524]
26. Wang J, et al. Role of individual subunits of the *Neurospora crassa* CSN complex in regulation of deneddylation and stability of cullin proteins. *PLOS Genet*. 2010; 6:e1001232.10.1371/journal.pgen.1001232 [PubMed: 21151958]
27. Zhou Z, Wang Y, Cai G, He Q. *Neurospora* COP9 signalosome integrity plays major roles for hyphal growth, conidial development, and circadian function. *PLOS Genet*. 2012; 8:e1002712.10.1371/journal.pgen.1002712 [PubMed: 22589747]
28. Gooch VD, et al. Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. *Eukaryot Cell*. 2008; 7:28–37.10.1128/EC.00257-07 [PubMed: 17766461]
29. Shi M, Larrondo LF, Loros JJ, Dunlap JC. A developmental cycle masks output from the circadian oscillator under conditions of choline deficiency in *Neurospora*. *Proc Natl Acad Sci USA*. 2007; 104:20102–20107.10.1073/pnas.0706631104 [PubMed: 18056807]
30. Larrondo LF, Loros JJ, Dunlap JC. 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*. 2012; 49:681–683.10.1016/j.fgb.2012.06.001 [PubMed: 22695169]
31. Guo J, Cheng P, Liu Y. Functional significance of FRH in regulating the phosphorylation and stability of *Neurospora* circadian clock protein FRQ. *J Biol Chem*. 2010; 285:11508–11515.10.1074/jbc.M109.071688 [PubMed: 20159972]
32. Shi M, Collett M, Loros JJ, Dunlap JC. FRQ-interacting RNA helicase mediates negative and positive feedback in the *Neurospora* circadian clock. *Genetics*. 2010; 184:351–361.10.1534/genetics.109.111393 [PubMed: 19948888]

33. Cheng P, He Q, He Q, Wang L, Liu Y. Regulation of the *Neurospora* circadian clock by an RNA helicase. *Genes Dev.* 2005; 19:234–241.10.1101/gad.1266805 [PubMed: 15625191]
34. Wei N, Serino G, Deng XW. The COP9 signalosome: More than a protease. *Trends Biochem Sci.* 2008; 33:592–600.10.1016/j.tibs.2008.09.004 [PubMed: 18926707]
35. Duffield G, Loros JJ, Dunlap JC. Analysis of circadian output rhythms of gene expression in *Neurospora* and mammalian cells in culture. *Methods Enzymol.* 2005; 393:315–341.10.1016/S0076-6879(05)93014-0 [PubMed: 15817297]
36. Froehlich AC, Liu Y, Loros JJ, Dunlap JC. White Collar-1, a circadian blue light photoreceptor, binding to the *frequency* promoter. *Science.* 2002; 297:815–819.10.1126/science.1073681 [PubMed: 12098706]
37. Mehra A, et al. A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. *Cell.* 2009; 137:749–760.10.1016/j.cell.2009.03.019 [PubMed: 19450520]
38. Görl M, et al. A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. *EMBO J.* 2001; 20:7074–7084.10.1093/emboj/20.24.7074 [PubMed: 11742984]
39. Lauinger L, Diernfellner A, Falk S, Brunner M. The RNA helicase FRH is an ATP-dependent regulator of CK1 α in the circadian clock of *Neurospora crassa*. *Nat Commun.* 2014; 5:3598.10.1038/ncomms4598 [PubMed: 24710172]
40. Cha J, Yuan H, Liu Y. Regulation of the activity and cellular localization of the circadian clock protein FRQ. *J Biol Chem.* 2011; 286:11469–11478.10.1074/jbc.M111.219782 [PubMed: 21300798]
41. Maywood ES, et al. Tuning the period of the mammalian circadian clock: Additive and independent effects of CK1 ϵ Tau and Fbx13Afh mutations on mouse circadian behavior and molecular pacemaking. *J Neurosci.* 2011; 31:1539–1544.10.1523/JNEUROSCI.4107-10.2011 [PubMed: 21273438]
42. Reischl S, et al. Beta-TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics. *J Biol Rhythms.* 2007; 22:375–386.10.1177/0748730407303926 [PubMed: 17876059]
43. Ohsaki K, et al. The role of beta-TrCP1 and beta-TrCP2 in circadian rhythm generation by mediating degradation of clock protein PER2. *J Biochem.* 2008; 144:609–618.10.1093/jb/mvn112 [PubMed: 18782782]
44. Maier B, et al. A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev.* 2009; 23:708–718.10.1101/gad.512209 [PubMed: 19299560]
45. Busino L, et al. SCFFbx13 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science.* 2007; 316:900–904.10.1126/science.1141194 [PubMed: 17463251]
46. Shi G, et al. Dual roles of FBXL3 in the mammalian circadian feedback loops are important for period determination and robustness of the clock. *Proc Natl Acad Sci USA.* 2013; 110:4750–4755.10.1073/pnas.1302560110 [PubMed: 23471982]
47. Hirano A, et al. FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell.* 2013; 152:1106–1118.10.1016/j.cell.2013.01.054 [PubMed: 23452856]
48. Yoo SH, et al. Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. *Cell.* 2013; 152:1091–1105.10.1016/j.cell.2013.01.055 [PubMed: 23452855]
49. Gao P, et al. Phosphorylation of the cryptochrome 1 C-terminal tail regulates circadian period length. *J Biol Chem.* 2013; 288:35277–35286.10.1074/jbc.M113.509604 [PubMed: 24158435]
50. Colot HV, et al. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc Natl Acad Sci USA.* 2006; 103:10352–10357.10.1073/pnas.0601456103 [PubMed: 16801547]
51. McCluskey K, Wiest A, Plamann M. The Fungal Genetics Stock Center: A repository for 50 years of fungal genetics research. *J Biosci.* 2010; 35:119–126.10.1007/s12038-010-0014-6 [PubMed: 20413916]

52. Belden WJ, et al. The band mutation in *Neurospora crassa* is a dominant allele of ras-1 implicating RAS signaling in circadian output. *Genes Dev.* 2007; 21:1494–1505.10.1101/gad.1551707 [PubMed: 17575051]
53. Froehlich AC, Loros JJ, Dunlap JC. Rhythmic binding of a WHITE COLLAR-containing complex to the *frequency* promoter is inhibited by FREQUENCY. *Proc Natl Acad Sci USA.* 2003; 100:5914–5919.10.1073/pnas.1030057100 [PubMed: 12714686]
54. Ruoff P, Christensen MK, Sharma VK. PER/TIM-mediated amplification, gene dosage effects and temperature compensation in an interlocking-feedback loop model of the *Drosophila* circadian clock. *J Theor Biol.* 2005; 237:41–57.10.1016/j.jtbi.2005.03.030 [PubMed: 15935389]
55. Aronson BD, Johnson KA, Loros JJ, Dunlap JC. Negative feedback defining a circadian clock: Autoregulation of the clock gene *frequency*. *Science.* 1994; 263:1578–1584.10.1126/science.8128244 [PubMed: 8128244]

Fig. 1. FRQ expression oscillates in the absence of FWD-1

(A) Strains were inoculated into 1-cm-diameter glass tubes partially filled with agar [race tubes, e.g., (4, 14)], grown for 24 hours in constant light and then transferred to darkness to elicit circadian control of growth and conidial development (“banding”). Assessment of rhythmic conidiation (banding) through this race-tube assay confirms the absence of overt circadian rhythmicity in *ras-1^{bd}, frq^{luc}*, *fwd-1* strains compared with a *ras-1^{bd}, frq^{luc}, fwd-1⁺* control. Nevertheless, quantification of FRQ-LUC bioluminescence throughout the entire race tube (A) or close to the beginning of the race tube (B to D) reveals the presence of oscillations in FRQ-LUC expression in the strains lacking FWD-1 as well as in FWD-1⁺. In (B) and (C), each line corresponds to the average of three different quantifications ($\pm$ SEM) of luminescence from within each demarked region across sequential time points.

Fig. 2. Lack of clear oscillations in abundance of FRQ in the absence of FWD-1 under standard culture conditions

(**A**) *ras-1^{bd}, frq^{luc}, fwd-1⁺* (top) and *ras-1^{bd}, frq^{luc}, fwd-1* (bottom) strains were grown in 2% LCM in darkness, and samples were harvested every 4 hours for 48 hours; proteins were extracted, and FRQ-LUC was detected with antibody to FRQ. (**B**) Densitometric analysis of the gels confirming elevated expression and loss of normal rhythmic FRQ expression in *fwd-1*. (**C**) Quantification of bioluminescence of static cultures, in 96-well plates with solid race-tube media, reveals the presence of oscillations in FRQ-LUC levels in the *fwd-1* strain. Each curve corresponds to the average of three independent wells.

Fig. 3. Rhythmicity in the absence of FWD-1 depends on the FRQ-WCC oscillator

(A) Scheme depicting the main features of FRQ, including domains and the position of the mutations of the main *frq* alleles and their period alterations. AUG_L and AUG_S, starts of long and short FRQ proteins; CC, coiled coil region; NLS, nuclear localization; FCD, CK1 interaction domains; PEST, domains enriched in proline, aspartate, glutamate, serine, and threonine associated with turnover; FFD, FRQ-FRH interaction. (B) A *frq_{c-box}-luc* reporter was used to monitor the status of the oscillator by following *frq* expression in strain containing different isolated *frq* alleles or (C) V5-tagged *frq* mutants as indicated on top of each graph, in the strains with (*fwd-1*⁺) or without (*Δfwd-1*) FWD-1. LUC activity was quantified for both WT and *fwd-1* strains. Each line corresponds to the average of three different wells $\pm$ SEM. Periods for each strain can be found in table S2.

Fig. 4. FRQ degradation rate does not predict period length in the absence of proper FRQ turnover

(A) Western blots, with α -V5, allow for the quantification of FRQ abundance after a light-to-dark transition in *frq^{KI}* (*frq^{V5H6}*) and *frq^{S900A}* strains in the presence or absence of FWD-1. (B) Densitometric analysis of (A) showing exponential fit used for FRQ half-life calculation. (C) The table compares the predicted periods, based on the experimental half-lives (9), with the ones obtained with the *frq_{C-box}-luc* reporter for each strain. (D) These values were plotted along with the data from Ruoff *et al.* (9). Experimental data for period length (gray dots) correlates well with protein half-life, leading to a model (gray line) in which periods can be predicted for each strain (gray diamonds). Period lengths for *frq^{S900A}* and *frq^{KI}* (black dots) nicely fit the model. In the absence of FWD-1, the model predicts these two strains to have a period of 37 hours (open circle). Nevertheless, luciferase analysis reveals a period of about 20 hours (black dots), close to the original *frq* allele present in each strain. Uncertainties in period and half-life determinations were considered as an average of 5 or 15%, respectively (9).

Fig. 5. A model describing distinct roles for clock protein phosphorylation and degradation through time

Essential events in the circadian cycle are shown within the shaded clock face. In the late night, WC-1 and WC-2 interact via PAS domains and assemble as the WCC at the Clock-box on the *frq* promoter to drive transcription, which leads to WC-1 destabilization. FRQ is expressed as a largely unstructured protein, adopting a defined and functional structure as it dimerizes and forms a complex with FRH (6). As part of this complex, CK1 is recruited (17). Hypophosphorylation of FRQ begins the CSP, whose kinetics determine the long time constant of the cycle, thereby setting circadian period length. FRQ promotes phosphorylation of WC-1 by CK1 and other kinases, eventually reducing *frq* expression to low levels and thereby stabilizing WC-1. Phosphorylated WCC leaves the *frq* promoter. As FRQ becomes hyperphosphorylated, it loses its ability to interact with CK1 (39) and WCC (17), becoming therefore a weak repressor of WCC (40). In addition, dephosphorylation of WCC, as well as new synthesis of these proteins, provide active WC-1 and WC-2 to reinitiate the cycle. Independently, and with kinetics that do not affect timekeeping, TSPs on FRQ lead to its recognition by FWD-1 and its associated cullin-SCF complex, leading to FRQ ubiquitylation and turnover in the proteasome.