Interconnected Feedback Loops in the *Neurospora* Circadian System

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In *Neurospora crassa*, white collar 1 (WC-1), a transcriptional activator and positive clock element, is rhythmically expressed from a nonrhythmic steady-state pool of *wc-1* transcript, consistent with posttranscriptional regulation of rhythmicity. Mutations in *frq* influence both the level and periodicity of WC-1 expression, and driven FRQ expression not only depresses its own endogenous levels, but positively regulates WC-1 synthesis with a lag of about 8 hours, a delay similar to that seen in the wild-type clock. FRQ thus plays dual roles in the *Neurospora* clock and thereby, with WC-1, forms a second feedback loop that would promote robustness and stability in this circadian system. The existence also of interlocked loops in *Drosophila melanogaster* and mouse clocks suggests that such interlocked loops may be a conserved aspect of circadian timing systems.

The circadian oscillator in *Neurospora* regulates the timing of development, dictating the time of day when hyphae growing on the surface of a substrate are endowed with the capacity to create aerial hyphae and, eventually, asexual spores. This eukaryotic circadian clock, like others that are known (*1–5*), comprises at least one autoregulatory negative feedback loop (*6*) of positive elements (heterodimers of PAS domain–containing proteins that act as transcription factors) and negative elements that down-regulate the activity of the positive elements. In *Neurospora*, the heterodimeric positive element is composed of proteins encoded by the *white wc-1* and *wc-2* genes (*7*); either of two forms of the FRQ protein (*6, 8*) are the negative element. Both mRNA and protein levels of *frq* are rhythmic in constant dark (*6, 8*). Light signals are transferred to the clock through the WC proteins to reset the clock via rapid induction of *frq* (*9*). Thus, the WC proteins play a dual role in the organism: In the context of light they are essential both for light induction of most light-induced genes (*10*), including *frq* and light resetting of the clock (*1–3, 9*), and in the dark they are essential for generation of circadian rhythms (*1–3, 7*). This dual function has suggested a possible evolutionary relationship between ancestors of the molecules responsible for light perception and the origins of circadian rhythmicity (*7*).

Proteins with circadian functions similar to those of the WC proteins include CLK and CYC in *Drosophila* (*11, 12*) and CLOCK and BMAL1 in mammals (*13*). Like the WC proteins, these pairs act as dimers, and through the circadian specific *cis*-element E-box (*13, 14*), they activate expression of *per/tim* in *Drosophila* and the family of negative element genes in mammals [e.g., (*15*)]. Many of these clock genes contain PAS domains (*7, 11, 16–18*), which are protein-protein interaction domains that are involved in a variety of biological pathways (*19*). Although sequence conservation has been noted among the PAS domains of the positively-acting elements WC-1, WC-2, CYC, CLK, BMAL, and CLOCK, the expression patterns of these clock components have appeared quite divergent: in mammals, *Bmal1* is rhythmically expressed with a peak at night and *CLOCK* appears constitutive (*18, 20*), in *Drosophila*, the reverse is true [*Clk* is rhythmic but with a dawn peak, whereas *cyc*, the ortholog of *Bmal1*, is constitutive (*12, 21*)], and in *Neurospora*, preliminary data suggest that neither *wc-1* nor *wc-2* were rhythmically expressed (*1, 3*). A major step forward in elucidating the logic behind this regulation was made by Glossup *et al.* (*22*) with the identification of interlocking feedback loops in the *Drosophila* clock, in which the rhythmic activating and repressing elements in one loop (CLK and PER, respectively) acted in the opposite way in an interconnected feedback loop (respectively, to repress and activate). Thus, clock proteins served dual functions at different times of day. We report here a similar

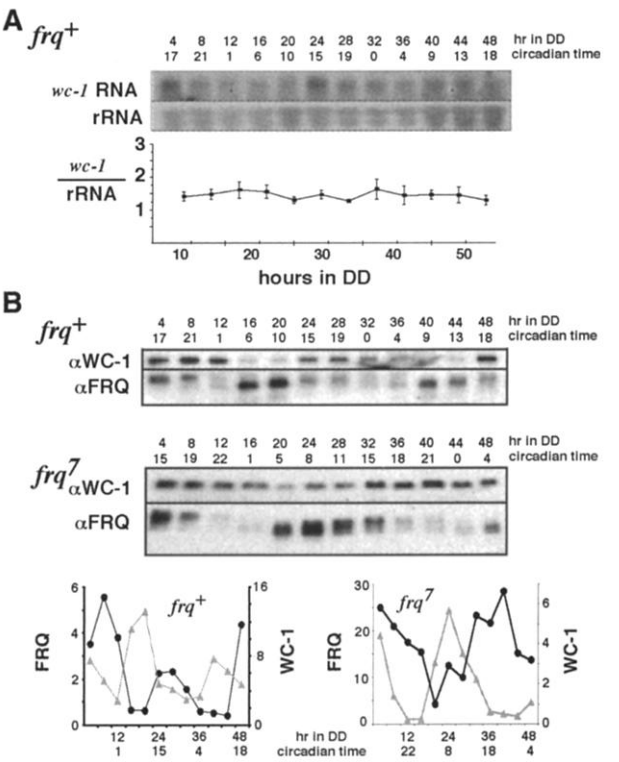

Fig. 1. WC-1 is circadianly regulated through a post-transcriptional mechanism. (**A**) The amount of *wc-1* transcript does not vary with time of day. Total RNA was harvested every 4 hours from cultures grown in constant darkness, and 40-μg samples were electrophoresed, blotted, and probed for the presence of *wc-1* transcript (*7, 8, 10*). The blot was then stripped and probed for rRNA as a loading control. A representative blot is shown and below this is plotted the average amount (±SEM) of *wc-1* transcript normalized to rRNA from five replicate experiments. Times are shown in real hours and in circadian time, where the circadian cycle is divided up into 24 equal parts with dawn set as circadian time 0. (**B**) WC-1 protein amounts are regulated in a circadian manner with a peak in the subjective night, out of phase with FRQ. Mycelia were collected every 4 hours in constant darkness for 2 days from both a clock wild-type (*frq*$^+$) and long-period clock mutant (*frq*^{7}) strain, the protein was extracted (*29*), and 100 μg of cell extract from each time point was loaded onto SDS-polyacrylamide gels, electrophoresed, and transferred to membranes. WC-1 was visualized with a specific antiserum (*30*). The blots were then stripped and reprobed for FRQ, and stained with Coomassie blue to measure protein transfer. The intensities of bands in the blot were calculated with densitometry, and a representative densitometric plot from one of three replicate experiments for both *frq*$^+$ and *frq*^{7} is shown at the bottom; WC-1 (black circles) cycles in both cultures are out of phase with FRQ (gray triangles). Times are shown in real hours and in circadian time.

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regulatory arrangement of interdigitating feedback loops, albeit with some divergent twists, in the *Neurospora* circadian system, suggesting that this arrangement may constitute a common aspect of functional organization underlying the structure of circadian oscillators in eukaryotes, one that would promote robustness and stability of the overt rhythms.

Although rhythms in transcript levels have been reported for *Bmal1* in mammals (*20*) and for *Clk* in flies (*12, 21*), we found no rhythms in *wc-1* transcript levels when measured over extended free runs under constant conditions (Fig. 1A). Surprisingly, however, when the amount of WC-1 protein was determined under identical conditions the steady-state level was robustly rhythmic (Fig. 1B). WC-1 displayed an approximately 5- to 10-fold oscillation of protein abundance with a peak in the late subjective night around circadian time (CT) 18 to 20. The time of the peak thus lags the peak in FRQ by about eight circadian hours. The circadian nature of this regulation was confirmed by showing that the period of the WC-1 oscillation was lengthened in the long-period mutant *frq*^{7} strain (Fig. 1B). The two forms of FRQ could be seen along with the characteristic rhythm in FRQ phosphorylation (*8*), but under these electrophoresis conditions, only a single WC-1 species was detected; under more stringent conditions for resolving mobility, we can detect phosphorylation of WC-1 [see below and (*23*)], but the phosphorylation does not appear to be rhythmic. The size of the protein, 127 kD, is consistent with the molecular weight predicted from its sequence, and the protein was absent in *wc-1* null strains.

The observation that constant *wc-1* transcript levels gave rise to rhythmic WC-1 protein amounts indicated posttranscriptional control, and the period changes in the *frq* mutant suggested a possible involvement of FRQ. To evaluate this, WC-1 levels were examined in *frq*-wild-type and mutant strains at two times (Fig. 2A). Under light:dark (L:D) cycles, WC-1 was detected in *frq*$^+$ strains and the elevated FRQ in the *frq*^{7} strain resulted in slightly elevated levels of WC-1, but in the absence of FRQ (in the null *frq*^{10} strain) WC-1 levels were extremely low. This suggested either that FRQ was preventing WC-1 degradation or promoting its synthesis. To evaluate the first alternative, we used 50 μM cycloheximide to block de novo protein synthesis in both constant light and in darkness and followed the kinetics of FRQ and WC-1 degradation (Fig. 2B). Although starting levels of FRQ varied as expected in light and dark and with time of day and the kinetics of the degradation of FRQ proteins were similar in all conditions, WC-1 degradation kinetics changed with the conditions. In the light, WC-1 was phosphorylated and degraded with kinetics similar to FRQ whereas, in the dark, WC-1 was more stable, its turnover was not coincident with increased phosphorylation, and the rate of degradation bore no relationship to the amount of FRQ, even though from 4 to 12 hours in the dark, WC-1 protein levels are approximately equal to or higher than FRQ (*24*). These data suggested that amount of FRQ is not influencing the speed of degradation of WC-1 protein in darkness.

To determine whether FRQ plays an active role in promoting WC-1 synthesis, we tested whether under noncircadian conditions an increase in FRQ would drive an increase in WC-1. We utilized a strain (*25*) (*frq*^{10}*::qa-2*pFRQ) in which the endogenous *frq* gene is replaced by the *hph* gene and which carries (at *his-3*, a neutral ectopic location) the FRQ open reading frame under the control of inducible *qa-2* promoter (*6, 9, 25*). When samples of *frq*^{10}*::qa-2*pFRQ were treated with 0.01M quinic acid (QA) for 6 hours in constant light or in darkness, there was a significant increase in the steady-state levels of both FRQ [in darkness, unpaired *t*-value = –3.8, $P = 0.019$, $n = 3$; in light unpaired *t*-value = –8.9, $P = 0.0009$, $n = 6$] and WC-1 [in darkness, $t = -4.1$, $n = 3$; in light $t = -6.48$, $P = 0.0001$, $n = 6$] (Fig. 2C).

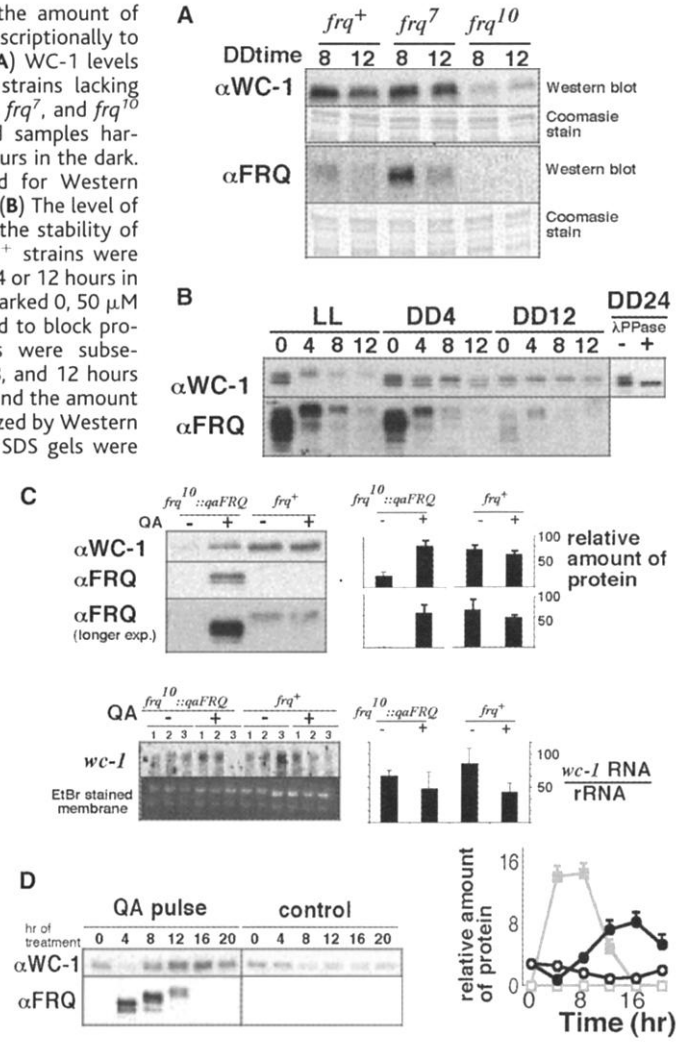

Fig. 2. FRQ influences the amount of WC-1 by acting posttranscriptionally to promote its synthesis. (**A**) WC-1 levels are greatly reduced in strains lacking FRQ. Mycelia from *frq*$^+$, *frq*^{7}, and *frq*^{10} strains were grown and samples harvested after 8 and 12 hours in the dark. Samples were processed for Western blot analysis as in Fig. 1. (**B**) The level of FRQ does not influence the stability of WC-1. Mycelia from *frq*$^+$ strains were grown in the light or for 4 or 12 hours in darkness, and at times marked 0, 50 μM cycloheximide was added to block protein synthesis. Samples were subsequently collected at 4, 8, and 12 hours after the drug addition, and the amount of FRQ and WC-1 visualized by Western blot as in Fig. 1. These SDS gels were electrophoresed longer than usual to better resolve mobility differences due to phosphorylation. A sample from a culture grown 24 hours in the dark without cycloheximide treatment, with (+) and without (–) subsequent dephosphorylation by λ-phosphatase (*9*), is shown on the right for comparison. (**C**) FRQ levels do not affect *wc-1* transcript levels but result in increased WC-1 protein production. Mycelia of the genotype noted were transferred to the dark, treated for 6 hours in the dark with (+) or without (–) 0.01 M quinic acid (QA). (Upper) Representative Western blots are on the left and densitometric analysis representing the average of three to six experiments (see text) are on the right with the maximum for each genotype set to 100%. (Lower) RNA blots (*7, 8, 10*) showing replicate experiments on the left are presented densitometrically as averages on the right (see text). (**D**) The QA-induced peak in FRQ precedes the peak in WC-1 by 8 hours. The inducible *frq*^{10}*:qa-2FRQ* strain was cultured in the dark for 28 hours, and a 4-hour pulse of 0.01 M QA was begun at time 0. It was washed out after 4 hours and replaced with fresh medium; control cultures received only fresh medium at time 0. Samples were taken every 4 hours starting at time 0 and processed for Western blotting as in Fig. 1. On the left is a representative Western blot, and on the right is densitometric analysis of three replicate experiments (SEM). Open gray squares, FRQ in the uninduced control; open black circles, WC-1 in the uninduced control; solid gray squares, FRQ in the induced cultures rapidly rising after addition of inducer; solid black circles, WC-1 in the induced culture rising after a 4-hour lag to a peak 16 hours after addition of inducer.

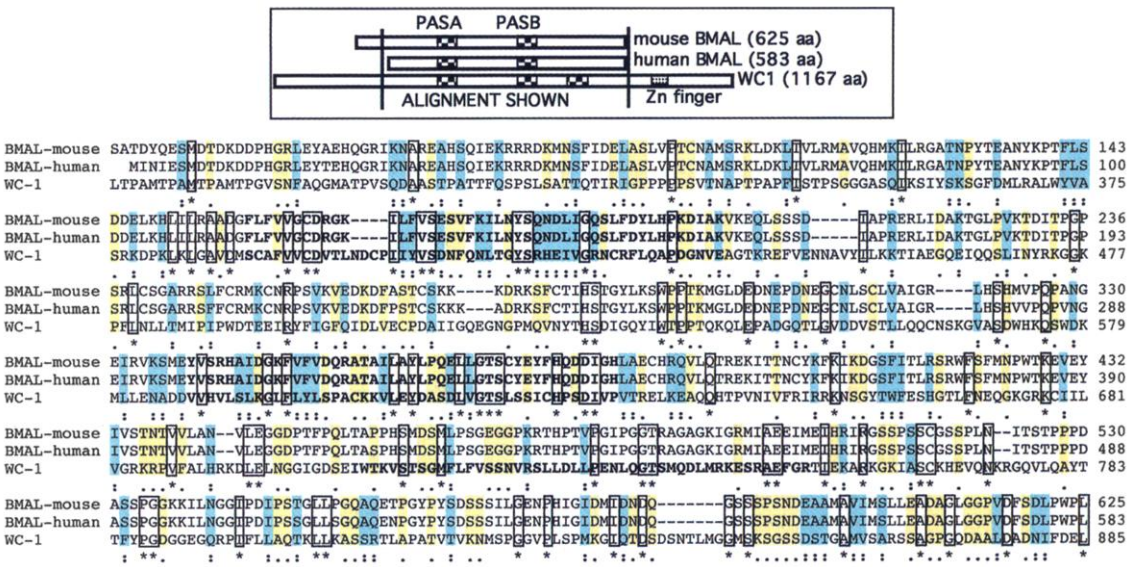

Fig. 3. WC-1 shows extended similarity to mammalian BMAL1 (*31*). Full-length human (accession no. O00327) and mouse (accession no. BAA81898) BMAL1 proteins were aligned with the central portion (amino acids 274 through 885) of WC-1 (accession no. CAA63964) by using the Blossum62 matrix and default parameters in CLUSTALW (EMBL Outstation, European Bioinformatics Institute; www.ebi.ac.uk/Tools/). The mouse BMAL1 and WC-1 sequences are shown only over the full extent of human BMAL1 and are truncated outside this region as shown at the top; hatched regions corresponding to the PAS A [also called LOV (*23*)], PAS B and C domains as identified by ProFileScan (ISREC Bioinformatics Group) are shown in bold in the alignment below, which notes identities (boxed, with "*" underneath), strong similarities (blue shading with ":" underneath) and weak similarities (yellow with "." underneath) as identified by CLUSTALW. The sequences shown are 48% identical or similar and yielded similarity scores from WU-BLAST2 (www.ebi.ac.uk/blast2/) of 3.3 e-06 (WC-1: human BMAL1) and 4.9 e-06 (WC-1: mouse BMAL1).

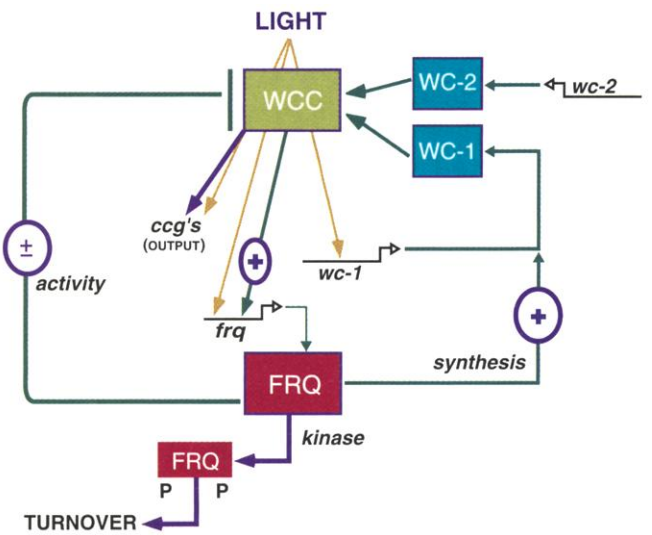

Fig. 4. Feedback loops within the *Neurospora* circadian system. Dual functions of FRQ proteins allow them to participate in multiple clock-related feedback loops, shown in green, in the *Neurospora* clock. The WC-1 and WC-2 proteins form a WCC (*11*) that activates gene expression in the dark from *frq* and possibly from *clock-controlled genes* (*ccg*'s) associated with output (*1–3*) and mediates light-induced transcription from *frq*, *ccg*'s, and *wc-1* (gold arrows). *frq* mRNA is translated to make FRQ proteins (*8*), which have two roles: (i) FRQ feeds back into the nucleus (*29*) to rapidly block the positive effect of the WCC in driving *frq* transcription and (ii) FRQ acts to promote the synthesis of new WC-1. Phosphorylation of FRQ triggers its turnover and is a major determinant of period length in the clock (*28*). Coupled to these loops in the circadian system (but not shown) is a FRQ-less oscillator or FLO (*3, 32, 33*).

The application of QA to the noninducible (*frq*$^{+}$) control strain had no effect on the levels of FRQ [in darkness, $t = 0.70$, $P = 0.52$; in light, $t = 1.13$, $P = 0.32$] or WC-1 [in darkness, $t = 0.79$, $P = 0.47$; in light $t = 53$, $P = 0.62$] (Fig. 2C). Furthermore, the steady-state levels of *wc-1* transcripts were not changed by QA application [one factor ANOVA, in darkness, $F = 1.04$, $P = 0.43$; in light $F = 0.34$, $P = 0.80$] (Fig. 2C), and no difference was noted between the *frq*^{10}*::qa-2*pFRQ and wild type in steady-state *wc-1* transcript levels. These data establish that FRQ up-regulates WC-1 through a posttranscriptional mechanism.

It was consistently observed that the peak of WC-1 lags the peak of FRQ in rhythmic oscillations in both wild-type and *frq*^{7} strains in darkness. To determine the extent to which this phase lag was the result of the promotion of WC-1 synthesis by FRQ, we followed the increase of both FRQ and WC-1 after induction of FRQ by a 4-hour pulse of QA (Fig. 2D). Addition of QA resulted in rapid strong induction of FRQ as has been seen before (*6*) with a sharp increase by 4 hours and a peak 4 to 8 hours after QA addition. Consistent with expectation, WC-1 synthesis also increased, lagging the increase in FRQ by about 8 hours and showing a peak in WC-1 about 16 hours after QA addition in a pattern that recapitulated the normal circadian oscillation in the two proteins. These data are consistent with FRQ playing a dual role in the clock, acting to depress its own synthesis and also to activate the synthesis of WC-1, the transcriptional co-activator of the *frq* gene.

The roles for WC-1 and its partner WC-2 in the *Neurospora* clock are reminiscent of those of CYC/CLK in *Drosophila* and BMAL1/CLOCK in mammals, and in all cases one of those partners is rhythmically expressed [WC-1, CLK (*12, 21*), and BMAL (*20*)], with peaks occurring in the opposite phase of the cycle from their targets. Given this similarity and the overall similarity in the appearance of the circadian feedback loops, we used BLAST2 to reanalyze a corrected WC-1 sequence (*26*) and search for similarities beyond simply the PAS domains previously reported (*8, 11, 17*). In addition to glutamine-rich activation domains, WC-1 has three PAS domains (Fig. 3) (*23*), and the sequence shows extended similarity to mammalian BMAL1 (48% of residues are identical or similar; smallest sum probability values of about 10^{-6}) that is not limited only to the PAS domains but extends over the full extent of all the mammalian BMAL1 sequences (Fig. 3). No other vertebrate proteins show comparable similarities; two insect (putative) potassium channels are as similar [reflecting the LOV domain in WC-1 (*23*)], but there is no functional connection to circadian rhythmicity as there is between WC-1 and BMAL, which play similar roles in circadian feedback loops. The similarities in time of expression, sequence, and activation function within the circadian feedback loop between WC-1 and BMAL1 suggests that the two

proteins may share other aspects of regulation also, and that the presence of interconnected loops found here for *Neurospora*, and previously in *Drosophila* and recently in mouse (*27*), may be a general property of circadian clocks.

These observations require a revision of the way we view the *Neurospora* circadian system (Fig. 4). WC-1 and WC-2 are made in the night and form a white collar complex (WCC) (*10, 23*) through PAS-domain mediated interactions. Late in the night the WCC activates transcription of *frq*, and at dawn the WCC mediates light signaling, which results in a rapid massive induction of *frq* transcript (*7, 9*) that sets the clock; meanwhile, WC-1 levels are declining. Beginning late at night and continuing in the morning, two forms of FRQ are translated from alternative ATG start codons (*8*). Their phosphorylation begins immediately (*8, 28*) and they also rapidly enter into the nucleus (*29*) where they act to block the activity of the WCC (now at close to its lowest level) thereby turning down the expression of their own gene. In the cytoplasm, FRQ translation continues from available but declining *frq* mRNA contributing to a lag between *frq* RNA and FRQ protein peaks. Also acting either directly or indirectly, FRQ promotes the synthesis of WC-1 from existing message so that levels of WC-1 begin to rise even as phosphorylation-promoted turnover of FRQ (*28*) begins. Thus, at close to the same time, FRQ is blocking activation by the WCC while promoting WC-1 synthesis to increase the level of the WCC. Finally phosphorylation of FRQ triggers its precipitous turnover (*8, 28*), FRQ promoted synthesis of WC-1 is balanced by WC-1 degradation, and WC-1 levels peak in the night near to when FRQ levels drop to their low point; the bolus of WC-1 created by the juxtaposition of FRQ promoted WC-1 synthesis and the blockage of WCC activation creates a sharp transition with high WCC activity to initiate the next cycle.

The unexpected identification of a post-transcriptional rhythm for WC-1 synthesis, dual roles for the FRQ proteins in depressing the level of their own transcript and promoting the synthesis of their transcriptional co-activator WC-1, and the resulting identification of an interconnected feedback loop within the *Neurospora* clock, show that there is much left to be learned about the circadian system of even a relatively simple model organism. The strong sequence similarities between WC-1 and BMAL1 suggest that the regulatory themes uncovered may have broad applicability. The interlocking connections among circadian feedback loops, connections that arise when clock molecules assume opposite roles in different phases of the clock cycle, should promote both robustness to the oscillation and stability to the output.

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31. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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An Empirical Assessment of Taxic Paleobiology

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The analysis of major changes in faunal diversity through time is a central theme of analytical paleobiology. The most important sources of data are literature-based compilations of stratigraphic ranges of fossil taxa. The levels of error in these compilations and the possible effects of such error have often been discussed but never directly assessed. We compared our comprehensive database of trilobites to the equivalent portion of J. J. Sepkoski Jr.'s widely used global genus database. More than 70% of entries in the global database are inaccurate; however, as predicted, the error is randomly distributed and does not introduce bias.

The publication of J. J. Sepkoski Jr.'s (*1*) factor-analytical description of the marine fossil record was an epochal event in modern evolutionary paleobiology. The compilation of marine families on which it was based (*2*, *3*) has served as the raw material for many influential papers, including studies of extinction (*4–7*), the periodicity of mass extinction (*8–10*), and evolutionary rates (*11–15*). Recognizing the need for a more detailed level of analysis, Sepkoski (*16*) began a more ambitious compilation of fossil genera (*17*), which now serves as the foundation for the majority of current work in the field.

There are critics of taxic paleobiology (*18*). Some have pointed out that taxa of a particular Linnean rank have no natural equivalence (*19*), and others (*20*, *21*) that traditional taxonomy contains a large number of polyphyletic or paraphyletic groups, which hamper the estimation of large-scale pattern (*22*). The most widespread complaint (*23*, *24*), however, has been that the basic accuracy of global databases is suspect because they are compiled by workers who are not systematic specialists. Because

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