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Assignment of circadian function for the *Neurospora* clock gene *frequency*

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Circadian clocks consist of three elements: entrainment pathways (inputs), the mechanism generating the rhythmicity (oscillator), and the output pathways that control the circadian rhythms. It is difficult to assign molecular clock components to any one of these elements. Experiments show that inputs can be circadianly regulated¹⁻³ and outputs can feed back on the oscillator^{4,5}. Mathematical simulations indicate that under- or overexpression of a gene product can result in arrhythmicity, whether the protein is part of the oscillator or substantially part of a rhythmically expressed input pathway⁶. To distinguish between these two possibilities, we used traditional circadian entrainment protocols^{7,8} on a genetic model system, *Neurospora crassa*.

Rather than being directly driven by environmental changes, the *zeitgeber*, circadian rhythms are controlled by endogenous oscillators, and synchrony with *zeitgeber* is achieved by entrainment of the oscillator. Unlike simple reactions to external signals, the complex mechanisms of entrainment reflect the robust momentum of the running clock as well as a time-of-day-specific responsiveness to *zeitgeber* signals. As a result, circadian clocks adopt specific stable phase relationships to *zeitgeber* cycles. In short cycles, entrained rhythms lag relative to the *zeitgeber*; in long cycles, they lead. When the cycle length is approximately half that of the endogenous free-running period (FRP, measured in constant conditions) then circadian rhythms (unlike driven rhythms) often 'frequency demultiply'⁹ (skip a cycle). The *Neurospora* clock is monitored by observing rhythmic conidiation (asexual spore formation) while it is growing in glass tubes. Null mutants of the *Neurospora* clock gene *frequency* (*frq*) are arrhythmic in constant conditions, induction of the protein FRQ resets the circadian phase, and FRQ is self-regulated by negative feedback. These findings are theoretically

Figure 1 Circadian entrainment of *frq*⁺ by 12 h temperature cycles (**a**, 16–32 °C; **b**, 22–27 °C; 6 h low (grey) and 6 h high (white) temperature in constant darkness). Conidiation (black areas) is double plotted: the data from 2 days are graphed in a continuum, starting at the top left of the graph with day 1, and moving down by one day on each line of the vertical axis. Double plotting the data in this way allows trends to be visualized across midnight. Note that conidiation occurs in warmth in high-amplitude cycles and in cold in low-amplitude cycles.

consistent with FRQ being either a rhythmically expressed input component⁶ or an oscillator component¹⁰⁻¹². Entraining conditions could distinguish between these possibilities. If FRQ is not essential for the oscillator, then both FRQ-sufficient (*frq*⁺, wild-type, FRP = 22 h; *frq*¹, short-period mutant, FRP = 16 h; *frq*⁷, long-period mutant, FRP = 29 h) and FRQ-deficient strains (*frq*⁹, frame shift by base-pair deletion resulting in premature stop codon; the phenotype is indistinguishable from the null mutant, *frq*¹⁰)¹³⁻¹⁶ should show typical circadian entrainment.

To test whether temperature *zeitgeber* cycles entrain or merely drive the conidiation rhythm, short cycles (6:6) were imposed on *frq*⁺. In high-amplitude cycles (16 to 32 °C), a driven component is present once each cycle (every 12 h, Fig. 1a). Circadian regulation is, however, apparent from the asymmetric conidiation in alternate cycles. In low-amplitude cycles (22 to 27 °C, Fig. 1b), the driven component disappears, and conidiation occurs every other cycle (once every 24 h; a frequency demultiplication). Phase changes due to different *zeitgeber* amplitudes (compare Fig. 1a and b) are typical of entrainment of circadian clocks⁷. Thus, temperature cycles result in true circadian entrainment of *Neurospora* and do not merely drive conidiation.

We compared the different *frq* mutants in temperature cycles of different lengths (see typical examples in Fig. 2a and b). All strains tested, including the FRQ-deficient mutant *frq*⁹, establish a stable phase relationship to the *zeitgeber* cycle, although *frq*¹ and *frq*⁹ are almost 180° out of phase. The two rhythmic strains, *frq*¹ and *frq*⁷ (FRPs of 16 and 29 h), also entrain in anti-phase (Fig. 2c). The different phase position of each strain over a wide range of *zeitgeber*

Figure 2 Temperature cycles and circadian entrainment of *frq* mutants. **a**, *frq*⁺ and *frq*⁹ in 19 h (9.5:9.5) temperature cycles (22–27 °C, shading as in Fig. 1). **b**, Average pixel densities of 6 tubes (thick line) for the experiment shown in **a** (±s.d. indicated by thin lines). **c**, Phase plots: Onsets of conidiation for *frq*⁹ (open circles), *frq*¹ (squares), *frq*⁺ (filled circles) and *frq*⁷ (triangles) are expressed in real hours ('phase') relative to the warm to cold (W → C) transition. Negative values indicate that onsets lag the temperature transition. Lines represent linear fits.

periods (T) depends on the strain's FRP (judged by those which are rhythmic in constant conditions). When T equals the FRP, conidiation starts 5–6 h (by extrapolation) after the warm-to-cold transition. As expected, conidiation leads in longer and lags in shorter cycles. This typical circadian entrainment^{7,17} produces the series of parallel lines. The phase plot of the arrhythmic strain (frq^9 , open circles) shows the same slope as the rhythmic strains (filled symbols), indicating that it is controlled by the same temperature-entrainable oscillator. Its relative position in Fig. 2c indicates a virtual FRP of about 12 h (by extrapolation, frq^9 conidiates 5–6 h into the cold when $T = 12$). Characteristic circadian entrainment is a property of all tested strains and, therefore, does not require functional FRQ protein. The light-blind *white collar* mutants^{18,19}, which are also arrhythmic in constant conditions, have a mutation in a gene that is required for frq transcription. Their responses to temperature cycles are superimposable with those of frq^9 (data not shown), probably because of the lack of FRQ. FRQ appears to determine FRP and hence also the phase position in temperature cycles.

Figure 3 frq mRNA levels in temperature cycles (22–27 °C, shading as in Fig. 1) for frq^+ (filled circles) and frq^9 (open circles). **a**, 22-h cycle; **b**, 16-h cycle. Rectangles at the top and bottom of the graphs indicate the timing of the entrained conidial bands in glass tubes (filled, frq^+ ; open, frq^9). Curves represent significant 2-harmonic-cosine fits ($r = 0.95$ for the 22-h cycle and 0.97 for the 16-h cycle). Curve fits for frq^9 were not significant ($r = 0.62$ and 0.60 , respectively).

Figure 4 Light:dark cycles do not produce circadian entrainment. **a**, 12-h light:dark cycles (dark phase in grey; fluences in $\text{nE m}^{-2} \text{s}^{-1}$ at left; constant temperature 22 °C). **b**, Phase plots for light cycles (400 $\text{nE m}^{-2} \text{s}^{-1}$, symbols as in Fig. 2c). Onsets of conidiation are related to the light-dark (L $\rightarrow$ D) transition, expressed in real

In free-running cultures of FRQ-sufficient strains, the overall abundance of frq messenger RNA oscillates with a periodicity that matches conidiation^{10,11}. mRNA accumulation in different *zeitgeber* periods (compare phase angles in Fig. 3a and b) also mirrors conidiation and shows that frq is controlled by the oscillator and not merely by temperature²⁰. In contrast, RNA levels in frq^9 are high and non-systematically variable throughout the temperature cycle, as they are in free-running cultures¹⁰. Thus, the temperature-entrainable oscillator requires FRQ protein to control frq accumulation (by negative feedback^{10,21}), but not to control rhythmic conidiation.

To compare light and temperature as *zeitgeber* signals for *Neurospora*, we used the demultiplication protocol described above. Light:dark cycles (6:6, 400 $\text{nE m}^{-2} \text{s}^{-1}$) drive conidiation in every cycle (Fig. 4a, top panel). The fluence rate was, therefore, titrated down to find conditions where light:dark cycles induce circadian entrainment as shown by frequency demultiplication and systematic phase-angle changes due to different *zeitgeber* amplitudes. At as low as 8 $\text{nE m}^{-2} \text{s}^{-1}$, conidiation is still driven every 12 h (Fig. 4a). At 4 $\text{nE m}^{-2} \text{s}^{-1}$ (equivalent to the light of a night with a full moon), a free-running rhythm appears in addition to the light-driven component. At 1 $\text{nE m}^{-2} \text{s}^{-1}$, conidiation runs free as in constant darkness. Irrespective of *zeitgeber* amplitude, neither frequency demultiplication nor phase angle changes occur in full photoperiod cycles, as they do in temperature cycles (Fig. 1). A third characteristic of circadian entrainment, phase angle changes due to different *zeitgeber* periods, is also absent in light:dark cycles (Fig. 4b). Onset of conidiation is independent of T , occurring a set amount of time after the light:dark transition (equal to 1/3 of the strain's FRP; compare with Fig. 2c). The lack of frequency demultiplication and the fixed phase angles show that full photoperiods drive, rather than entrain, conidiation of the FRQ-sufficient strains (frq^+ , frq^1 , frq^7). The threshold level above which light drives conidiation is the same as the level that renders *Neurospora*²² and *Drosophila pseudoobscura*²³ arrhythmic in constant light.

Molecular and physiological light responsiveness *per se* is not impaired in frq -null strains^{12,13,24}, but the temperature-entrainable oscillator is unresponsive to light:dark cycles in frq^9 (Fig. 4c; see also ref. 25) and frq^{10} (data not shown). We predict, therefore, that FRQ is part of a clock-regulated light input pathway to the temperature-

hours. **c**, frq^+ and frq^9 in a 19-h light:dark cycle (constant temperature 26 °C, 400 $\text{nE m}^{-2} \text{s}^{-1}$ white light). Statistical analysis revealed no rhythmicity in frq^9 . Experiments at 22 °C gave the same result.

entrainable oscillator. Note that the different rhythmic *frq* mutants show strain-specific responses to short light pulses^{25,26}.

In laboratory experiments, the effect of light on the *Neurospora* clock, even at moonlight levels, is so strong that it apparently stops the oscillator²². In nature, such a clock would be no more than an hourglass timer, functioning only during the darkest nights. The light-cycle experiments shown here were done in constant temperature, but when light and temperature are given in anti-phase in a 24 h cycle (cold with light and warm with dark)²⁰, temperature dominates as the *zeitgeber*. When *frq*⁺ is exposed to temperature cycles (22–27 °C) in constant light (which results in arrhythmicity in constant temperature), conidiation is robustly rhythmic (data not shown). Thus, temperature cycles 'gate' light transduction, enabling the *Neurospora* clock to continue throughout the day. Our results show that FRQ is unnecessary for entraining an oscillator with generic circadian properties in temperature cycles. However, without FRQ, the circadian clock cannot synchronize to light:dark cycles and self-sustained rhythmicity is almost never seen, except under special conditions^{13,14,27}. As part of a circadianly regulated light input pathway^{1–3}, FRQ apparently supplies the clock with sufficient amplitude for self-sustained rhythmicity (*frq*⁰ remains arrhythmic after release from temperature entrainment) and sets the period in the circadian range. This role of FRQ is especially relevant in view of the recent reports of clock-regulated light transduction (via cryptochrome) to the clock in flies and mammals^{1,28,29}. □

Methods

Strains and media. *bd*, *bd frq*¹ and *bd frq*⁰ are standard lab strains (provided by the Dunlap Lab, Dartmouth Medical School). *bd frq*⁷ was obtained from the Fungal Genetics Stock Center. Solid media for glass tubes contained 1× Vogels salts¹⁴, 0.3% glucose, 0.5% L-arginine, 2% agar and 10 ng biotin per ml. Liquid media was the same, except for 0.5% glucose and the lack of agar.

Zeitgeber cycles. Depending on the *zeitgeber*, half of each cycle was spent in low temperature or darkness, the other half in high temperature or light (Lumilux Interna, Osram). Temperature cycles were created in custom-made incubators. For increases in temperature, 90% of the end point (22 to 27 °C) was attained in 48 min. For decreases (27 to 22 °C) 108 min was required for 90% of the change. Light cycles were administered at constant temperature (26 or 22 °C). Fluence was titrated with neutral density filters (Rosco). Glass tubes were inoculated and incubated for about 1 day in constant light and ambient lab temperature (23 ± 1 °C) before transfer to the *zeitgeber* cycle (low temperature or darkness).

mRNA analysis. Glass tubes and liquid cultures (3.7 × 10⁸ conidia per 10 ml of media, in a 50 ml Erlenmeyer flask) were simultaneously inoculated and transferred (see above) to the experimental set-up. Samples were collected over the course of the third full cycle, by which time conidiation rhythms are generally stably entrained. RNA was prepared and analysed by standard methods^{12,21}. Loading differences were normalized by relating *frq* to ribosomal RNA. For each individual data set, the average quantification in the *frq*⁰ series is 1 and all other values (*frq*⁺ and *frq*⁰) are expressed as proportions thereof.

Data analysis. Images of glass tubes were digitized with an Apple Color One scanner, stored as PICT files and analysed with CHRONO³⁰. Conidiation was quantified by the number of white pixels in each vertical line of the image and expressed as deviation around the non-rhythmic trend. 3–6 tubes were analysed for each time series and variable. Onsets of conidiation were defined as upward transition through the non-rhythmic trend (see zero lines in Figs 2b and 4c) of the averaged time series.

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Control of organ shape by a secreted metalloprotease in the nematode *Caenorhabditis elegans*

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The molecular controls governing organ shape are poorly understood. In the nematode *Caenorhabditis elegans*, the gonad acquires a U-shape by the directed migration of a specialized 'leader' cell, which is located at the tip of the growing gonadal 'arm'. The *gon-1* gene is essential for gonadal morphogenesis: in *gon-1* mutants, no arm elongation occurs and somatic gonadal structures are severely malformed². Here we report that *gon-1* encodes a secreted protein with a metalloprotease domain and