SI Text

Methods

Measuring the lifetime of the *wc-1* mRNA. A *bd, his3 a N. crassa* strain (87-84) kindly provided by J. C. Dunlap and J. J. Loros (Department of Biochemistry, Dartmouth Medical School, Hanover, NH) was crossed with a *wc-1, met-7 A* strain (FGSC 3914). Offspring 87-84-6 was confirmed to be *his-3 wc-1* at 34°C and was transformed with a plasmid pDE3dBH-*qa-2:wc-1* (targeting the *his-3* locus) kindly provided by Y. Liu (1) using the spheroplast method and histidine selection (2). Transformant 87-84-6-8 was tested to band at 0.001 M quinic acid (QA) + 0.17% arginine + Fries medium and not to band on 0.1% glucose + 0.17% arginine + Fries medium in race tubes (3); transformant 87-84-6-8 was also confirmed to be *wc-1,a* by growth at 34°C on 2% glucose + Fries medium and a cross to a *bd A* (FGSC 1858). In each lifetime, experiment transformant 87-84-6-8 was grown in 16 or more replicate 500-ml flasks with 120 ml of QA (0.0192% or 0.3%) + Fries medium liquid culture for 4 h at 25°C in a shaker (New Brunswick Scientific, Edison, NJ, Series 25) at 150 rpm under 70 μm/s per m^2 light source and then shifted to 2% galactose (or 2% glucose) + Fries medium in the dark (or light) (other conditions same). For each time point, a culture was harvested by vacuum filtration through a Buchner funnel onto 541 Whatman paper and frozen at -70°C for later RNA isolation using the High Pure RNA isolation kit (Roche). The quality and quantity of the RNAs were assessed by using an RNA Nano LabChip (Agilent Technologies). RNA was analyzed by real-time PCR (rt-PCR). cDNAs were synthesized from 1.6 μg of total RNA with the High-Capacity cDNA Archive (synthesis) kit (Applied Biosystems). *wc-1* and *rRNA* cDNAs were detected by rt-PCR (ABI-Prism 7500, Applied Biosystems) according to the manufacturer's directions by using *Taq*Man probes. Triplicate reactions (50 μl) were analyzed by using the $\Delta\Delta C_T$ method (Applied Biosystems). As a control, five zero-time points were collected before shift to the preferred carbon source (induced) as well as 11 time points under shift from glucose to glucose (2%) (noninduced) and quantitated for the level of *wc-1* mRNA. The control enabled us to determine the full range of response of the QA-inducible switch.

Ensemble identification of genetic networks. In trying to model the foregoing experimental data in terms of a genetic network such as shown in Fig. 1, we are faced with a fundamental and ubiquitous difficulty of systems biology: essentially all of the relevant model parameters (including, e.g., molecular species initial concentrations and reaction rate coefficients) are unknown, and there is a large number of such unknown parameters whereas the available experimental data are sparse and noisy. Even a relatively simple genetic network can require many unknown model parameters. For example, 47 parameters (including 16 initial species concentrations, 26 rate coefficients and 5 unknown concentration unit conversion factors) are required to model the above-described experimental data sets with the simple circuit in Fig. 1. The unknown parameters are typically poorly constrained only by a sparse set of noisy profiling data, available only for a limited number of molecular species (e.g., 183 data points for altogether five different species in the *N. crassa* clock system in the dark). To obtain a meaningful comparison of the model to the available data (3-6), we have used a novel ensemble method (7, 8) of circuit identification which was developed for the context of sparse, noisy time-dependent profiling data without requiring, e.g., any stationary state assumption concerning the reactants and products in the genetic network (9). Instead of trying to identify one unique model parameter set, our goal in this ensemble method is to generate a large, random sample of models, i.e., an ensemble of models, consistent with the available RNA and protein profiling data, implemented as a Monte Carlo (MC) simulation technique (7). In the ensemble method, a random walk is initiated in the 47-dimensional space of model parameters, and a likelihood function Q (defined below) is used to guide the walk into a parameter region of near-maximum Q values. The Q value in this context is the likelihood that the genetic network model in Fig. 1 could have given rise to the observed profiling data (3-6), calculated as a function of the model parameters (i.e., the rate coefficients, initial concentration values of all species, and unit conversion factors of observed species in the genetic network). We now give a more detailed, formal description of the ensemble approach, as applied here specifically to the *N. crassa* biological clock system.

Let the M-dimensional vector $\boldsymbol{\theta} := (\theta_1, .., \theta_M)$ denote the unknown parameters, comprising the natural logarithms (ln) of the rate coefficients, of the initial species concentrations and of any unknown unit conversion factors in the model, so that, e.g., M = 47 for the full model in Fig. 1. All species concentrations are measured and given here in a common, but unknown "model unit" of concentration (*cu*) and all rate coefficients in units of $1/(\text{hour} \times cu^{k-1})$ for reactions of *k*th order (i.e., having *k* reactants). Our ensemble of models is then formally described in terms of a probability distribution, the likelihood function $Q(\boldsymbol{\theta})$, on the "model space" of all "model vectors" $\boldsymbol{\theta}$. To construct such a $Q(\boldsymbol{\theta})$, suppose that in a series of M_E experiments, labeled by $e = 1,.. M_E$, in each experiment the concentrations $[s]$ of certain species *s* are measured at time points *t*, let $Y_l := \ln([s]^{(x)}_{t,e})$ for each data point labeled by $l := (t,s,e)$. Here, the superscript "(*x*)" in $[s]^{(x)}_{t,e}$ denotes that concentration is measured in some experimental or "detector units" of concentration, such as photon or radioactive decay count units or ratio of induction units. Next, let $\mathbf{Y} := (Y_1, \ldots, Y_D)$ denote the *D*-dimensional vector of all those Y_l. Likewise, let $\mathbf{F}(\boldsymbol{\theta}) := (F_1(\boldsymbol{\theta}),..,F_D(\boldsymbol{\theta}))$ denote the corresponding predicted values for these observables $\mathbf{Y}$ for a given model $\boldsymbol{\theta}$. For the above-described set of observables $\mathbf{Y}$, the predicted values $\mathbf{F}(\boldsymbol{\theta})$ are calculated from $\boldsymbol{\theta}$ by numerically solving the network's system of rate equations with the rate coefficients and initial conditions comprised by $\boldsymbol{\theta}$ and then calculating from that solution the predicted log-concentration $F_l(\boldsymbol{\theta}) := \ln(\phi_{s,t,e}\, [s]_{t,e})$ for each observed species *s* at each observation time point *t* in each experiment *e*. Here, $[s]_{t,e}$ denotes the predicted species concentrations, given in the model unit "cu", and $\phi_{s,t,e}$ denotes the unknown unit conversion factor from the model unit to the various detector units used to represent the experimental data. Subsets of experimental data points (*s,t,e*) which have been measured under identical conditions in the same experiment with the same experimental detection method share the same $\phi_{s,t,e}$-value; for the experimental data sets analyzed here, only five independent, unknown $\phi_{s,t,e}$-parameters are required. Experimental data points shown in Fig. 2 have been converted to model units, i.e., are shown as ensemble averages of the decadic log, $\lg([s]^{(x)}_{t,e} / \phi_{s,t,e})$.

It is reasonable to assume (but not fundamental to our ensemble method!) that the probability distribution $P(\mathbf{Y})$ of the data are representable as a multivariate Gaussian $P(\mathbf{Y}) \equiv P(\mathbf{Y}; \boldsymbol{\mu}, \boldsymbol{\Sigma}) = \text{const} \; x \; exp\,[-\chi^2/2]$ with

$$\chi^2(\mathbf{Y}; \boldsymbol{\mu}, \boldsymbol{\Sigma}) = (\mathbf{Y}-\boldsymbol{\mu})^{\mathrm{T}} \boldsymbol{\Sigma}^{-1} (\mathbf{Y}-\boldsymbol{\mu})$$

and $\boldsymbol{\mu}$ and $\boldsymbol{\Sigma}$ denote the mean and variance-covariance matrix of the observation vector $\mathbf{Y}$, with $\Sigma_{lk} := \sigma_l^{\,2} \delta_{lk}$ and an assumed standard deviation of $\sigma_l \cong 0.14$ for all log-concentration data points Y_l. A given $P(\mathbf{Y}; \boldsymbol{\mu}, \boldsymbol{\Sigma})$ does of course *not* uniquely determine the model ensemble $Q(\boldsymbol{\theta})$. There is an infinite manifold of $Q(\boldsymbol{\theta})$ which is consistent with the data distribution $P(\mathbf{Y})$, and we have to make "reasonable" choices. The simplest choice which we have adopted here is to take $P(\mathbf{Y}; \boldsymbol{\mu}, \boldsymbol{\Sigma})$ as the likelihood (in which the experimental data $\mathbf{Y}$ are viewed as fixed) to determine the ensemble $Q(\boldsymbol{\theta})$. Thus the parameters $\boldsymbol{\theta}$ are distributed according to the following likelihood:

$$Q(\boldsymbol{\theta}) = \Omega^{-1} P(\mathbf{Y}; \mathbf{F}(\boldsymbol{\theta}), \boldsymbol{\Sigma}) = \Omega^{-1} W(\boldsymbol{\theta})$$

with a weight $W(\boldsymbol{\theta}) := P(\mathbf{Y}; \mathrm{F}(\boldsymbol{\theta}), \boldsymbol{\Sigma})$ and normalization factor $\Omega := \sum_{\boldsymbol{\theta}} W(\boldsymbol{\theta})$ where $\sum_{\boldsymbol{\theta}}$ denotes integration over all M components of $\boldsymbol{\theta}$. We restrict the allowed θ_m – domain for unknown log initial concentrations and log rate coefficients to $\exp(\theta_m) > 10^{-8}$ (measured in the model units stated above).

To generate a random sample of $\boldsymbol{\theta}$-points distributed according to $Q(\boldsymbol{\theta})$, we use a standard *Metropolis algorithm* (7)**:** starting from some initial $\boldsymbol{\theta}^{(ini)}$, we generate a Markovian random walk (7) through $\boldsymbol{\theta}$-space. For each step of this walk, we propose a random change to either one randomly selected $\boldsymbol{\theta}$-component ("local update") or simultaneously to all $\boldsymbol{\theta}$-components ("global update"). The so-proposed $\boldsymbol{\theta}'$ is then probabilistically either accepted (i.e., the walk moves from $\boldsymbol{\theta}$ to the new $\boldsymbol{\theta}'$) or rejected (i.e., the walk remains at the old $\boldsymbol{\theta}$). The probability for acceptance (7) of proposed new

$\boldsymbol{\theta}'$-points is designed such that the terminal distribution of $\boldsymbol{\theta}$-points visited by the random walk after a large number of such updating steps ("equilibration"), is the desired ensemble distribution $Q(\boldsymbol{\theta})$ (7). Only the weight function $W(\boldsymbol{\theta})$, but *not* the normalization factor Ω, needs to be evaluated in each such a Metropolis updating step, because only ratios of probabilities, $Q(\boldsymbol{\theta}')/Q(\boldsymbol{\theta}) = W(\boldsymbol{\theta}')/W(\boldsymbol{\theta})$ enter into the calculation (7). However, each Metropolis step does require a completely new solution of the reaction network kinetic rate equations to evaluate the weight $W(\boldsymbol{\theta}')$ for the proposed new model $\boldsymbol{\theta}'$. The kinetic rate equation solutions were obtained by the adaptive Runge-Kutta method with a global error tolerance of 10^{-8}, and verified with other higher-order stiff methods (10).

In our actual simulation runs, we did *not* update all $\boldsymbol{\theta}$-components, θ_m, according to the foregoing procedure. Rather, we chose the logs of unknown, independent unit conversion factors $\ln(\phi_{s,t,e})$ so as to maximize $Q(\boldsymbol{\theta})$, given the *M' = 42* remaining (nonunit-conversion-factor) $\boldsymbol{\theta}$-components. Only the remaining $\boldsymbol{\theta}$-components were subjected to the random Metropolis updating steps described above, by using the so-maximized $Q(\boldsymbol{\theta})$ as the terminal distribution. Because of the Gaussian dependence of the original $Q(\boldsymbol{\theta})$ on the log unit conversion factors, $\ln(\phi_{s,t,e})$, this "reduced" MC procedure is mathematically equivalent to the "full" MC procedure of subjecting *all M* $\boldsymbol{\theta}$-components, including all $\ln(\phi_{s,t,e})$, to random Metropolis updates. The corresponding "reduced" values of $\chi^2(\mathbf{Y}; \mathbf{F}(\boldsymbol{\theta}), \boldsymbol{\Sigma})$, minimized with respect to the independent $\ln(\phi_{s,t,e})$, are what is shown in SI Fig. 5 and Fig. 3.

For the model in Fig. 1 with $n = m = 4$, we first chose some set of rate coefficients and initial concentrations to give us a (weakly damped or undamped) oscillatory solution. We then rescaled the rate coefficients and initial concentrations and shifted the initial time value so that the period, maximal amplitude and phase of the oscillation for the *[CCG]* protein species in the model roughly matched those of the experimental *[CCG]* data. The resulting model parameter vector served as the initial $\boldsymbol{\theta}^{(ini)}$ in our MC equilibration run for the $n = m = 4$ model as well as other models considered in Fig. 3. We used a 1:1

random mixture of local and global updating moves, with the maximum proposed step widths automatically adjusted after every 20th sweep (where one sweep = *M* Metropolis updating steps) so as to keep the average Metropolis acceptance probability in both local and global updating steps around 50%, e.g., between 0.34 and 0.66 for results reported in Figs. 2 and 3. After about 4-6 $\times 10^4$ equilibration MC sweeps, about 4×10^4 accumulation MC sweeps were performed, and the components of the resulting $\boldsymbol{\theta}$ at the end of each accumulation sweep were included into our MC random sample.

In SI Fig. 5, the progress of such a MC random walk toward its "equilibrium state" is shown. This "equilibrium" is reached when the probability for a given parameter set to be visited equals the likelihood, Q, and, consequently, when the walk mainly explores regions of near-maximal Q-values or, equivalently, near-minimal values of χ^2. The "model ensemble" is then the collection of models "$\boldsymbol{\theta}$" which are visited after the random walk has settled into its equilibrium state. Results of this walk to an equilibrium state from different random number seeds are reported in (11).

In conventional maximum-likelihood methods one seeks to identify a unique model $\boldsymbol{\theta}^{(opt)}$ by maximization of some likelihood function Q($\boldsymbol{\theta}$). This is then sometimes complemented by a sensitivity analysis, based on the local behavior of Q($\boldsymbol{\theta}$) in close proximity to $\boldsymbol{\theta}^{(opt)}$, or based on an *ad hoc*, brute force exploration of a few wider, but dimensionally limited parameter regions. Such an approach is justified if experimental data are abundant, available for essentially all molecular species, and low in noise, resulting in a Q($\boldsymbol{\theta}$) sharply peaked at $\boldsymbol{\theta}^{(opt)}$. By contrast, in our current situation, experimental data are sparse, noisy and available for only a few of the many potentially relevant molecular species. As a consequence, there may then exist vast expanses of $\boldsymbol{\theta}$-space where Q($\boldsymbol{\theta}$) is maximal, or nearly so, and any unique, "optimal" choice of $\boldsymbol{\theta}$ (if one exists, by whatever choice of likelihood!) may seriously misrepresent the information actually contained in the data. The crucial advantage of the ensemble method is that it systematically explores those expanses of $\boldsymbol{\theta}$-space. In doing so, it allows us to get a more complete and systematic understanding of what can be known, inferred or predicted on the basis of the existing data and, of equal importance, what is not known and cannot be

predicted. Thus, the method allows us to make some quite definitive, experimentally testable model predictions for some model parameters and some observable properties, even though many other parameters and properties may be very poorly constrained. Furthermore, the presently most poorly constrained properties are those whose future measurement will provide the most stringent additional constraints. Hence the ensemble can systematically guide the design of maximally informative "new" experiments, based on the available "old" data.

Results and Discussion

Stability analysis of genetic network. The model in Fig. 1 can be translated into a system of 16 differential equations describing the rate of change of each of the 16 species in the genetic network as a function of time *t*. The 16 species concentrations *[wc-1^1], [wc-1^{r0}], [wc-1^{r1}], [WC-1], [wc-2^1], [wc-2^r], [WC-2], [WCC], [frq^0], [frq^1], [frq^{r1}], [FRQ], [ccg^0], [ccg^1], [ccg^{r1}],* and *[CCG]* are abbreviated here to u_1, u_{r0}, u_{r1}, u_p, v_1, v_r, v_p, w, f_0, f_1, f_r, f_p, g_0, g_1, g_r, and g_p, respectively, with constant total *frq*-gene concentration $f_G := f_0 + f_1$. The reaction labels in Fig. 1 double as the rate coefficients in the reaction network. This 16-dimensional model can be reduced to a 7-dimensional one by several simplifications. The clock-controlled gene and its products (g_0, g_1, g_r, and g_p) can be dropped from the rate equations because their dynamics are driven entirely by the clock genes (*wc-1*, *wc-2*, and *frq*) and their products, and the *ccg* products do not feed back on the clock genes in Fig. 1. The *WC-2* protein is in 5-fold molar excess over *FRQ* and *WC-1* in the nucleus (12), and hence *wc-2* and its products (v_1, v_r, and v_p) can be treated approximately as constants. The total amount of each gene, e.g., $f_0 + f_1 =: f_G$ is constant, allowing us to eliminate f_0. Likewise, the concentration of the unregulated *wc-1* gene, u_1, is a constant (4). These simplifications lead to a reduced model with a "dynamical vector" $\mathbf{y} := (f_1, f_r, f_p, w, u_p, u_{r1}, u_{r0})$ obeying the following 7 rate equations, of the general form $\dot{\mathbf{y}} = \mathbf{\Gamma}(\mathbf{y})$, with the seven components of the "reaction rate vector" $\mathbf{\Gamma}(\mathbf{y})$ given by the right-hand sides of the rate equations:

$$\dot{f}_1 = A(f_G - f_1)w^n - \overline{A}f_1$$

$$\dot{f}_r = S_3(f_G - f_1) - S_4 f_1 - D_3 f_r$$

$$\dot{f}_p = L_3 f_r - D_6 f_p$$

$$\dot{w} = E_2 u_p - D_8 w - nA(f_G - f_1)w^n + n\overline{A}f_1 - Pwf_p{}^m$$

$$\dot{u}_p = L_1 u_{r1} - D_4 u_p - E_2 u_p$$

$$\dot{u}_{r1} = C_1 u_{r0} f_p - D_7 u_{r1}$$

$$\dot{u}_{r0} = V_1 - D_1 u_{r0} - C_1 u_{r0} f_p$$

Here, e.g., $\dot{w} \equiv dw/dt$ denotes the time derivative of *w(t)*; $E_2 \coloneqq C_2 v_p =$ constant and $V_1 \coloneqq S_1 u_1 =$ constant. The Hill coefficients *n* and *m* are, respectively, the number of WCC molecules needed to cooperatively activate *frq* and *ccg*; and the number of FRQ molecules needed to degrade cooperatively WCC.

To explore the long-time dynamics of our clock model, we analyze its stationary states or "fixed points" (FP), denoted by $\mathbf{y}^*$, where all species' time derivatives would vanish, i.e., the solution(s) of the 7 coupled equations $\mathbf{\Gamma}(\mathbf{y}^*) = \mathbf{0}$. We can show that the model has at most three FPs. By linearizing the rate equations near the FP, we can find out whether or not the FP is *stable* (i.e., for slight departures the system returns to the FP) (13, 14). If all FPs of the model are *unstable*, then a variety of nontrivial dynamical behaviors are possible, including oscillations. So, a necessary and sufficient condition for the model to exhibit only sustained oscillations, regardless of initial conditions, is that all its FPs be

unstable (13). Stability or instability of a FP is governed by the "stability matrix" **J,** the Jacobian of $\mathbf{\Gamma}(\mathbf{y})$ with matrix elements $J_{ij} := \partial \Gamma_i / \partial y_j$ evaluated at $\mathbf{y}^*$ which, for our 7-dimensional model, has the general form:

$$\begin{pmatrix}
-\gamma_1 & 0 & 0 & d_1 & 0 & 0 & 0 \\
\bar{b}_1 & -\gamma_2 & 0 & 0 & 0 & 0 & 0 \\
0 & \bar{b}_2 & -\gamma_3 & 0 & 0 & 0 & 0 \\
\bar{d}_1 & 0 & \bar{b}_3 & -\gamma_4 & b_4 & 0 & 0 \\
0 & 0 & 0 & 0 & -\gamma_5 & b_5 & 0 \\
0 & 0 & \bar{d}_3 & 0 & 0 & -\gamma_6 & b_6 \\
0 & 0 & \bar{e}_3 & 0 & 0 & 0 & -\gamma_7
\end{pmatrix}$$

The nonzero **J**-matrix elements are given by $\gamma_1 = Aw^n + \bar{A}$, $\gamma_2 = D_3$, $\gamma_3 = D_6$, $\gamma_4 = D_8 + n^2 A w^{n-1}(f_G - f_1) + P f_p{}^m$, $\gamma_5 = D_4 + E_2$, $\gamma_6 = D_7$, $\gamma_7 = D_1 + C_1 f_p$, $\bar{b}_1 = S_4 - S_3$, $\bar{b}_2 = L_3$, $\bar{b}_3 = -mPw f_p{}^{m-1}$, $\bar{d}_1 = n(Aw^n + \bar{A})$, $\bar{d}_3 = -\bar{e}_3 = C_1 u_{r0}$, $b_4 = E_2$, $b_5 = L_1$, $b_6 = C_1 f_p$, and $d_1 = nAw^{n-1}(f_G - f_1)$, with all concentrations set to their respective FP values (e.g., $f_p = f_p{}^*$). The sparseness and regularity of this matrix **J** is due to the closed feedback loop in the genetic network in Fig. 1 and mathematically resembles the linearized system of the synthetic oscillator known as the repressilator (15).

A FP $\mathbf{y}^*$ *is* unstable if and only if at least one of the (in general complex) eigenvalues of **J** acquires a positive real part. The eigenvalues of **J**, denoted by λ, are the roots of the 7th

order characteristic polynomial $\Phi(\lambda) := \det(\mathbf{J} - \lambda\mathbf{E})$ where **E** denotes the unit matrix (16). By factorization of $\Phi(\lambda)$ into lower-order subpolynomials and a Routh-Hurwitz analysis (17) of these subpolynomials, we can prove that an FP is unstable [i.e., a complex λ exists with $\Phi(\lambda) = 0$ and $\mathrm{Re}(\lambda)>0$] *if and only if*

$$R := a_3^2 + a_1^2 a_4 - a_1 a_2 a_3 > 0$$

where the a_n are coefficients of a 4th order subpolynomial of $\Phi(\lambda)$, given in terms of the **J**-matrix elements by

$$a_1 = \gamma_1 + \gamma_2 + \gamma_3 + \gamma_4,\ a_2 = (\gamma_1 + \gamma_2)(\gamma_3 + \gamma_4) + \gamma_1\gamma_2 + \gamma_3\gamma_4 - d_1\bar{d}_1,$$
$$a_3 = \gamma_1\gamma_2(\gamma_3 + \gamma_4) + \gamma_3\gamma_4(\gamma_1 + \gamma_2) - d_1\bar{d}_1(\gamma_2 + \gamma_3),\ a_4 = \gamma_1\gamma_2\gamma_3\gamma_4 - d_1\bar{b}_1\bar{b}_2\bar{b}_3 - d_1\bar{d}_1\gamma_2\gamma_3.$$

SI Fig. 6 shows a projection of a MC-generated model ensemble into a 3D parameter subspace. Different colors indicate whether the model FPs are all unstable ($R>0$, in red) or whether at least one FP of the model is stable ($R<0$, in blue) according to our Routh-Hurwitz analysis.

We can also prove that the foregoing FP instability criterion ($R>0$) can be satisfied *if and only if* the level of cooperativity in the model exceeds a threshold given by

$n\,m > 4$.

If the $n\,m > 4$ cooperativity condition is satisfied there will exist regions in the model's rate coefficient parameter space where the system can sustain undamped oscillations; if the cooperativity condition is violated, no sustained oscillation will be found anywhere in the model's parameter space.

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