Temperature compensation and temperature sensation in the circadian clock

Philip B. Kidd[a,b], Michael W. Young[a], and Eric D. Siggia[b,1]

[a]Laboratory of Genetics, Rockefeller University, New York, NY 10065; and [b]Center for Physics and Biology, Rockefeller University, New York, NY 10065

Edited by Susan S. Golden, University of California, San Diego, La Jolla, CA, and approved October 8, 2015 (received for review June 8, 2015)

All known circadian clocks have an endogenous period that is remarkably insensitive to temperature, a property known as temperature compensation, while at the same time being readily entrained by a diurnal temperature oscillation. Although temperature compensation and entrainment are defining features of circadian clocks, their mechanisms remain poorly understood. Most models presume that multiple steps in the circadian cycle are temperature-dependent, thus facilitating temperature entrainment, but then insist that the effect of changes around the cycle sums to zero to enforce temperature compensation. An alternative theory proposes that the circadian oscillator evolved from an adaptive temperature sensor: a gene circuit that responds only to temperature changes. This theory implies that temperature changes should linearly rescale the amplitudes of clock component oscillations but leave phase relationships and shapes unchanged. We show using *timeless* luciferase reporter measurements and Western blots against TIMELESS protein that this prediction is satisfied by the *Drosophila* circadian clock. We also review evidence for pathways that couple temperature to the circadian clock, and show previously unidentified evidence for coupling between the *Drosophila* clock and the heat-shock pathway.

circadian clock | temperature compensation | mathematical models

Circadian rhythms are daily oscillations in gene expression and protein concentration that regulate sleep (1, 2), metabolism (3, 4), and a host of other biological processes (5–8). Circadian oscillations are known to be present in nearly all animals and plants, as well as some fungi and bacteria (9). In all organisms in which circadian oscillations have been observed, circadian oscillations satisfy three defining properties (10). First, circadian oscillations are self-sustained and spontaneously maintain a period of about 24 h. Second, the circadian rhythm is sensitive to light and temperature and can be synchronized to external oscillations in the quantity of either. Third, the period of the circadian oscillation is "temperature-compensated"; that is, the endogenous period of the oscillation is relatively insensitive to temperature.

The genetic basis of circadian clocks has been elucidated in considerable detail over the past two decades, particularly in the fruitfly *Drosophila melanogaster*, the mouse *Mus musculus*, and the bread mold *Neurospora crassa* (11). In *Drosophila*, a self-sustained circadian oscillation in neurons is generated when a pair of proteins, PERIOD (PER) and TIMELESS (TIM), dimerize and, after some delay, translocate into the nucleus, where the proteins repress their own transcription by inactivating a transcription factor dimer composed of the proteins CLOCK and CYCLE. Light sensitivity is mediated by the blue light photoreceptor CRYPTOCHROME (CRY), which upon activation binds TIMELESS and promotes TIMELESS degradation. A remarkably similar mechanism underlies circadian clocks in mouse and *Neurospora*. The circadian clock of cyanobacteria has also been studied in detail but has a significantly different structure (12, 13).

Much is therefore known about the first two defining properties of circadian clocks. Temperature compensation, however, remains poorly understood despite having been a key problem in chronobiology for nearly the entire history of the field (14, 15). In part, this lack of understanding is because the mechanisms that set the 24-h circadian period are not fully known. Many transcriptional regulatory programs are carried out in a matter of minutes (see, for example, refs. 16 and 17), so generating a 24-h circadian rhythm requires extensive regulation to generate long time delays. In wild-type (WT) *Drosophila*, the translocation of the PER-TIM dimer into the nucleus takes about 6–8 h (18, 19) and relies on a complex set of protein interactions and chemical modifications. The delay between peak expression of *per* and *tim* mRNA and protein is also long, about 6 h in constant darkness in WT (see Fig. 2 and Fig. S1), and mRNA stability and processing are also subject to circadian regulation (20). Although many details about these processes are known (21), the full set of reactions setting the associated time scales is not. Additionally, the 12- to 14-h length of the combined transcriptional/translational delay and nuclear translocation time does not fully account for the 24-h period of the clock.

Not knowing the full set of chemical reactions that contribute to determining the circadian period has made research into the temperature compensation difficult. However, some period-determining reactions are known, and experiments on those reactions have provided some insight, albeit fragmentary, into the nature of temperature compensation. Nuclear translocation of PER and TIM relies on many complicated chemical modifications, in particular, daily rhythms of phosphorylation (22, 23) involving the enzymes casein kinase 2 (CK2) (24, 25), DOUBLETIME [a casein kinase 1 (CK1) homolog] (26–28), SHAGGY [a glycogen synthase kinase (GSK-3) homolog] (23), and others (21). An experiment that altered gene dosages in *Neurospora* has shown that the activity of CK2 has a strong influence on the period of the circadian rhythm (29). Additionally,

Significance

Circadian clocks in animals, plants, and fungi possess the remarkable property of temperature compensation: the clock has a temperature-insensitive period, while retaining the ability to synchronize to temperature cycles. The conservation of temperature compensation across clades means it is likely critical to the function of circadian clocks. Temperature compensation also places the circadian clock in contrast to other biological pathways, which generally have temperature-sensitive time scales. However, the mechanism of temperature compensation remains unknown. We present a general scheme by which a circadian oscillator can be temperature-compensated while still synchronizing to environmental temperature cycles. In particular, we give experimental evidence that the circadian clock consists of a temperature-insensitive core oscillator coupled to a specific adaptive temperature signaling pathway.

Author contributions: P.B.K., M.W.Y., and E.D.S. designed research; P.B.K. performed research; P.B.K., M.W.Y., and E.D.S. analyzed data; and P.B.K., M.W.Y., and E.D.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: siggiae@rockefeller.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511215112/-/DCSupplemental.

increasing CK2 levels several-fold by gene duplication leads to a decreasing period at higher temperature, whereas decreasing CK2 levels leads to an increasing period at higher temperature. Another experiment in mammalian cell culture used a chemical screen to identify CK1 as a key period-determining enzyme (30). Knockdown of CK1 leads to a loss of temperature compensation, and, most interestingly, the phosphorylation rate of CK1 was independently temperature-compensated in an in vitro assay. It should be pointed out, however, that the substrate in this assay was an artificial peptide, not a physiologically relevant CK1 target, making the in vivo implications of the experiment unclear. In the flowering plant *Arabidopsis*, it has been shown that temperature-dependent changes in the concentration of CK2 and CCA1 are required for temperature compensation (31). In *Drosophila*, the equilibrium level of PER/TIM binding is temperature-compensated (32). These experiments provide interesting hints into how temperature compensation is achieved at the biochemical level, but no clear picture has emerged.

Temperature compensation of the circadian period does not imply that the circadian clock simply ignores temperature, and it is known that the clock can be entrained by temperature oscillations as small as 1° C (33) in *Drosophila*, and that temperature has an important role in regulating circadian physiology (34). The traditional solution to this problem has been to posit that the period-determining processes of the clock are all temperature-sensitive but that the changes in their rates cancel out to leave the overall period unaffected by changes in temperature. This process results in an oscillator that is both temperature-entrainable and temperature-compensated. This type of procedure underlies nearly all of the theoretical literature on temperature compensation (see ref. 35 for an early example and ref. 36 for a review); here, we refer to it as the "network model." There are several problems with the network model approach. First, as Hong et al. (37) have pointed out, many period-affecting mutations in circadian genes do not affect temperature compensation, which is difficult to explain in a model where careful balancing of rates is required to allow temperature compensation. Second, some experiments have suggested that specific signaling pathways are present for mediating circadian temperature sensation (38–44) and that removing these pathways eliminates phase shifting of the clock in response to temperature changes. These results cast doubt on the idea that the entire circadian clock is temperature-sensitive.

Here, we provide experimental support for an entirely different view of the mechanism of temperature compensation. We show that the period-determining processes in the core circadian clock are all independently temperature-compensated and that a dedicated signaling pathway must therefore be responsible for circadian temperature sensation. We refer to this scheme as the "pathway model." This type of model was first suggested by François et al. (45), based on the computational evolution of circadian networks with the twin properties of temperature entrainment and period compensation. The computation built the circadian clock around a temperature-sensing module that was adaptive, responding only to temperature changes, and in that way, encoded the two desired properties. The pathway models make two main predictions. First, there should be a specific pathway (or perhaps a few pathways) for temperature sensation by the circadian clock. Second, the concentration of the protein and mRNA components of the clock should scale in a simple fashion with temperature. In particular, the overall amplitude or average value of an oscillation in any given component can change with temperature, but the shape of the oscillation and the phase relationships between different oscillating components should remain approximately the same at any temperature (at least within a physiological range).

In this respect, the pathway model is consistent with (although independent of) another model for temperature compensation, the "amplitude model." In this model, first developed by Lakin-Thomas et al. (46), temperature compensation is achieved by a cancelation between an increase in amplitude and an increase in rate at high temperatures. The relationship between these models is examined further in the Discussion.

In this paper, we present compelling evidence against the network model and in favor of the pathway model for temperature compensation. We first argue for the generality of the scaling and pathway predictions using a simple mathematical model. We then present experimental evidence from quantitative Western blot and luciferase reporter experiments demonstrating that the simple scaling prediction is satisfied by the circadian clock in *Drosophila*. We provide support for the prediction of specific signaling pathways by showing that knockouts in the heat-shock pathway affect both circadian temperature phase shifting and temperature regulation of sleep behavior. Finally, we discuss the implications of these results and suggest some ideas for continuing work on circadian temperature compensation.

Results

Temperature Scaling in Some Simple Examples. We begin by developing a simple mathematical example of the pathway model. The model features three main components: an mRNA X that is translated into a protein Y, which has an alternate modification state Z. Each main component actually consists of a pair of reversibly interconverted isoforms, X/X^*, Y/Y^*, and Z/Z^* (diagrammed in Fig. 1*A*). All of the kinetics are linear except the repression of X/X^* by Z/Z^*. In the simple limit where each isoform is replaced by a single species, the model is equivalent to the Goodwin oscillator model (47) and is described by the following equations:

$$\begin{aligned}\dot{X} &= \frac{k_X}{Z^n} - d_X X \\ \dot{Y} &= k_Y X - d_Y Y \\ \dot{Z} &= k_Z Y - d_Z Z,\end{aligned} \qquad [1]$$

for some Hill coefficient n, production rates k_i, and degradation rates d_i. Note we have assumed that the Michaelis–Menten kinetics in the first term in $\dot{X}$ are totally unsaturated, a slight modification from ref. 47 that does not affect the behavior because Z^n is large. François et al. (45) showed that by a simple rescaling of the units for X, Y, and Z, one can obtain the modified system

$$\begin{aligned}\dot{x} &= \frac{1}{z^n} - d_X x \\ \dot{y} &= x - d_Y y \\ \dot{z} &= y - d_Z z,\end{aligned} \qquad [2]$$

in which the production coefficients k_i have vanished without changing the d_i and without rescaling time. This result implies that a change in any of the k_i can be reversed by a rescaling of concentration units, leaving the shape of the orbit and its period unchanged. The Goodwin oscillator can thus be made into a temperature-compensated/temperature-entrainable oscillator by making the d_i independent of temperature and at least one of the k_i temperature-sensitive. The effect of changes in temperature will then be described by the simple rescaling given above, just as predicted by the evolved models in ref. 45. To see how the degradation rates d_i can be rendered temperature-independent in a natural way, we restore the isoform pairs shown in Fig. 1*A*. This adjustment leads to a pair of equations for X/X^*:

$$\begin{aligned}\dot{X} &= \frac{k_X}{(Z+Z^*)^n} - dX - f_X X + r_X X^* \\ \dot{X}^* &= \frac{k_X}{(Z+Z^*)^n} - d^* X^* - r_X X^* + f_X X,\end{aligned} \qquad [3]$$

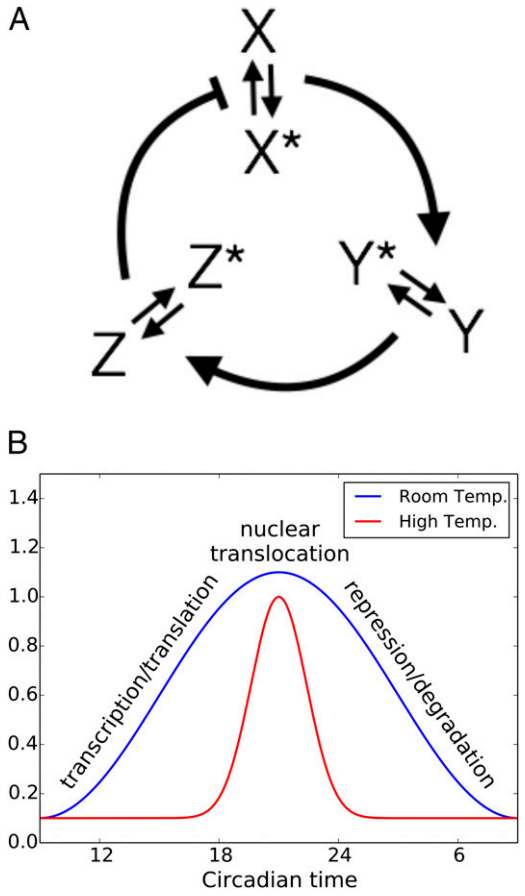

Fig. 1. (*A*) Diagram of a simple model for oscillator temperature compensation. mRNA isoforms *X*/*X** are translated into proteins *Y*/*Y**, which are converted into a second form *Z*/*Z**, which represses transcription of *X*/*X**. Each half of an isoform pair can be reversibly converted into and plays the same role as the other but has a different degradation rate. (*B*) Circadian oscillation of TIM protein, with various phases of the daily circadian cycle marked on corresponding locations on the oscillation. In red, a hypothetical scenario is shown in which a change in temperature causes a shortening of nuclear translocation time and a compensating increase in transcription time, leading to a change in shape of the oscillation.

where f_X and r_X represent the forward and reverse rates of conversion between X and X^*, respectively (assumed much faster than the period-determining circadian rates). Similar equations can be written for Y/Y^* and Z/Z^*. Adding the above equations together gives back the simplified form in **1**, except with X and Z replaced by sums $(X+X^*)$ and $(Z+Z^*)$, and an effective degradation rate given by

$$d_X = d\frac{K}{1+K} + d^*\frac{1}{1+K}, \qquad [4]$$

where $K = r_X/f_X$ is the equilibrium constant describing the balance between X and X^*. The effective rate d_X can be rendered temperature-independent by letting $d^*/K \gg d$ and having K increase with temperature, so that increases in d and d^* are cancelled by a shift in the equilibrium toward the more slowly degraded isoform. In particular, we can set $d = 0$ always and describe the temperature dependence of rates d^* and $K = r_X/f_X$ by an Arrhenius form $\exp(-E/kT)$ with energies E_{d^*} and $E_K = E_r - E_f$, respectively. The effective rate d_X will be temperature-independent as long as $K \gg 1$ at room temperature and $E_K = E_{d^*}$.

In this case, the model will behave exactly as the temperature-compensated Goodwin oscillator discussed above and will show a simple scaling of oscillation shape with temperature. Additionally, although the model is abstract, it shares a number of features with the real circadian clock. In particular, it is known that in *Drosophila*, *per* and *tim* mRNA are present in multiple isoforms (48) and that PER and TIM proteins are subject to reversible biochemical modifications that alter the proteins' degradation rates (49, 50). The model serves as a useful paradigmatic example of the pathway model and additionally shows that independent temperature compensation of all reactions in a biological oscillator can be accomplished without an excessively complicated network. Of course, temperature compensation in the Goodwin model can be achieved by a constraint imposed on the d_i, but in this case, the shape of the orbits will change with temperature, as is easily seen by simulation.

We can extend this example using a simple qualitative argument, which is depicted in Fig. 1*B*. Consider a time course of TIM protein levels over the course of 24 h in constant darkness. TIM protein will be at a minimum at subjective midday and a maximum at subjective midnight. The rising phase of TIM concentration corresponds to transcription and translation of *tim* mRNA during the late day, the peak phase corresponds to nuclear translocation of the PER-TIM dimer, and the falling phase corresponds to repression of TIM production and degradation of the dimer. If all of these subprocesses of the clock have temperature-dependent rates, such that a shortening of one process is compensated by a lengthening in another, then we should expect the shape of the curve of TIM protein oscillation to change with temperature. For example, if at higher temperatures, a shortening in nuclear translocation time is canceled by a lengthening in transcription/translation times, then we should expect a narrowing of the portion of the curve where TIM levels are high (because of more rapid repression of *tim* transcription) and a corresponding widening of the part of the curve where TIM levels are low (because of slowing in TIM production). This possibility is shown in Fig. 1*B*.

Western Blot and Luciferase Measurements. To test for the shape invariance of the circadian oscillations, we measured the oscillations of different components of the circadian clock in *D. melanogaster* at three different temperatures: 18°C, 25°C, and 29° C. We chose to measure the oscillation in TIM protein levels (by Western blot) and transcription rate (by observing appearance of a luciferase reporter protein) to get a picture of both the positive (transcriptional) and negative (posttranslational) sides of the clock feedback loop (Fig. 1*B*). In both cases, WT flies were prepared by synchronization in at least 3 d of a light-dark (LD) cycle at the requisite temperature, followed by transfer into constant darkness (DD) at the same temperature. Measurements were made on the second day in constant darkness to minimize the residual effects of light while not allowing too much desynchronization of the population. For Western blots, *yw* flies were sampled by flash freezing every hour, and TIM concentrations were measured relative to cadherin (CDH). Results are shown in Fig. 2. For luciferase measurements, groups of 30–50 flies carrying a *timeless* luciferase (*tim-luc*) reporter (shown to recapitulate the dynamics of *tim* expression reported in ref. 51) were put into a 35-mm dish filled with luciferin-containing cornmeal food and observed in a Hamamatsu top-counting luminescence detector. Results are shown in Fig. 3.

Representative blot images are shown in Fig. 2*A*, from which it is clear that TIM levels increase at higher temperatures. Fig. 2*B* shows samples from two different temperatures (18° C and 29° C) run side-by-side on the same gel. A quantification combining the data from each of these gels (with three biological replicates) is shown in Fig. 2*C*. This quantification shows that both the amplitude and average of TIM oscillations increase with temperature. In Fig. 2*D*, the curves are shown after a rescaling by subtracting a linear trend (so that the mean is 0 throughout)

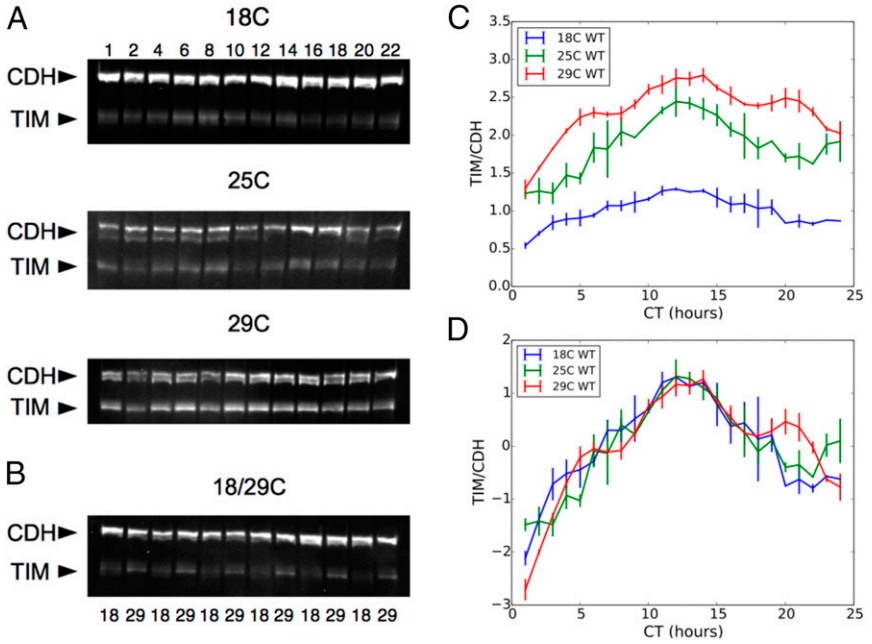

Fig. 2. TIM protein Western blots at three different temperatures. Representative blot images are shown on the left. In *A*, bands correspond to time points 2 h apart as shown, covering a full day at the indicated temperature. *B* shows bands from the first 6 h of the day at both 18° C and 29° C (as labeled) run side-by-side. *C* shows quantification of TIM protein concentrations relative to CDH, normalized so that the mean of the 18° C time series is 1. *D* shows that the same curves effectively coincide after rescaling to a mean of 0 and an rms deviation of 1. All error bars are SEM for three biological replicates.

and normalizing the root-mean-square (rms) deviation from the mean to 1. This process leads to a precise collapse of the curves onto each other, confirming the simple scaling prediction of the model discussed above.

In Fig. 3*A*, representative raw luciferase data at 25° C are shown. The decaying trend is attributable to depletion of the luciferin substrate (52) but can be easily subtracted. Fig. 3*B* shows the same data after detrending by subtracting a 30-h moving average and smoothing in a 1-h window (time points are taken every 15 min). Fig. 3*C* shows the oscillations at the three temperatures, on the second day in constant darkness, after rescaling by mean subtraction and normalization of the rms deviation. Again the curves collapse onto each other, confirming the simple scaling prediction. One might object that luciferase

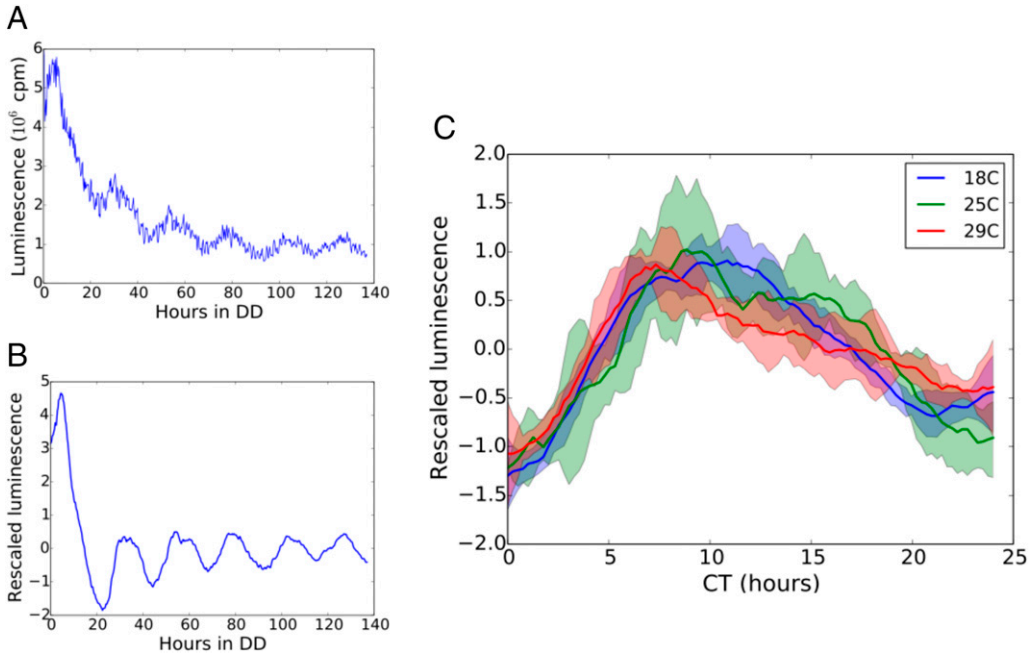

Fig. 3. Luminescence from *tim-luc* flies at 18°C, 25°C, and 29° C. (*A*) Sample of raw luminescence data plotted in millions of photon cpm. (*B*) The same data after detrending and smoothing. (*C*) Rescaled luminescence curves from *tim-luc* flies at different temperatures, taken from the second day in constant darkness. Shaded areas indicate SEM across three repetitions of the experiment.

activity and stability are temperature dependent. We performed quantitative PCR (qPCR) measurements of *tim* mRNA levels at two different temperatures to show that mRNA oscillations scale similarly to luminescence oscillations and that the delay between peak mRNA concentration and luminescence levels is independent of temperature. Data are shown in Fig. S1, confirming that luminescence and qPCR data are consistent, with a delay of about 4–5 h at both 18° C and 25° C.

It is useful in assessing these data to have a control experiment in which one would expect to see changes in the shape of the curve of TIM oscillation at different temperatures. Our arguments in *Temperature Scaling in Some Simple Examples* imply that any mutant with a temperature-dependent change in rate for some circadian process should show a corresponding change in the shape of its oscillations. The per^L mutant has a substantial defect in temperature compensation [the period increases from 27 h at 18°C to 31 h at 29° C (53)]. Fig. 4*A* shows the results of Western blots from per^L flies, and Fig. 4*B* shows luciferase measurements from per^L; *tim-luc* flies, after rescaling in both cases. A shift in the peak of the curves with temperature is evident and has a magnitude significantly larger than what would be expected from the difference in periods alone. It is worth pointing out that molecular circadian oscillations in per^L are noisier and have lower relative amplitude than in WT (Fig. S2 and data in refs. 52 and 54). However, the shift in peaks is statistically significant.

Fig. 4*C* shows the position of the peaks in protein and luminescence oscillations for both WT and per^L at all three temperatures. Peak times are measured relative to the natural period for each strain, so for WT flies, 0.5 corresponds to 12 h. Wedges are centered at the position of the peaks, with widths corresponding to the uncertainty in position of the peaks found from fitting a sine function. From these data, it is clear that in the per^L background, there is not only a shift in the peaks at different temperatures, but the phase difference between luminescence and protein peaks is also temperature-dependent. This finding confirms that a change in shape is present, as predicted by our model, and that our method is able to detect such changes reliably. It is important to keep in mind that (in WT flies) the peak in luminescence lags peak mRNA concentration by about 5 h (Fig. S1), so that the true *tim* mRNA peak precedes the TIM protein peak at all temperatures.

It is interesting that a yeast two-hybrid experiment (55), as well as stains of circadian neurons (32), suggests that the defect in per^L temperature compensation may be attributable to a temperature-dependent effect on PER-TIM binding, which in turn leads to a delay in nuclear translocation. The increasing delay in nuclear translocation likely explains the progressive delay in the peak of protein and mRNA expression and explains the narrowing of the gap between protein concentration and transcription levels, because a defect in repression of *tim* transcription by PER/TIM protein would allow relatively high levels of protein and mRNA to be present simultaneously. The effect of PER/TIM binding on TIM stability (56) may also play a role in determining how temperature influences the shape of the curves in Fig. 4, but in any case it is clear that strong temperature effects are present in the per^L background that are absent in WT.

The other prediction of our model is the presence of a specific signaling pathway for coupling the circadian oscillation to temperature. As briefly mentioned earlier, a significant amount of evidence for this proposition has already been found. Glaser et al. (41) and Sehadova et al. (43) have found that mutations associated with the function of mechanosensitive chordotonal organs affect circadian temperature entrainment. In particular, morning anticipation in hot–cold cycles is eliminated and luciferase reporter oscillations damp out rapidly under temperature entrainment in mutants of the genes *nocte* and $norp^A$. Animals lacking functional temperature-sensitive TrpA channels have been shown to entrain more slowly to temperature cycles and to have noisier molecular rhythms under temperature

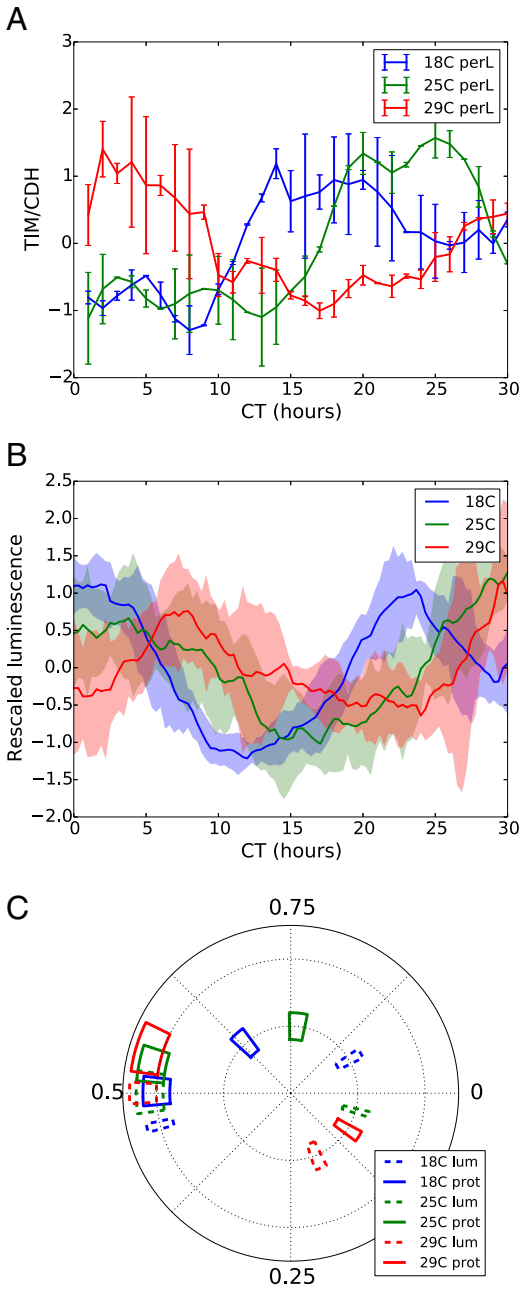

Fig. 4. (*A*) TIM protein oscillations in per^L flies, rescaled to have mean 0 and rms deviation 1. CT corresponds to a 30-h period with hour 0 being subjective morning on the second day in DD. Error bars are SEM for three biological replicates. (*B*) Rescaled luminescence curves from per^L; *tim-luc* flies at different temperatures, taken from the second day in constant darkness, showing a change in shape. Shaded areas indicate SEM across three different experiments. (*C*) Peaks of protein (solid lines) and luminescence (dotted lines) oscillations show a change in relative phase in per^L. Each wedge is centered on the peak phase, with the width of the wedges giving the error in the location the peak (determined by a sinusoidal fit). Wedges on the inner circle come from per^L flies, and wedges on the outer circle come from WT flies. Phase labels around the two concentric circles correspond to fractions of the period for each strain. The wedges corresponding to WT are offset slightly to aid visibility.

entrainment (40). One experiment has shown that flies carrying a null mutation in the photoreceptor *cryptochrome* (cry^b) show reduced phase shifts in response to a 30-min 37° C heat shock (42). However, experiments performed in our own laboratory (Fig. S3) have failed to find this effect, and subsequent work by other groups has cast doubt on the role of cryptochrome in circadian temperature entrainment (57, 58). New data from the laboratory of Patrick Emery (39) suggest that calcium signaling is essential for circadian temperature responses and that the above-mentioned neural pathways converge by calcium-dependent degradation of TIM.

Given that there are clearly multiple pathways present for communicating temperature to the clock, it may be difficult to eliminate circadian temperature entrainment in an intact mutant animal. However, virtual elimination of temperature phase-shifting has been achieved in mouse fibroblasts subjected to pharmacological inhibition of the heat-shock pathway (38). It has also been shown that a knockout of the HSF1 transcription factor affects circadian expression in fibroblasts under temperature cycles and that there is a physical association between HSF1 and the mammalian circadian transcription factor BMAL1 (44). We were therefore interested in looking for an effect of the heat-shock pathway on circadian temperature sensation in *Drosophila*.

The *Drosophila* heat-shock response is mediated by the single transcription factor HSF. HSF is required for embryonic development, but viable heterozygous knockouts and a temperature-sensitive mutant (inactive above 30° C) are available (59). We performed phase-resetting curve (PRC) experiments on these mutants, using a temperature step from 18°C to 29° C as the stimulus. The results are shown in Fig. 5*A*. The phase shifts are computed relative to the circadian time (CT) 13 sample, rather than a control population. This is because the shape of activity oscillations in WT flies is significantly different at 18°C and 29° C, making a principled phase comparison between temperatures impossible. The CT 13 time point was chosen as a reference because at that time point the phase shift relative to a control appeared small by eye, but the choice is essentially arbitrary. Both heterozygous and temperature-sensitive heat-shock mutants show increased phase-shifting in response to temperature steps. This is different from what is seen in mammalian fibroblasts, and the difference is likely explained by the presence of neural temperature signaling pathways in *Drosophila*, which presumably interact with the cell autonomous heat-shock pathway in complex ways.

We performed a control experiment by repeating the above procedure but replacing the stimulus with a temperature step down, from 25°C to 18° C. In this case (Fig. S4), the phase shifts are comparable in WT and heterozygous HSF knockouts, as one might expect, because the heat-shock pathway should not be activated at these temperatures. It is interesting to note that the shape of the curves are different in the two experiments, suggesting that the mechanisms of circadian temperature sensation are different for warm and cold temperatures. This was also suggested by experiments in ref. 60, which found that a specifically cold-sensitive RNA-binding protein influences circadian transcription in mice, as well as prior work examining circadian responses to cold temperatures (61, 62).

Intriguingly, the heat-shock pathway also appears to have an effect on the daily distribution of sleep at different temperatures. WT *Drosophila* tend to sleep more during the day and less at night at high temperatures (63), as shown in Fig. 5*B*. However, heterozygous HSF knockouts show a similar distribution of sleep at both 18°C and 29° C (Fig. 5*C*). This finding suggests that, in *Drosophila* at least, the heat-shock pathway may also have an unexpected role in regulation of sleep by temperature.

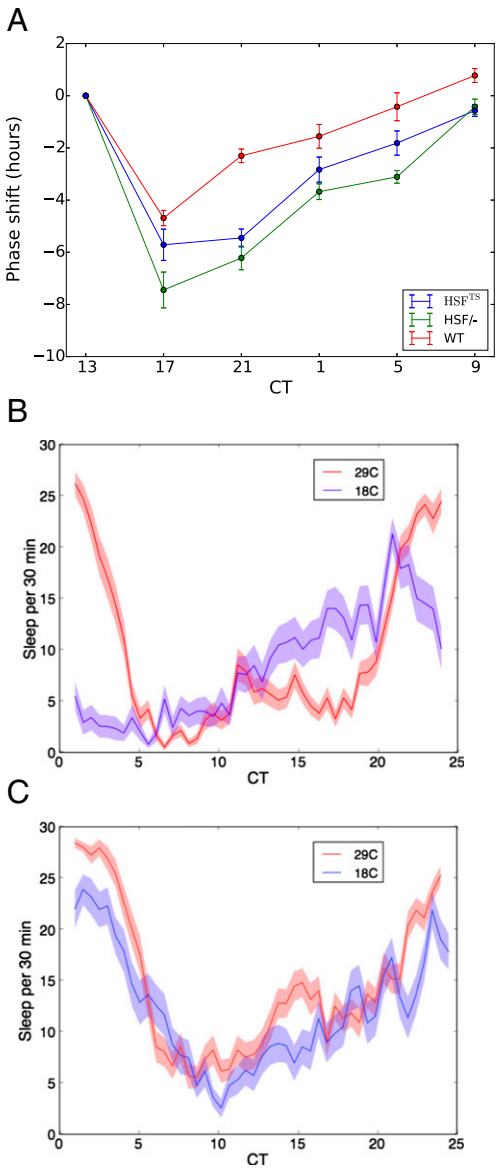

Fig. 5. (*A*) Results of a PRC experiment applying a temperature step from 18° C to 29° C to three strains of flies. HSF/- indicates a heterozygous knockout of the heat-shock transcription factor, and HSF^{TS} indicates a temperature-sensitive HSF mutant. Phase shifts are plotted against the hour of the subjective day at which the step occurred and are calculated relative to the phase of the group at CT 13. Error bars are SEM. (*B* and *C*) Effect of temperature on sleep profile in WT and heat-shock mutant flies. Sleep per 30 min is shown over the course of the circadian day at 18° C and 29° C in WT flies (*B*) and heterozygous heat-shock transcription factor knockouts (*C*). Shaded regions indicate SEM across at least 15 individuals.

Discussion and Conclusion

We have presented experimental evidence supporting a pathway model for the effects of temperature on the circadian oscillator based on the theory in reported in ref. 45. By observing two components of the core clock which oscillate with different phases (TIM protein and *tim* expression reported by a luciferase construct), we have shown that only the amplitude but not the shape of the circadian oscillation changes with temperature. Parallel experiments in per^L mutant flies whose circadian period varies from 27 to 31 h over the accessible temperature range were an important control. In this case, the oscillations changed shape substantially at different temperatures, demonstrating our experiments had sufficient sensitivity.

For a typical nonlinear oscillator, whether mechanical or biochemical, it is completely unexpected to observe oscillations at

different parameter values that can be superimposed by shifting means and linearly scaling amplitudes. In ref. 45, this property emerged by in silico evolution that favored temperature entrainment and period compensation. The computation began from an initial gene network that responded adaptively to changes in temperature. That is, the computation registered changes in temperature but returned to the same operating point irrespective of temperature in the absence of change. Adaptation is a common property of sensory systems. Computational evolution then built a delayed negative-feedback oscillator around the adaptive temperature sensor and with it the properties selected for, namely temperature compensation and temperature entrainment. There is certainly parameter tuning in this model as there is in the network model, which is perfectly reasonable because natural evolution has plausibly shaped the phenotype of the circadian oscillator. However, there was no direct computational selection for the linear rescaling of orbits between different temperatures, and that property has to be considered as an emergent consequence of the basic assumptions of entrainment and period compensation. In the simple example we proposed, the scaling of orbits results from the specific form of the model equations, and it would be interesting to understand more abstractly why the same property arose from the evolutionary computation. In general, our results indicate that the linear superposition of orbits at different temperatures is a generic property of circadian oscillators.

It is much less clear how to experimentally test the starting assumption reported in ref. 45: that an adaptive temperature sensor is the dominant coupling responsible for temperature entrainment. The suppression of the heat-shock pathway was shown to eliminate temperature phase shifting in mouse fibroblasts (38, 44). However, an organism may be much more complicated in this regard than single cells. We demonstrated a coupling between the heat-shock pathway and the circadian clock in *Drosophila* but found that heat-shock pathway mutants display increased sensitivity to temperature. This suggests that other pathways are present for temperature signaling in *Drosophila*. We also showed that heat-shock mutants lack the normal temperature dependence of daily sleep distribution, suggesting a role for the heat-shock pathway in sleep regulation.

It is important to note that we do not suggest that temperature signaling via the heat-shock pathway is a universal mechanism of circadian clocks. Indeed, our own data (Fig. S4) suggest that separate mechanisms are present for hot and cold responses even with *Drosophila*. New data from the laboratory of Patrick Emery (39) suggest that the various neural pathways involved in circadian temperature signaling all act via calcium-dependent degradation of TIM through the SOL protease pathway. The data show that inhibiting calcium signaling significantly reduces circadian phase shifts in response to temperature changes and, most interestingly, that the calcium response to a temperature step appears to be adaptive, just as our model predicts.

Our claim that each period-influencing process in the circadian clock is independently temperature-compensated raises the question of how such compensation could be achieved. We presented a mathematical model showing how the pathway model scheme can be implemented in a variant of the Goodwin model. In our model, temperature entrainment was achieved by allowing the production rates of proteins or mRNA to be temperature-dependent, because these rates affect the quantity of oscillating components but not the period. Although the model does not insist on coupling temperature to the clock via transcription rates, the model is consistent with evidence that transcriptional signaling via the heat-shock pathway is responsible for temperature phase shifting in mammalian fibroblasts (38, 44). It has also been shown that the period of the circadian clock is buffered against changes in transcription rates in both mammalian cells (64) and *Drosophila* (65), which is also consistent with the assumptions of our model. Additionally, our model implements temperature compensation by balancing changing degradation rates against a shifting between paired isoforms for each mRNA and protein in the model. Such interconvertible isoforms with varying degradation rates are known to be present in the circadian clock, both in mRNA (48) and proteins (49, 50).

It is also worth noting that the mathematical model predicts that the increase in transcription/translation rates with temperature leads to a change in the amplitude of oscillation. This model has the expected feature that when the production rate of the *i*th variable increases, the corresponding amplitude of oscillation increases as well. In that sense it, is consistent with the predictions of the amplitude model for temperature compensation proposed in ref. 46. This model proposes that an increase in rates at higher temperature can be canceled by a proportional increase in amplitude, without specifying precisely how the compensation occurs. The amplitude model is supported by data on zebrafish circadian transcription (66), as well as by *Drosophila* phase-resetting curve experiments, both recent (67) and canonical (62). Evidence for increase in amplitude at higher temperatures has also been found in *Neurospora* (68) and cyanobacteria (69). Interestingly, this increase in circadian amplitude with temperature may also provide a mechanism for seasonal adaptation of the clock in *Drosophila* (70). Our own data (Fig. 2*C*) show that the amplitude of TIM oscillations roughly doubles between 18° and 29° C, which is consistent with the typical dependence of biological reaction rates on temperature (71). Insofar as the amplitude model remains agnostic about exactly how compensation is achieved, the model can be regarded as complimentary to, and in some sense independent of, the pathway model. In sum, our data, combined with model of François et al. (45), provide the first experimental support to our knowledge for a theory of the complete temperature compensation of a temperature-entrainable oscillator.

Our results have broad implications for future research into the mechanisms of temperature compensation. We have shown that temperature compensation is not a network-wide process but that each period-setting process of the clock must be temperature-compensated on its own. Experimental results on temperature compensation must be interpreted in this context. Future work is needed to understand the temperature compensation of nuclear translocation, transcriptional repression, and nuclear export and degradation, among other processes. Nuclear translocation is a particularly promising avenue for future research. A great deal about the biochemistry of nuclear translocation has been discovered in the last decade (19), and much of this knowledge can be leveraged into experiments on temperature compensation. Additionally, the multifarious network of reversible phosphorylations known to regulate nuclear translocation of the PER-TIM dimer bears an intriguing resemblance to models of temperature compensation in the literature (72, 73). Another promising avenue for research is single-cell time lapse observations. Work in *Drosophila* S2 cells has contributed significantly to the understanding of PER-TIM dynamics (cf. ref. 19). The advent of fluorescent protein-tagging experiments on the circadian clock in *Neurospora* (74, 75), as well as reporter experiments in mammalian fibroblasts (76), will allow observation of the effect of temperature changes on multiple subprocesses of the circadian clock in a single experiment. Elucidating the biochemical mechanisms of temperature compensation in the circadian clock remains an open problem.

Methods

Strains. Western blots were performed on *Drosophila* strains *yw* and per^L. Luciferase experiments used *tim-luc* flies obtained from Ralf Stanewsky, University College London, London (51), and per^L; *tim-luc* flies generated by crossing *tim-luc* to a per^L;*If/CyO;sb/TM6* balancer line. Temperature PRC experiments used *iso31* and strains hsf^1 and hsf^4 (59), obtained from the Bloomington Stock Center.

Western Blots and qPCR. Flies were entrained for at least 3 d in a 12 h:12 h LD cycle at a given temperature, then moved into constant darkness at the same temperature. Measurements began at CT 0 on the second day in DD (36 h after the last lights off). Flies were flash frozen on dry ice every hour for 24 h (or 30 for per^L), and heads were isolated for protein or RNA extraction.

For protein, heads were homogenized in an equal volume of radioimmunoprecipitation (RIPA) buffer (50 mM Tris, pH 8; 150 mM NaCl; 1 mM EDTA; 1% Triton-X 100 by volume; 0.5% sodium deoxycholate; 20% glycerol by volume; 0.02% NaN_3), supplemented with 1 mM DTT and protease inhibitor mixture (Calbiochem). Homogenate was mixed with a double volume of dilution buffer (RIPA buffer without Triton-X, sodium deoxycholate, DTT, and protease inhibitor), and centrifuged twice for 10 min at 4° C. Extracts were quantitated and resolved by SDS/PAGE. Blots were probed with rat anti-TIM (77) at 1:1,000 and rat anti-CDH (Santa Cruz Biotechnology) at 1:200. HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were applied at 1:10,000 and imaged with ECL 2 (enhanced chemiluminescence) substrate (Thermo Scientific) on a Bio-Rad ChemiDoc.

For RNA, extracts were homogenized with TRIzol (Invitrogen) and then column-purified with an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, including DNase treatment (Qiagen). RNA solutions were quantitated and then reverse-transcribed with iScript reverse transcriptase (Bio-Rad). QCPRs on the resulting cDNA were performed with PerfeCTa SYBR Green FastMix (Quanta Biosciences) in a LightCycler 480 (Roche) and analyzed by the ΔΔ Ct method. The primers for *tim* were taken from ref. 78, and the primers for the housekeeping gene *gapdh* were from ref. 79.

Luciferase Measurements. Flies were entrained in the manner described above, and during the day on the last day of entrainment, about 50 male flies were placed in a 30-mm plate containing about 1–2 mL of standard cornmeal/molasses fly food supplemented with 15 mM D-luciferin (potassium salt; Biosynth) layered on top of 7 mL of standard food. At the time of lights out (zeitgeber time 12), the plates were placed in the wells of a LM-2400 photon detection unit (Hamamatsu) for measurement. The resulting raw data were detrended by subtracting a 30-h moving average and smoothed by averaging time points in a 1-h window.

Behavior Measurements. For temperature step phase-shifting experiments, flies in a *Drosophila* activity monitor (Trikinetics) were entrained for at least 3 d in a 12 h:12 h LD cycle at 18° C and then moved into DD at 18° C. On the second day in DD, monitors were moved at the indicated time to a different incubator at 29° C. Behavior was measured for at least 5 d subsequently, and phase shifts were determined by computing a cross-correlation function between activity traces. For heat-shock phase-shifting experiments, WT and cry^b flies [the laboratory of Michael Rosbash, Brandeis University, Waltham, MA (80)] were entrained similarly and then placed in constant darkness in a custom-built small incubator designed to provide rapid and precise temperature stimuli from a Peltier effect heating/cooling element (Custom Thermoelectric). Temperature was increased from 25° C to 37° C for 30 min at CT 15 on the first day in DD. Phase shifts were computed as before, by comparing to a control group kept at 25° C.

Sleep measurements were made in DD using flies entrained in the same manner and judging sleep as beginning after a 5-min period of inactivity (81, 82).

ACKNOWLEDGMENTS. We thank Paul François, Stuart Brody, and members of M.W.Y.'s laboratory for helpful discussions. We thank Ralf Stanewsky and Michael Rosbash for providing strains and Ralf Stanewsky for helpful advice related to luciferase measurements. We thank Patrick Emery for providing us with a manuscript of his in-press paper on calcium signaling. This research was funded by National Science Foundation Grants PHY-0954398 and PHY-1502151 (to E.D.S.) and National Institutes of Health Grant GM054339 (to M.W.Y.).

1. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G (2000) Correlates of sleep and waking in Drosophila melanogaster. *Science* 287(5459):1834–1837.
2. Toh KL, et al. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 291(5506):1040–1043.
3. Bass J, Takahashi JS (2010) Circadian integration of metabolism and energetics. *Science* 330(6009):1349–1354.
4. Peek CB, et al. (2013) Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice. *Science* 342(6158):1243417.
5. Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* 12(18):1574–1583.
6. Druzd D, de Juan A, Scheiermann C (2014) Circadian rhythms in leukocyte trafficking. *Semin Immunopathol* 36(2):149–162.
7. Sellix MT, Menaker M (2010) Circadian clocks in the ovary. *Trends Endocrinol Metab* 21(10):628–636.
8. Xu K, Zheng X, Sehgal A (2008) Regulation of feeding and metabolism by neuronal and peripheral clocks in Drosophila. *Cell Metab* 8(4):289–300.
9. Dunlap JC (2003) *Chronobiology: Biological Timekeeping* (Sinauer Associates, Sunderland, MA).
10. Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb Symp Quant Biol* 25:159–184.
11. Young MW, Kay SA (2001) Time zones: A comparative genetics of circadian clocks. *Nat Rev Genet* 2(9):702–715.
12. Nakajima M, et al. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* 308(5720):414–415.
13. Nishiwaki T, et al. (2007) A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J* 26(17):4029–4037.
14. Pittendrigh CS (1954) On temperature independence in the clock system controlling emergence time in Drosophila. *Proc Natl Acad Sci USA* 40(10):1018–1029.
15. Hastings JW, Sweeney BM (1957) On the mechanism of temperature independence in a biological clock. *Proc Natl Acad Sci USA* 43(9):804–811.
16. O'Brien T, Lis JT (1993) Rapid changes in Drosophila transcription after an instantaneous heat shock. *Mol Cell Biol* 13(6):3456–3463.
17. Rougvie AE, Lis JT (1990) Postinitiation transcriptional control in Drosophila melanogaster. *Mol Cell Biol* 10(11):6041–6045.
18. Jang AR, Moravcevic K, Saez L, Young MW, Sehgal A (2015) Drosophila TIM binds importin α1, and acts as an adapter to transport PER to the nucleus. *PLoS Genet* 11(2): e1004974.
19. Saez L, Meyer P, Young MW (2007) A PER/TIM/DBT interval timer for Drosophila's circadian clock. *Cold Spring Harb Symp Quant Biol* 72(1):69–74.
20. Lim C, Allada R (2013) Emerging roles for post-transcriptional regulation in circadian clocks. *Nat Neurosci* 16(11):1544–1550.
21. Mehra A, Baker CL, Loros JJ, Dunlap JC (2009) Post-translational modifications in circadian rhythms. *Trends Biochem Sci* 34(10):483–490.
22. Edery I, Zwiebel LJ, Dembinska ME, Rosbash M (1994) Temporal phosphorylation of the Drosophila period protein. *Proc Natl Acad Sci USA* 91(6):2260–2264.
23. Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell* 105(6):769–779.
24. Akten B, et al. (2003) A role for CK2 in the Drosophila circadian oscillator. *Nat Neurosci* 6(3):251–257.
25. Smith EM, Lin JM, Meissner RA, Allada R (2008) Dominant-negative CK2alpha induces potent effects on circadian rhythmicity. *PLoS Genet* 4(1):e12.
26. Bao S, Rihel J, Bjes E, Fan JY, Price JL (2001) The Drosophila double-timeS mutation delays the nuclear accumulation of period protein and affects the feedback regulation of period mRNA. *J Neurosci* 21(18):7117–7126.
27. Cyran SA, et al. (2005) The double-time protein kinase regulates the subcellular localization of the Drosophila clock protein period. *J Neurosci* 25(22):5430–5437.
28. Price JL, et al. (1998) double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. *Cell* 94(1):83–95.
29. Mehra A, et al. (2009) A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. *Cell* 137(4):749–760.
30. Isojima Y, et al. (2009) CKIepsilon/delta-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. *Proc Natl Acad Sci USA* 106(37):15744–15749.
31. Portolés S, Más P (2010) The functional interplay between protein kinase CK2 and CCA1 transcriptional activity is essential for clock temperature compensation in Arabidopsis. *PLoS Genet* 6(11):e1001201.
32. Huang ZJ, Curtin KD, Rosbash M (1995) PER protein interactions and temperature compensation of a circadian clock in Drosophila. *Science* 267(5201):1169–1172.
33. Winfree AT (1972) Acute temperature sensitivity of circadian rhythm in Drosophila. *J Insect Physiol* 18(2):181–185.
34. Boothroyd CE, Wijnen H, Naef F, Saez L, Young MW (2007) Integration of light and temperature in the regulation of circadian gene expression in Drosophila. *PLoS Genet* 3(4):e54.
35. Ruoff P, Rensing L (1996) The temperature-compensated goodwin model simulates many circadian clock properties. *J Theor Biol* 179(4):275–285.
36. Kurosawa G, Iwasa Y (2005) Temperature compensation in circadian clock models. *J Theor Biol* 233(4):453–468.
37. Hong CI, Conrad ED, Tyson JJ (2007) A proposal for robust temperature compensation of circadian rhythms. *Proc Natl Acad Sci USA* 104(4):1195–1200.
38. Buhr ED, Yoo SH, Takahashi JS (2010) Temperature as a universal resetting cue for mammalian circadian oscillators. *Science* 330(6002):379–385.
39. Tataroglu O, et al. (2015) Calcium and SOL protease mediate temperature resetting of circadian clocks. *Cell*, in press.
40. Lee Y, Montell C (2013) Drosophila TRPA1 functions in temperature control of circadian rhythm in pacemaker neurons. *J Neurosci* 33(16):6716–6725.
41. Glaser FT, Stanewsky R (2005) Temperature synchronization of the Drosophila circadian clock. *Curr Biol* 15(15):1352–1363.
42. Kaushik R, et al. (2007) PER-TIM interactions with the photoreceptor cryptochrome mediate circadian temperature responses in Drosophila. *PLoS Biol* 5(6):e146.
43. Sehadova H, et al. (2009) Temperature entrainment of Drosophila's circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain. *Neuron* 64(2):251–266.
44. Tamaru T, et al. (2011) Synchronization of circadian Per2 rhythms and HSF1-BMAL1: CLOCK interaction in mouse fibroblasts after short-term heat shock pulse. *PLoS One* 6(9):e24521.

45. François P, Despierre N, Siggia ED (2012) Adaptive temperature compensation in circadian oscillations. *PLOS Comput Biol* 8(7):e1002585.
46. Lakin-Thomas PL, Brody S, Coté GG (1991) Amplitude model for the effects of mutations and temperature on period and phase resetting of the Neurospora circadian oscillator. *J Biol Rhythms* 6(4):281–297.
47. Goodwin BC (1965) Oscillatory behavior in enzymatic control processes. *Adv Enzyme Regul* 3:425–438.
48. Low KH, Lim C, Ko HW, Edery I (2008) Natural variation in the splice site strength of a clock gene and species-specific thermal adaptation. *Neuron* 60(6):1054–1067.
49. Ko HW, Jiang J, Edery I (2002) Role for Slimb in the degradation of Drosophila Period protein phosphorylated by Doubletime. *Nature* 420(6916):673–678.
50. Koh K, Zheng X, Sehgal A (2006) JETLAG resets the Drosophila circadian clock by promoting light-induced degradation of TIMELESS. *Science* 312(5781):1809–1812.
51. Stanewsky R, Lynch KS, Brandes C, Hall JC (2002) Mapping of elements involved in regulating normal temporal period and timeless RNA expression patterns in Drosophila melanogaster. *J Biol Rhythms* 17(4):293–306.
52. Plautz JD, et al. (1997) Quantitative analysis of Drosophila period gene transcription in living animals. *J Biol Rhythms* 12(3):204–217.
53. Price JL (1997) Insights into the molecular mechanisms of temperature compensation from the Drosophila period and timeless mutants. *Chronobiol Int* 14(5):455–468.
54. Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J Neurosci* 10(8):2749–2762.
55. Gekakis N, et al. (1995) Isolation of timeless by PER protein interaction: Defective interaction between timeless protein and long-period mutant PERL. *Science* 270(5237): 811–815.
56. Grima B, Dognon A, Lamouroux A, Chélot E, Rouyer F (2012) CULLIN-3 controls TIMELESS oscillations in the Drosophila circadian clock. *PLoS Biol* 10(8):e1001367.
57. Gentile C, Sehadova H, Simoni A, Chen C, Stanewsky R (2013) Cryptochrome antagonizes synchronization of Drosophila's circadian clock to temperature cycles. *Curr Biol* 23(3):185–195.
58. Yoshii T, Hermann C, Helfrich-Förster C (2010) Cryptochrome-positive and -negative clock neurons in Drosophila entrain differentially to light and temperature. *J Biol Rhythms* 25(6):387–398.
59. Jedlicka P, Mortin MA, Wu C (1997) Multiple functions of Drosophila heat shock transcription factor in vivo. *EMBO J* 16(9):2452–2462.
60. Tang C, et al. (2015) Analysis of gene expression profiles reveals the regulatory network of cold-inducible RNA-binding protein mediating the growth of BHK-21 cells. *Cell Biol Int* 39(6):678–689.
61. Chandrashekaran MK (1974) Phase-shifts in drosophila pseudoobscura circadian rhythm evoked by temperature pulses of varying durations. *J Interdiscipl Cycle Res* 5(3-4):371–380.
62. Zimmerman WF, Pittendrigh CS, Pavlidis T (1968) Temperature compensation of the circadian oscillation in drosophila pseudoobscura and its entrainment by temperature cycles. *J Insect Physiol* 14(5):669–684.
63. Ishimoto H, Lark A, Kitamoto T (2012) Factors that differentially affect daytime and nighttime sleep in Drosophila melanogaster. *Front Neurol* 3:24.
64. Dibner C, et al. (2009) Circadian gene expression is resilient to large fluctuations in overall transcription rates. *EMBO J* 28(2):123–134.
65. Cooper MK, Hamblen-Coyle MJ, Liu X, Rutila JE, Hall JC (1994) Dosage compensation of the period gene in Drosophila melanogaster. *Genetics* 138(3):721–732.
66. Lahiri K, et al. (2005) Temperature regulates transcription in the zebrafish circadian clock. *PLoS Biol* 3(11):e351.
67. Varma V, Mukherjee N, Kannan NN, Sharma VK (2013) Strong (type 0) phase resetting of activity-rest rhythm in fruit flies, Drosophila melanogaster, at low temperature. *J Biol Rhythms* 28(6):380–389.
68. Liu Y, Merrow M, Loros JJ, Dunlap JC (1998) How temperature changes reset a circadian oscillator. *Science* 281(5378):825–829.
69. Sweeney BM, Hastings JW (1960) Effects of temperature upon diurnal rhythms. *Cold Spring Harb Symp Quant Biol* 25:87–104.
70. Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* 24(1):219–230.
71. Hazel JR, Prosser CL (1974) Molecular mechanisms of temperature compensation in poikilotherms. *Physiol Rev* 54(3):620–677.
72. Daniels BC, Chen YJ, Sethna JP, Gutenkunst RN, Myers CR (2008) Sloppiness, robustness, and evolvability in systems biology. *Curr Opin Biotechnol* 19(4):389–395.
73. Rajan K, Abbott LF (2007) Temperature-compensated chemical reactions. *Phys Rev E Stat Nonlin Soft Matter Phys* 75(2 Pt 1):022902.
74. Castro-Longoria E, Ferry M, Bartnicki-Garcia S, Hasty J, Brody S (2010) Circadian rhythms in Neurospora crassa: Dynamics of the clock component frequency visualized using a fluorescent reporter. *Fungal Genet Biol* 47(4):332–341.
75. Khatun R, Lakin-Thomas P (2011) Activation and localization of protein kinase C in Neurospora crassa. *Fungal Genet Biol* 48(4):465–473.
76. Feillet C, et al. (2014) Phase locking and multiple oscillating attractors for the coupled mammalian clock and cell cycle. *Proc Natl Acad Sci USA* 111(27):9828–9833.
77. Myers MP, Wager-Smith K, Rothenfluh-Hilfiker A, Young MW (1996) Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. *Science* 271(5256):1736–1740.
78. Menet JS, Abruzzi KC, Desrochers J, Rodriguez J, Rosbash M (2010) Dynamic PER repression mechanisms in the Drosophila circadian clock: From on-DNA to off-DNA. *Genes Dev* 24(4):358–367.
79. Ozturk N, VanVickle-Chavez SJ, Akileswaran L, Van Gelder RN, Sancar A (2013) Ramshackle (Brwd3) promotes light-induced ubiquitylation of Drosophila Cryptochrome by DDB1-CUL4-ROC1 E3 ligase complex. *Proc Natl Acad Sci USA* 110(13): 4980–4985.
80. Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 95(5):669–679.
81. Huber R, et al. (2004) Sleep homeostasis in Drosophila melanogaster. *Sleep* 27(4): 628–639.
82. Stavropoulos N, Young MW (2011) insomniac and Cullin-3 regulate sleep and wakefulness in Drosophila. *Neuron* 72(6):964–976.