ACKNOWLEDGMENTS

The authors thank C. Webster for improving the figure and A. Greenleaf, D. Wassarman, J. Pearson, C. Bennett, and T. Robinson for comments on the figure and manuscript.

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Keeping the Beat in the Rising Heat

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DOI 10.1016/j.cell.2009.04.051

Circadian clocks use temperature compensation to keep accurate time over a range of temperatures, thus allowing reliable timekeeping under diverse environmental conditions. Mehra et al. (2009) and Baker et al. (2009) now show that phosphorylation-regulated protein degradation plays a key role in circadian temperature compensation.

Rising temperatures result in rising reaction rates for most chemical and biochemical processes. Fish in icy Michigan lakes have slower metabolic rates than their brethren in Southeast Asia, and hibernating animals slow their basal metabolic rates by lowering their core temperature. Although for some biological systems there is an adaptive advantage in having reaction rates that change with temperature, other processes must maintain the same reaction rate regardless of temperature (Figure 1A). In particular, the 24 hr circadian clock accurately keeps time regardless of temperature. This phenomenon, known as temperature compensation, has fascinated circadian biologists for years (Hastings and Sweeney, 1957; Pittendrigh, 1954). Two new studies from Jay Dunlap's group, published in *Cell* (Mehra et al., 2009) and *Molecular Cell* (Baker et al., 2009), shed light on the molecular basis of temperature compensation.

Temperature compensation is especially important for poikilotherms (organisms whose internal temperature depends on the environment) but is also preserved in homeotherms like us and can be detected in hibernating mammals, laboratory animals, and cultured mammalian cells (for example see Izumo et al., 2003). The preservation of temperature compensation even in nonhibernating mammals suggests that it is an intrinsic and universally conserved feature of the circadian clock mechanism. The most likely basis for this mechanism, proposed long before the biochemical details of circadian timekeeping were known, is that a series of counterbalanced biochemical reactions in the core of the clock undergo equal and opposing changes with alterations in temperature (Hastings and Sweeney, 1957) (Figures 1B and 1C). However, despite more than 50 years of theories and experimentation, as well as enormous gains in the molecular understanding of the circadian clock, the mechanism of temperature compensation has remained a black box.

The basic mechanism of all known circadian timekeepers involves the rhythmic accumulation of key transcriptional repressors, such as the PERIOD protein in the fly *Drosophila melanogaster*, the CRYPTOCHROME proteins in mammals, and the FREQUENCY (FRQ) protein in the fungus *Neurospora crassa*. The abundance of these proteins is regulated largely by a balance, which changes over the course of the day, between phosphorylation and dephosphorylation events that regulate protein stability. One might suspect, then, that temperature compensation could occur if the rising temperature simultaneously increased the opposing activities of kinases, phosphatases, and the protein degradation machinery. In their new study, Mehra et al. (2009) show using a combined genetic and biochemical approach that

alterations in phosphorylation-regulated protein degradation affect temperature compensation in *Neurospora*. They find that two independent *Neurospora* mutants known to have altered (but not defective) temperature compensation harbor mutations in two genes encoding the α and β1 subunits of the casein kinase 2 (CK2) $\alpha_2\beta_2$ tetrameric kinase. This parallel genetic identification of two CK2 subunits firmly places this kinase within the temperature compensation mechanism. The authors confirm that FRQ is a substrate of CK2. They further show that the mutations in CK2 are loss-of-function mutations that cause decreased phosphorylation of FRQ. The normal role of CK2 appears to be accelerating FRQ degradation at high temperatures. In wild-type *Neurospora* cells, the FRQ protein half-life is unchanged (that is, temperature compensated) when the temperature rises from 22°C to 28°C. However, when CK2 is mutated or absent, FRQ is more stable (that is, overcompensated) as the temperature rises to 28°C. The authors also identified the phosphorylation sites that destabilize FRQ at high temperatures. Mutation of these sites phenocopies the effect of the CK2 mutations by producing a more stable FRQ protein with altered temperature compensation. Thus, Mehra et al. uncover a mechanism that regulates FRQ protein stability at higher temperatures.

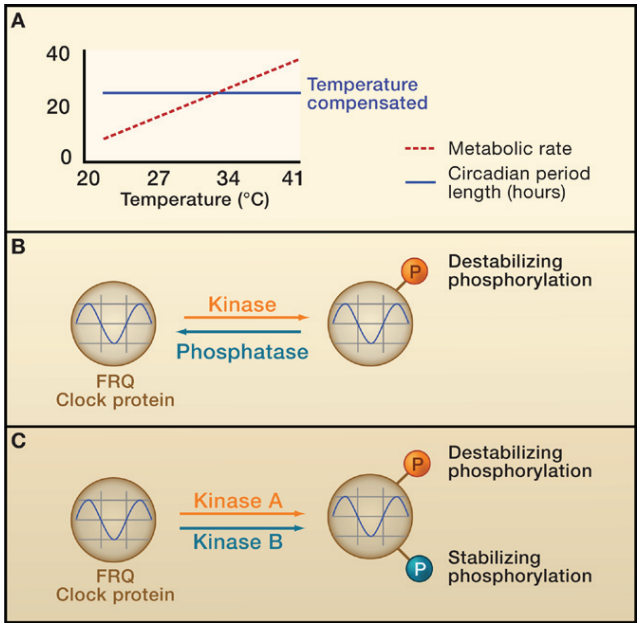

Figure 1. Temperature Compensation in the *Neurospora* Circadian Clock

(A) The rate of most biological processes, reflected in the metabolic rate (red dotted line), doubles with every 10°C increase in body temperature. However, the circadian clock is temperature compensated such that it remains stable as the temperature changes. The length of the clock period (blue solid line) may even decrease with increasing temperature.

(B) A kinase-phosphatase model for temperature compensation. Phosphorylation of a specific site on the clock protein FREQUENCY (FRQ) leads to its degradation. As the temperature rises, there is an increase in activity of both casein kinase 2 (CK2; Mehra et al., 2009) and any enzyme that opposes phosphorylation, such as a phosphatase or a deubiquitinating enzyme, leading to no net change in the degradation rate of FRQ.

(C) A dual kinase model for temperature compensation. Kinase A (such as CK2) phosphorylates a destabilizing site (orange phosphate group), whereas a different kinase (kinase B) phosphorylates a stabilizing site (blue phosphate group). As the temperature rises, both sites are more frequently phosphorylated, again leading to no net change in the degradation rate of FRQ.

Mehra et al.'s findings are consistent with the model that temperature compensation is mediated by the balanced sum of opposing processes. In this instance, the greater stability of FRQ at elevated temperatures is countered by increased CK2 activity. But what mechanism, in the absence of CK2, decreases the FRQ degradation rate as the temperature increases? The suspects include deubiquitination and dephosphorylation. Indeed, the increased activity of deubiquitinating enzymes and protein phosphatases at increased temperatures could slow the phosphorylation-regulated degradation of FRQ (Figure 1B). Mehra et al. tested this possibility by altering the abundance of protein phosphatase 1 and protein phosphatase 2A catalytic subunits but found no resulting changes in temperature compensation. Changing the abundance of casein kinase 1, another major circadian regulator, also had no effect. Future work might implicate more specific phosphatase regulators in temperature compensation (Virshup and Shenolikar, 2009).

The regulation of FRQ by CK2 is only the beginning of a complex story. Mutations in CK2 and FRQ alter temperature compensation at higher temperatures, but at lower temperatures, other mechanisms, also based on balancing opposing reactions such as phosphorylation and dephosphorylation, must be at work. Efforts to illuminate the role of phosphorylation in the *Neurospora* clock will be greatly aided by the new findings of Baker et al. (2009). They analyzed FRQ phosphorylation in *Neurospora* by mass spectrometry every 4 hr throughout one circadian cycle. Demonstrating the extreme complexity of phosphorylation in regulating circadian proteins, they found more than 80 serine/threonine phosphorylation sites in FRQ. Not unexpectedly, the phosphorylation state of a number of these sites changes throughout the circadian cycle. To determine the importance of these phosphorylation sites, the authors assessed 48 multisite mutant FRQ proteins for their effects on period length in the circadian cycle. Of these, eight mutant proteins increased period length, whereas three mutant proteins decreased period length. This is consistent with a model in which increased kinase activity can both accelerate and decelerate the clock by simultaneously affecting two different phosphorylation sites (Figure 1C). Further investigation of the functions of these phosphorylation sites and their kinases should produce additional insights into clock regulation.

The Mehra et al. study raises broader questions. For example, do the metazoan clock proteins PERIOD and CRYPTOCHROME show similar temperature compensation? Is CK2 the most important kinase in this process, or just the one that was most amenable to discovery by genetic methods? Also, because temperature compensation persists in *Neurospora* CK2 and FRQ mutants, it will be important to test whether changes in FRQ phosphorylation are also a part of the temperature compensation mechanism at lower temperatures. In addition, it should be considered whether other temperature-dependent processes such as transcriptional regulation have a similar bidirectional effect

on the clock period, although both theoretical and experimental work point to the rates controlling phosphorylation and protein degradation as having a larger effect on the clock period than the rates of transcriptional regulation (Dibner et al., 2009; Gallego et al., 2006). More broadly, could temperature compensation be a property of the circadian network architecture that appears to be conserved across many species? Does temperature compensation occur in a similar way in organisms (such as, cyanobacteria and the model plant *Arabidopsis thaliana*) that keep time using clocks with a very different network structure than that found in *Neurospora*? These questions aside, circadian phosphorylation cycles are clearly essential for clocks from cyanobacteria, insects, and mammals (Gallego and Virshup, 2007). In fact, in a reconstituted cell-free cyanobacterial clock, the circadian cycle of KaiC phosphorylation is temperature compensated in the absence of transcription and new protein synthesis (Nakajima et al., 2005). The findings of Mehra et al. and Baker et al. now demonstrate the importance of phosphorylation in temperature compensation in *Neurospora*. These exciting studies provide mechanistic insight into a fascinating but poorly understood process and explain how *Neurospora* can keep an accurate beat as life heats up.

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The CULt of Caspase-8 Ubiquitination

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DOI 10.1016/j.cell.2009.03.015

Caspase-8 is activated at the plasma membrane by the death-inducing signaling complex (DISC). Jin et al. (2009) show that polyubiquitination of caspase-8, rather than targeting it for proteasomal degradation, is critical for sustaining caspase-8 activity after dissociation from the DISC.

The fate of many cells in vertebrates is governed by the extrinsic apoptosis pathway in which the binding of proapoptotic ligands to death receptors at the cell surface causes receptor clustering and recruitment of the adaptor protein FADD. This leads to activation of the initiator caspases-8 and -10 to form the death-inducing signaling complex (DISC). Substantial biochemical and structural data support a model in which the DISC traps FADD in a conformation that allows binding of caspase-8 (Scott et al., 2009), leading to the activation of caspase-8 by dimerization (Keller et al., 2009). Activated caspase-8 then removes its own recruitment domains and translocates to the cytosol, where it is thought to deliver the knockout blow by activating the downstream apoptotic machinery. However, the caspase-8 dimer is too unstable to exist for long once released from the DISC (Donepudi et al., 2003, Pop et al., 2007). This raises a key question: how is the active caspase-8 dimer maintained for long enough to activate its downstream targets once removed from the DISC? Ashkenazi and coworkers now propose an unusual and elegant mechanism for the stabilization of caspase-8 that involves polyubiquitination (Jin et al., 2009).

The mechanism described by Jin et al. provides an intriguing new connection between ubiquitination and the apoptotic pathway. According to their findings, stabilization of caspase-8 is dependent on its polyubiquitination by the cullin3 ubiquitin E3 ligase. This modification does not lead to caspase-8 degradation, but instead promotes its aggregation and stabilization. Therefore, ubiquitination is a direct gain-of-function event for caspase-8 activity. A more conventional role for the ubiquitin system in regulating caspase activity through degradation of caspases or their modulators has been explored by many investigators (Vaux and Silke, 2005). Ubiquitination of caspases in the fruit fly *Drosophila* has recently been shown to negatively regulate caspase activity independent of degradation (Ditzel et al., 2008), but direct activation by ubiquitination is a new idea, and the article by Jin et al. provides extensive corroboration.