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PERSPECTIVE

The right place at the right time: regulation of daily timing by phosphorylation

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Each day we perform a ritual: We sleep for ~8 h, awaken and rise, and ~16 h later, we sleep again. This ritual is based on a temporal program that continues, although not necessarily exactly every 24 h, even if we are shielded from environmental signals such as light and dark, scheduled meals, or any social "rituals" such as work schedules. This endogenous, ~24-h oscillation, is called a circadian rhythm. Circadian clocks regulate processes from gene expression to behavior, and have been observed in all phyla. As such, the circadian system is a fundamental biological process—a part of life like reproduction or cell division. Because daily temporal structure is fundamental to, for instance, medicine or work schedules, an understanding of the underlying biochemical mechanism is highly relevant to human health and quality of life.

A genetic approach to the clock mechanism has revealed a network of genes (clock genes) that function as a transcriptional–translational negative feedback loop, with at least one protein feeding back to inhibit the transcriptional activator of its own gene (Hardin et al. 1990). In mice, CRYPTOCHROME (CRY) and PERIOD (PER) form heterodimers and down-regulate their activators, CLOCK (CLK) and BRAIN AND MUSCLE ARNT-LIKE-1 (BMAL-1) (see Fig. 1A). It is an elegantly simple molecular mechanism implemented in animals, plants, fungi, and cyanobacteria by sets of clock genes unique to each group. Common to all these circadian systems is phosphorylation of one or several of the clock proteins (Vanselow et al. 2006). This post-translational modification is apparently critical to all circadian clock mechanisms—in some cases even more important than regulated transcription.

Persistent, unabated circadian rhythms in constant conditions are, of course, not typical in nature; rather, they are usually synchronized to the 24-h rotation of the earth. This so-called "entrainment" is thought to work via regular perturbations of at least one clock component by a Zeitgeber (an environmental signal such as light) or by a downstream component of its signal transduction pathway. Light is generally the strongest Zeitgeber for organisms, but food, temperature, and various chemicals can also act as synchronizers.

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Thus, a complete circadian system—be it at the level of the whole organism or single cells—includes Zeitgeber input pathways, oscillator components (often called the core mechanism), and output pathways (Fig. 1B). Because the expression of different genes is potentially involved in any step along this pathway, the resulting circadian rhythm is a highly complex genetic trait. Despite everything that is known, many questions remain to be solved, even concerning the system's most essential features. For example, it is not clear how the molecular network can produce an oscillation as long as ~24 h. One hypothesis is that the circadian period derives from negative feedback formed by a collection of incremental processes, incorporating delays. These include subcellular localization, protein production, and degradation, which all can be controlled by post-translational modification via phosphorylation.

Circadian clocks in real life

In laboratory experiments, clocks are commonly studied in constant conditions, where differences in free-running period are used to tease apart the function of clock components. However, given that this temporal program evolved under natural (i.e., entrained) conditions, important insights into clock mechanisms, be it at the systemic, cellular or molecular level, come from studying the system under entrainment. Early observations showed that characteristics of a clock observed in constant conditions are also found when the clock is entrained (Hoffmann 1963; Pittendrigh and Daan 1976). Similar to mechanical oscillators, a rhythm with a short free-running period will entrain with an earlier phase than a rhythm with a longer period (although they both adopt the period of the Zeitgeber under entrainment). As can be deduced from Figure 1, alterations in input and output pathways (not only in the oscillator) can change the entrained phase. For example, it could be changed by an alteration in Zeitgeber strength or in the sensitivity to the Zeitgeber signal, as well as by alterations in the coupling of the output pathway to the oscillator, even if the oscillator components remained the same.

Entrainment is our most accessible clue to human circadian behavior (e.g., when does one sleep), and it can be assessed quantitatively (e.g., for the purposes of genetics). Although it is common to think of humans as either "early" or "late" types (larks and owls), chronotypes ac-

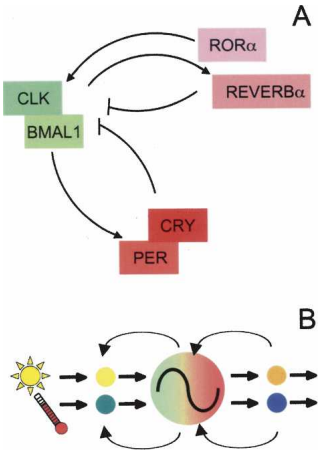

Figure 1. Cartoon drawings of circadian clock mechanisms. (*A*) The genetic network controlling circadian rhythms involves many components. In mice, CLOCK and BMAL1 activate expression of PER and CRY (at least three PER and two CRY genes). PERs and CRYs feed back to down-regulate CLOCK and BMAL1 activity. They also dampen activity of REVERBα, a nuclear orphan receptor family member that has negative effects on the activator complex. (*B*) Every circadian system, be it a cell or an entire organism, receives Zeitgeber signals (shown on the *left* here as light and/or temperature), which are transmitted via signal transduction pathways to a rhythm-generating mechanism (*center*, see *A*). (*Right*) Rhythmic signals are transduced via output pathways, to a large number of clock-regulated processes. Both input and output pathways can be regulated (at multiple loci) by the clock.

tually form a normal distribution with relatively few extreme early or late individuals at the ends (Roenneberg et al. 2004). Among the extreme chronotypes are a subset that suffer from advanced or delayed sleep timing, as classified according to strict criteria (Jones et al. 1999). Analysis of two families with familial advanced sleep phase syndrome (FASPS) showed mutations (apparently not common polymorphisms) concerning phosphorylation of the hPER2 protein, one in a phosphorylation site of hPER2 itself, and the other in a kinase, casein kinase Iδ (CKIδ), that phosphorylates hPER2 (Toh et al. 2001; Xu et al. 2005). The relationship between early entrained phase and short free-running period due to the hPER2 mutation is supported, although only based on a single subject in a temporal isolation experiment.

In this issue of *Genes & Development*, a new approach is described that has been used to understand phosphorylation of the mouse PER2 (mPER2) protein, and it has led to novel experiments that show how alteration of phosphorylation state can lead to either a clock that is slow and late or fast and early (Vanselow et al. 2006).

The importance of being phosphorylated (and dephosphorylated)

Clock proteins in all genetic model systems show striking and temporally distinct phosphorylation patterns. It is common to see a progressive increase in electrophoretic mobility (due to phosphorylation, as indicated by phosphatase treatment) that is associated with a subsequent decrease in overall clock protein amounts and followed by production of new (unphosphorylated) species (Zerr et al. 1990; Garceau et al. 1997). The period of this phosphorylation cycle is consistent with that of other circadian rhythms; e.g., with behavior. Phosphorylation in circadian timing is not just a downstream clock output event. This is best illustrated by the CKIε (*tau*) mutant hamster, a spontaneous mutation that remains among the most striking clock mutant phenotypes with a very short free-running period and a consistently early entrained phase (Lowrey et al. 2000). Furthermore, genetic (both forward and reverse) and pharmacological experiments confirm the fundamental nature of phosphorylation in circadian timing (Kloss et al. 1998; Yang et al. 2003; Nawathean and Rosbash 2004; Yang et al. 2004; Brunner and Schafmeier 2006).

Last year, in a milestone paper, a circadian oscillation with fundamental clock properties (e.g., self-sustainment in constant conditions, among others) was measured in a test tube with only three clock proteins and ATP as major components (Nakajima et al. 2005). The readout was the phosphorylation status of one of the proteins, demonstrating, at least in this simple system, that a metabolic beat—a "phoscillator"—could be at the heart of the circadian oscillation, devoid of any transcription and/or translation. Notably, this work concerned the cyanobacterial circadian mechanism. In the eukaryotic clock, it has been proposed that phosphorylation supports circadian timing through regulation of the proteins of the transcription–translation feedback loop via their subcellular localization and also their half-life, by targeting them to proteasome-mediated degradation (Young and Kay 2001; Brunner and Schafmeier 2006).

Experiments by the Kramer group (Vanselow et al. 2006) have systematically investigated phosphorylation sites in the mPER2 protein. This is an excellent candidate clock protein for such questions because of the association of hPER2 with FASPS, as well as the striking phenotype in knockout mouse mutants (Zheng et al. 1999), its putative role in entrainment (Albrecht et al. 1997), and the wealth of information concerning *period* in *Drosophila*, where it was *the* pioneer clock gene (Bargiello et al. 1984; Reddy et al. 1984). Vanselow et al. (2006) used purified, proteolysed mPER2 and then subjected it to phosphopeptide enrichment and tandem mass spectrometry. No less than 21 phosphorylation sites were revealed on mPER2, in a method that sets a new standard for phosphopeptide mapping. This seems like a large number, but the sites tended to be clustered around regions associated with nuclear localization, for instance, suggesting that several phosphate groups might contribute to the same function. One of the phosphorylated serine residues corresponds to that which was mutated to glycine in one of the FASPS families (Toh et al. 2001), demonstrating that, at least in a cell culture system, this potential phosphorylation site is actually phosphorylated.

Vanselow et al. (2006) used reverse genetics and pharmacological approaches in combination with cell culture

methods to define discrete events in the metabolism of wild-type and mutated mPER2. In particular, they used the FASPS mutation to probe chronotype in a cell culture. Several important findings emerged: First, it is clear that expression of the PER2 FASPS mutation (which is not specifically a CKIε target site but rather a priming site, necessary for CKIε to phosphorylate neighboring residues), as well as a mPER2 that is mutated in neighboring CKIε target sites, can phenocopy FASPS on the cellular level. Both of these mutant mPER2-expressing cell lines show an advanced phase (earlier expression vs. wild-type mPER2 cells) of a clock promoter-regulated reporter gene after synchronization by dexamethasone and after entrainment in temperature cycles. Second, cell cultures expressing the mutant proteins show a short free-running period (although it damps rapidly and is not easy to quantify), suggesting that the cause of the early phase of entrainment in FASPS is indeed associated with a shorter period on the cellular level.

The cell culture system was used to probe which aspects of the molecular cycle were aberrant in cells carrying the FASPS and downstream CKIε site mutations. Vanselow et al. (2006) teased apart two opposite effects of phosphorylation of the mPER2 protein. On one hand, phosphorylation at sites distinct from the (S659) FASPS site destabilized the protein, promoting its degradation via the proteasome. On the other hand, phosphorylation at the FASPS site led to its stabilization through retention in the nucleus, essentially protecting mPER2 from proteasomal degradation. These opposed effects were modeled, and the results of the model were in turn tested in cell culture experiments, to show how changes in phosphorylation kinetics concerning the same component, but at different sites, could lead to opposing phenotypes.

Another recent paper also combines modeling and cell-based systems to investigate the phosphorylation of the mPER1 and mPER2 proteins, specifically with respect to CK1ε and CK1δ (Gallego et al. 2006a). Interestingly, Gallego et al. (2006a) present an alternative viewpoint, namely that the CK1ε *tau* mutation (the *tau* phenotype is similar to FASPS, in terms of advanced entrained phase) is a gain of function rather than a loss of function. Furthermore, this effect is specific to the PER proteins, and contrasts the effects of *tau* mutant CK1ε on other clock proteins (e.g., BMAL1) and on the Wnt signaling pathway, where it is a loss-of-function mutation. Gallego et al. (2006a) posit that the basis for the *tau* or FASPS phenotype is increased degradation due to hyperphosporylation, whereas Vanselow et al. (2006) suggest that increased degradation follows premature nuclear clearance.

There are many roads leading to Rome. Even the phosphorylation of a single protein may involve a chain of events: A kinase phosphorylates a certain target site in a protein; the phosphorylation state of this site triggers phosphorylation of other sites (not necessarily by the same kinases). The end-product of this chain is a certain functional form of the protein leading to a characteristic phenotype; for example, FASPS. Thus, FASPS can theoretically be caused by at least three different mutations: (1) in the initial kinase, (2) in the protein's target site, and (3) in the kinase that "reads" the state of this site. And because clock proteins act as a network and regulate each other's levels, mutations in a number of other genes or kinases may result in an extremely early chronotype.

Are there indications of a phoscillator in animals?

The "clock-in-a-tube" paper of last year (Nakajima et al. 2005), describing how a phoscillator can generate an ~24-h rhythm without transcription and translation, invites re-evaluation of our assumptions about clock mechanisms. Dynamic regulation of reversible phosphorylation plays a fundamental role in most signal transduction pathways connected with essential cellular processes, ranging from cell cycle to differentiation to apoptosis (Knippschild et al. 2005). The phosphorylation state and even the activity of many proteins is fine-tuned by a balance between kinases and phosphatases. An unbiased query into targets of kinases showed that transcription factors are the largest functional group of proteins to be phosphorylated (Ptacek et al. 2005), and the known clock components function as transcriptional activators and repressors. The first indication of the role of CKIε (called DOUBLETIME or DBT in *Drosophila*) in the circadian system was in *Drosophila,* marking the beginning of a genetic approach to phosphate metabolism and the clock (Kloss et al. 1998). Ten years earlier, physiological experiments had shown that by the addition of creatine, a phosphate sink, to the media of a unicellular algae, the circadian clock was accelerated (Roenneberg et al. 1988).

There has been an avalanche of information published recently concerning the regulation of phosphorylation in the circadian clock mechanism. In *Drosophila*, CKIε also regulates dCLK, the activator of PER, in an on/off balance with PP2A (Kim and Edery 2006). dPER, one of the proteins that acts negatively on dCLK function, is also regulated by both DBT (dCKIε) and dPP2A (Sathyanarayanan et al. 2004) and dPER promotes phosphorylation of dCLK (and its subsequent degradation) by DBT (Yu et al. 2006). The dimerization partner of dPER, dTIM, is phosphorylated by SHAGGY (GSK3), thereby controlling its stability and subcellular localization (Young and Kay 2001). In the fungal model system *Neurospora*, there have been numerous kinases and phosphatases linked to the central clock mechanism, recently reviewed in this journal by Brunner and Schafmeier (2006). In the mammalian clock system, a recent report showed CKIε activity modified by PROTEIN PHOSPHATASE 5 (PP5), which is in turn regulated by mCRY (Partch et al. 2006).

Let us put this into a non-clock-centric perspective. Annotation of the human genome indicates that there are between 500 and 2000 kinases (Ficarro et al. 2002; Milanesi et al. 2005). In the more tractable cellular experimental system, *Saccharomyces cerevisiae*, there are ~120. It is estimated that ~30% of the 6100 yeast proteins are phosphoproteins. In a first attempt to characterize the phosporylome, Snyder and coworkers (Ptacek et al. 2005) have assayed 87 yeast kinases on a yeast

proteome chip, asking which proteins they phosphorylate. The experiment established a roadmap, with kinases modifying from one to many substrates, and with substrates being modified by one to several kinases.

By mapping out the phosphorylome, sets of formulaic interactions between kinases and substrates become apparent, based on known biological activities (Ptacek et al. 2005). These include "kinase cascades," where an upstream kinase phosphorylates a downstream one. There are "transcription factor-regulated substrate–kinase pairs," whereby the same transcription factor activates expression of both upstream kinase and downstream phosphatase. We inspected the most common regulatory modules and determined that the molecular clock network resembles a "kinase–substrate pair feedback loop II module" (Fig. 2A): mCKIε phosphorylates both the activating transcription factor (mBMAL1) and its downstream products (mPER2 and mPER1, mCRY1 and CRY2, as well) (see Fig. 2B). Furthermore, the kinase activity of CKIε itself depends on dephosphorylation (Gietzen and Virshup 1999). This activation is accomplished in the context of the circadian clock by multimerization of CKIε, mCRY1, and/or mCRY2 with PP5 (Partch et al. 2006). These relationships suggest that

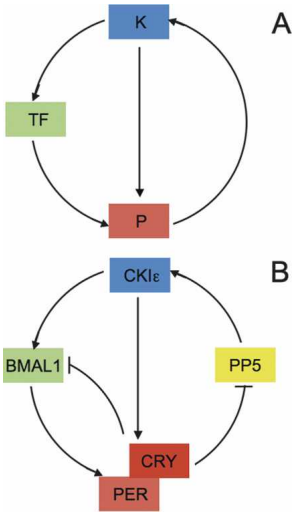

Figure 2. Feedback loops based on phosphorylation. (*A*) A kinase–substrate pair feedback loop II module (Ptacek et al. 2005). (K) Kinase; (TF) transcription factor; (P) protein. In this commonly found configuration, the kinase phosphorylates both the transcription factor and the respective gene product, which feeds back to regulate activity of the kinase. (*B*) A hybrid cartoon of the mouse clock mechanism with two interlocked loops. One of them is the backbone of the transcription–translation feedback loop from Figure 1B, with BMAL1 activating expression of the negative effectors, PER and CRY. PER and CRY also participate in a feedback loop—a phoscillator—involving CKIε activity. CKIε is inactive when autophosphorylated (Gietzen and Virshup 1999). It can be activated by PP5 (Partch et al. 2006). CKIε controls half-life and subcellular localization of PER, and also phosphorylates CRY. PP5 activity is, in turn, negatively regulated by CRY. Thus, cycles in CRY abundance would effect a rhythm in CKI activity, creating an oscillation that appears independent of regulated transcription.

clock components could also form a metabolic, nontranscriptional feedback loop, a phoscillator in mammals. Could this be a core clock mechanism and, if so, what is its relationship to the transcription–translation feedback loop (see also Roenneberg and Merrow 1998, 2002)? Could such a phoscillator explain rhythms in behavior and clock protein levels despite constitutive expression of *period* and *timeless* RNA in *Drosophila* (Yang and Sehgal 2001)? It may underpin the residual clock properties that have been demonstrated in clock gene mutant strains (Merrow et al. 1999; Roenneberg et al. 2005).

Although PP2A has clock-regulated rhythmic expression in *Drosophila* (Sathyanarayanan et al. 2004), most kinases and phosphatases are either uncharacterized for their expression or are expressed constitutively. In the case of CKIε and PP5, kinase and phosphatase activity would be predicted to be clock regulated via mCRY. This configuration creates a loop that could oscillate without the benefit of regulated transcription. There are many more kinases and phosphatases that are indicated as regulators of the molecular pathways of the circadian clock. For instance, PP1 slows down degradation of mPER2 (Gallego et al. 2006b), thus potentially forming an additional feedback module.

We conclude by proposing an alternative hypothesis, namely that an extended "kinase–substrate pair feedback loop II module" is also part of the core clock mechanism generating the ~24-h oscillation at the molecular level. This would be an ancestral clock mechanism, with the specialized transcription/translation regulatory components evolving uniquely for animals, plants, and fungi after the branches in the tree of life were established. These transcription factors have been thoroughly incorporated into the clock mechanism so that clocks function poorly (or not as clocks) without their contributions (e.g., optimal and accurate phase control). This hypothesis thus should not be a challenge to the old one; rather, it should provide an additional framework for designing creative experiments.

Acknowledgments

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References

Albrecht, U., Sun, Z.S., Lee, C.C., Eichele, G., and McLean, V.M. 1997. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* **91:** 1055–1064.

Bargiello, T.A., Jackson, F.R., and Young, M.W. 1984. Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature* **312:** 752–754.

Brunner, M. and Schafmeier, T. 2006. Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and *Neurospora*. *Genes & Dev.* **20:** 1061–1074.

Ficarro, S.B., McCleland, M.L., Stukenberg, P.T., Burke, D.J.,

Ross, M.M., Shabanowitz, J., Hunt, D.F., and White, F.M. 2002. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **20:** 301–305.

Gallego, M., Eide, E.J., Woolf, M.F., Virshup, D.M., and Forger, D.B. 2006a. An opposite role for tau in circadian rhythms revealed by mathematical modeling. *Proc. Natl. Acad. Sci.* **103:** 10618–10623.

Gallego, M., Kang, H., and Virshup, D.M. 2006b. Protein phosphatase 1 regulates the stability of the circadian protein PER2. *Biochem. J.* doi: 10.1042/BJ20060678.

Garceau, N.Y., Liu, Y., Loros, J.J., and Dunlap, J. 1997. Alternative initiation of translation and time specific phosphorylation yield multiple forms of essential clock protein FREQUENCY. *Cell* **89:** 469–476.

Gietzen, K.F. and Virshup, D.M. 1999. Identification of inhibitory autophosphorylation sites in casein kinase Iε. *J. Biol. Chem.* **274:** 32063–32070.

Hardin, P.E., Hall, J.C., and Rosbash, M. 1990. Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. *Nature* **343:** 536–540.

Hoffmann, K. 1963. Zur Beziehung zwischen Phasenlage und Spontanfrequenz bei der endogenen Tagesperiodik. *Z. Naturforschg.* **18b:** 154–157.

Jones, C.R., Campbell, S.S., Zone, S.E., Cooper, F., DeSano, A., Murphy, P.J., Jones, B., Czajkowski, L., and Ptacek, L.J. 1999. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. *Nat. Med.* **5:** 1062–1065.

Kim, E.Y. and Edery, I. 2006. Balance between DBT/CKIε kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. *Proc. Natl. Acad. Sci.* **103:** 6178–6183.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. 1998. The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase ε. *Cell* **94:** 97–107.

Knippschild, U., Gocht, A., Wolff, S., Huber, N., Lohler, J., and Stoter, M. 2005. The casein kinase 1 family: Participation in multiple cellular processes in eukaryotes. *Cell. Signal.* **17:** 675–689.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288:** 483–491.

Merrow, M., Brunner, M., and Roenneberg, T. 1999. Assignment of circadian function for the *Neurospora* clock gene frequency. *Nature* **399:** 584–586.

Milanesi, L., Petrillo, M., Sepe, L., Boccia, A., D'Agostino, N., Passamano, M., Di Nardo, S., Tasco, G., Casadio, R., and Paolella, G. 2005. Systematic analysis of human kinase genes: A large number of genes and alternative splicing events result in functional and structural diversity. *BMC Bioinformatics* (Suppl. 4) **6:** S20.

Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T., and Kondo, T. 2005. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* **308:** 414–415.

Nawathean, P. and Rosbash, M. 2004. The doubletime and CKII kinases collaborate to potentiate *Drosophila* PER transcriptional repressor activity. *Mol. Cell* **13:** 213–223.

Partch, C.L., Shields, K.F., Thompson, C.L., Selby, C.P., and Sancar, A. 2006. Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5. *Proc. Natl. Acad. Sci.* **103:** 10467–10472.

Pittendrigh, C.S. and Daan, S. 1976. A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: Pacemaker as clock. *J. Comp. Physiol. A* **106:** 291–331.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., et al. 2005. Global analysis of protein phosphorylation in yeast. *Nature* **438:** 679–684.

Reddy, P., Zehring, W.A., Wheeler, D.A., Pirrotta, V., Hadfield, C., Hall, J.C., and Rosbash, M. 1984. Molecular analysis of the period locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. *Cell* **38:** 701–710.

Roenneberg, T. and Merrow, M. 1998. Molecular circadian oscillators—An alternative hypothesis. *J. Biol. Rhythms* **13:** 167–179.

———. 2002. Life before the clock—Modeling circadian evolution. *J. Biol. Rhythms* **17:** 495–505.

Roenneberg, T., Nakamura, H., and Hastings, J.W. 1988. Creatine accelerates the circadian clock in a unicellular alga. *Nature* **334:** 432–434.

Roenneberg, T., Kuehnle, T., Pramstaller, P.P., Ricken, J., Havel, M., Guth, A., and Merrow, M. 2004. A marker for the end of adolescence. *Curr. Biol.* **14:** R1038–R1039.

Roenneberg, T., Dragovic, Z., and Merrow, M. 2005. Demasking biological oscillators: Properties and principles of entrainment exemplified by the *Neurospora* circadian clock. *Proc. Natl. Acad. Sci.* **102:** 7742–7747.

Sathyanarayanan, S., Zheng, X., Xiao, R., and Sehgal, A. 2004. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell* **116:** 603–615.

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J., and Fu, Y.H. 2001. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291:** 1040–1043.

Vanselow, K., Vanselow, J.T., Westermark, P.O., Reischl, S., Maier, B., Korte, T., Herrmann, A., Herzel, H., Schlosser, A., and Kramer, A. 2006. Differential effects of PER2 phosphorylation: Molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes & Dev.* (this issue).

Xu, Y., Padiath, Q.S., Shapiro, R.E., Jones, C.R., Wu, S.C., Saigoh, N., Saigoh, K., Ptacek, L.J., and Fu, Y.-H. 2005. Functional consequences of a CKIδ mutation causing familial advanced sleep phase syndrome. *Nature* **434:** 640–644.

Yang, Z. and Sehgal, A. 2001. Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. *Neuron* **29:** 453–467.

Yang, Y., Cheng, P., He, Q., Wang, L., and Liu, Y. 2003. Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the *Neurospora* circadian clock. *Mol. Cell. Biol.* **23:** 6221–6228.

Yang, Y., He, Q., Cheng, P., Wrage, P., Yarden, O., and Liu, Y. 2004. Distinct roles for PP1 and PP2A in the *Neurospora* circadian clock. *Genes & Dev.* **18:** 255–260.

Young, M.W. and Kay, S.A. 2001. Time zones: A comparative genetics of circadian clocks. *Nat. Rev. Genet.* **2:** 702–715.

Yu, W., Zheng, H., Houl, J.H., Dauwalder, B., and Hardin, P.E. 2006. PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes & Dev.* **20:** 723–733.

Zerr, D.M., Hall, J.C., Rosbash, M., and Siwicki, K.K. 1990. Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* **8:** 2749–2762.

Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C., and Bradley, A. 1999. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* **400:** 169–173.