Kinases and Circadian Clocks: PER Goes It Alone

In the January 30, 2004, issue of *Molecular Cell*, Nawathean and Rosbash describe how kinases cooperate to create a small but active pool of PER that can localize to the nucleus and act on its own in a transcriptional feedback loop underlying the *Drosophila* circadian clock.

One of the nice things about the field of circadian rhythms is that the problem has had real staying power. It's not just that the family of players keeps growing and that similar themes keep turning up in different organisms, but also that there appear on a regular basis novel activities or relationships among those components that we already thought we understood. And as often as not the new knowledge allows us to explain yet a little better how a cell builds a 24 hr clock. A recent paper (Nawathean and Rosbash, 2004) did just this by examining the role(s) of kinases in modulating the activity of one of the original circadian clock proteins, the PER protein of *Drosophila*.

It's common to begin pieces like this (as did Nawathean and Rosbash) by restating the fact that, "In all genetically studied model organisms, a negative feedback loop of gene expression makes a major contribution to the circadian rhythm mechanism." In animals and fungi, this core negative feedback loop involves a transcription factor, comprising a heterodimer of proteins that interact via PAS domains, that activates expression of genes encoding proteins that feed back to depress this activation (reviewed in Dunlap, 2003). To better appreciate the present story, you can start more or less a decade ago and recall how we all thought the *Drosophila* circadian clock worked: there was PER and its sidekick TIM; they both had to be there and had to dimerize, both to get themselves into the nucleus and to stabilize PER (reviewed in Hall, 2003). The necessity of TIM for clock operation in vivo led to the assumption that it was required for nuclear entry of the PER/TIM dimer and for the molecular events surrounding repression. (Hold that thought.) PER was also known to become phosphorylated, which was believed to dictate its turnover (subsequently shown to be true), but other possible roles for this phosphorylation, e.g., modulation of activity, were gradually displaced if not forgotten. In the meantime, outside the fly, casein kinase II (CKII) was shown by Tobin and colleagues to modulate the DNA binding ability of CCA1 (Sugano et al., 1998), later acknowledged to be a component of the *Arabidopsis* clock, and also to be essential for the Neurospora clock (reviewed in Dunlap, 2003). More *Drosophila* clock components emerged too, including the kinases responsible for PER phosphorylation, a casein kinase I isoform known as doubletime (DBT), and CKII, and additional interlocked feedback loops took shape (reviewed in Hall, 2003). These are the players in our tale.

The present work that reshapes the original core feedback loop can be traced to prior studies using clockless *Drosophila* S2 cells to examine, without the complication of a ticking biological clock, the ability of PER and TIM to repress. Originally (Darlington et al., 1998), PER was quite feeble as a repressor on its own, but over the last 5 years PER seems to have been doing better (Rothenfluh et al., 2000) and better (Ashmore et al., 2003; Chang and Reppert, 2003) in going it alone, without TIM. Its strength as a sole repressor was verified again in the first figures of the present work, so even in the hotbed of clocks we may tentatively assume that the following is true: PER does not need TIM to repress, and therefore also does not need TIM to enter the nucleus. In the present work, though, we see something new: although TIM still acted to stabilize PER (we knew that), this increased level of nondegraded PER led to no greater repression (that's a surprise). That is, now it's the quality of PER and not just the quantity that is important. A likely avenue for qualitative modulation of PER activity, of course, is phosphorylation, and in confirmation of this, RNAi-derived inhibition of either or both DBT and CKII led to more PER but correspondingly to less repressive activity—the kinases additively potentiated the repressive activity of PER in vivo. This could be due to a requirement of phosphorylation for nuclear entry or for the actual biochemistry of repression. To parse this conundrum, the investigators resorted to drug use, specifically to leptomycin B (LMB) as well as to RNAi, to block CRM-1-mediated nuclear export, and showed that phosphorylation promotes nuclear localization; i.e., reduced kinase activity resulted in reduced nuclear PER even in the presence of LMB. But if the sole role of the kinases is to get PER into the nucleus, then, by allowing nuclear PER accumulation, LMB should suppress the effects of kinase inhibition on PER activity. However, it does not—LMB treatment combined with RNAi-inhibited kinases yielded more nuclear PER than is seen in untreated cells, but it displays much less PER activity. Therefore, although the kinases promote nuclear entry of PER, their major role is to potentiate its repressive activity.

An inescapable conclusion from the data, stated as a prediction by the authors, is that there must be both active and inactive pools of PER. Happily, this realization may go a long way toward resolving some nasty residual loose ends in the *Drosophila* clock story that have arisen over the years from trying to tie together the cell and molecular biologies of clock components. For instance, one can now rationalize and dismiss the likelihood of a novel clock mechanism in the brain of silk moths that was conjectured following a failure to see cycles of PER nuclear localization in that tissue (Sauman and Reppert, 1996): this would be explained now by the hypothesis that a pool of active PER is moving to the nucleus but comprises a minor (and therefore largely invisible) component compared to the inactive pool. Likewise, as described here by the authors, perfectly good molecular rhythms have been documented in fly eyes in the absence of immunocytochemically observable rhythms in PER nuclear localization, presumably because only a small active pool needed to move. And last, early appearance of this small but active pool of PER would join

early appearance of VRI (Cyran et al., 2003) as mechanisms to explain why *per* and *tim* mRNA levels begin to fall before PER is seen moving into the nucleus (reviewed in Hall, 2003). In terms of understanding real clocks in animals, if there is a caveat to the application of this well-crafted story, it arises only from the fact that it was necessary here to use clockless S2 cells (lacking TIM) to tease apart cause and effect. However, flies keeping time have the full armature of clock proteins: although PER is potentially capable of functioning in the early night as a repressor on its own, does it really do the heavy lifting without TIM? What are the relative contributions of PER versus PER/TIM versus VRI to the evening decline in *per* expression? I suspect we may not have seen the last of this discussion, but Nawathean and Rosbash have taken a major step in focusing it. And in any case, the hard trench work will now follow as the intracellular concentrations of the pertinent molecules are scrutinized, the molecular and temporal nature of the specific phosphorylations are revealed, and we come to understand how nuances in the time and place of phosphorylation can modulate the biochemistry of PER and the structural biology of its interactions at the core of the fly clock.

Jay C. Dunlap
Department of Genetics
Dartmouth Medical School
7400 Remsen
Hanover, New Hampshire 03755

Selected Reading

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Endosomal Protein Traffic Meets Nuclear Signal Transduction Head On

Rab5 plays a key role in controlling protein traffic through the early stages of the endocytic pathway. Previous studies on the modulators and effectors of Rab5 protein function have tied the regulation of several signal transduction pathways to the movement of protein through endocytic compartments. In the February 6, 2004, issue of *Cell*, Miaczynska et al. describe a surprising new link between Rab5 function and the nucleus by uncovering two new Rab5 effectors as potential regulators of the nucleosome remodeling and histone deacetylase protein complex NuRD/MeCP1.

Moving proteins through the endocytic pathway is a complex process. Proteins can enter the endocytic pathway by several routes, each of which is regulated at multiple points. Once in the cell, protein constituents then face a number of trafficking choices, including recycling back to the cell surface or traveling deeper into the endocytic pathway. Over the past 10 years, it has become increasingly evident that at many steps in the endocytic trafficking pathway endocytic cargo can interface with and influence many cellular processes (Di Fiore and De Camilli, 2001; Sorkin and Von Zastrow, 2002; von Zastrow, 2003). Differential trafficking of cell surface receptors is one way the movement of proteins through the endocytic pathway can influence cellular signal transduction processes. Once activated by ligand binding, many receptors are internalized via clathrin-coated pits, and their journey through the endocytic pathway begins. In the case of G protein-coupled receptors (GPCRs), internalization results in one of two fates, rapid recycling to the cell surface or delivery to the lysosome and degradation. These fates are largely determined by the receptor itself and the proteins with which it associates (Spiegel, 2003; von Zastrow, 2003). Differences in these interactions and trafficking can have a strong influence on signaling by GPCRs as well as type III TGF-β receptors (Chen et al., 2003).

Ligand-induced clathrin-mediated endocytosis of the epidermal growth factor receptors (EGFRs) also impacts cell signaling. Attenuation of EGFR signaling can be accomplished by the internalization and degradation of the receptor (Di Fiore and De Camilli, 2001). However, like the GPCRs and the type III TGF-β receptor, it has become clear that internalization of EGFR and other receptor tyrosine kinases (RTKs) does not necessarily result solely in simple downregulation of signaling cascades by the removal of activated receptors from the cell surface. It has been suggested that ligands for some RTKs, such as EGFR, may remain associated with the receptors as they travel through the early stages of the endocytic pathway, resulting in receptors that remain in their active, signaling-competent form. A number of signaling molecules can be found on endocytic structures, including Ras, Raf, and MEK, suggesting that these intracellular endocytic compartments have signaling potential (Sorkin and Von Zastrow, 2002).

Rab5 is a small GTPase of the Ras superfamily that is responsible for mediating membrane trafficking events through the early stages of the endocytic pathway, and represents a key point at which endocytic trafficking can be regulated. In its GTP-bound state, Rab5 facilitates the fusion of the plasma membrane-derived endocytic vesicles with the early endosome and is also involved