A PER/TIM/DBT Interval Timer for *Drosophila*'s Circadian Clock

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Circadian rhythms in *Drosophila* are supported by a negative feedback loop, in which PERIOD (PER) and Timeless (TIM) shut down their own transcription as they translocate once a day from the cytoplasm of clock-containing cells to the nucleus. Period length is partially determined by an interval of cytoplasmic retention of the TIM and PER proteins. To study this process, we examined PER/TIM/Doubletime (DBT) physical interactions and nuclear translocation by imaging individual cultured *Drosophila* cells. Using live cell video microscopy and green fluorescent protein (GFP) tags, we observed dynamic patterns of stability and localization for DBT, PER, and TIM that resembled those previously found in vivo. These studies suggest that a cytoplasmic interval timer regulates nuclear translocation of these proteins. The cultured cell assay provides a potent system to study interactions among new and known genes involved in the generation of circadian behavior.

INTRODUCTION

That single genes could make a substantial contribution to circadian rhythms was first demonstrated by Konopka and Benzer (1971). Upon screening mutagenized *Drosophila* for changes in patterns of daily eclosion, they identified three mutations affecting a single X-chromosomal locus, *period* (*per*). One mutant, *per⁰*, was arrhythmic, whereas *per-short* (*per^S*) and *per-long* (*per^L*) mutants were rhythmic with 19-hour and 29-hour periods, respectively. Locomotor activity was also affected, and each *per* mutation produced equivalent effects on eclosion and locomotor activity rhythms. More than a decade passed before the molecular identity of *period* was established (Bargiello and Young 1984; Reddy et al. 1984; Jackson et al. 1986; Citri et al. 1987). Yet, the primary sequence of the encoded protein provided little information about its function in circadian rhythm generation.

In further work, the spatial expression of *per* was found to be relatively widespread, localized to the nucleus and cytoplasm of several tissues (Siwicki et al. 1988; Saez and Young 1988), and was not limited to the head, which was known to be the site of *per*'s regulation of circadian locomotor behavior (Konopka et al. 1983). Most importantly, PER abundance in photoreceptor cells varied, with lowest levels observed in the middle of the day and highest levels in the middle of the night (Siwicki et al. 1988). This observation prompted an examination of the temporal expression of *per* mRNA. RNA abundance cycled with a circadian rhythm in fly heads, with specifically altered periods in the mutants (Hardin et al. 1990).

TIMELESS AND TEMPORAL DELAYS IN THE CIRCADIAN CLOCK

Because high levels of PER protein were correlated with times of low *per* expression, it was proposed that PER proteins negatively regulate *per* RNA accumulation (Hardin et al. 1990). A basis for oscillating feedback, and

two new classes of molecular cycling (affecting protein stability and subcellular localization), emerged with the discovery of a second-chromosome clock gene called *timeless* (*tim*) (Sehgal et al. 1994; Vosshall et al. 1994). The *tim* null mutation (*tim⁰*) eliminated *per* RNA and protein oscillations in addition to causing arrhythmic eclosion and locomotor activity (Sehgal et al. 1994). Like *per*, *tim* mRNA and protein were found to oscillate in a circadian fashion and these oscillations were abolished in *per⁰* and *tim⁰* mutants (Sehgal et al. 1995). Although the peak of *tim* mRNA accumulation was slightly earlier than that of *per*, PER and TIM proteins accumulated with the same phase, indicating a link between these two genes (Sehgal et al. 1995). Subsequently it was shown that the PER and TIM proteins physically interact in vitro, in yeast and *Drosophila* cells (Gekakis et al. 1995; Saez and Young 1996). Interdependent functions of *per* and *tim* were also indicated by the finding that PER and TIM coexpression was required for the nuclear accumulation of either protein, and that PER stability was dependent on TIM (Vosshall et al. 1994; Price et al. 1995; Hunter-Ensor et al. 1996; Myers et al. 1996). Thus, with the discovery of *tim* and the regulated nuclear accumulation of PER and TIM (see also Curtin et al. 1995), a model was proposed that incorporated the concept of a specific temporal delay to explain how negative feedback could generate oscillations in gene expression. This delay was originally thought to be determined by the slow kinetics of association of PER and TIM in the cytoplasm, such that once dimerized, PER/TIM complexes would enter the nucleus and negatively regulate their own expression (cf. Sehgal et al. 1995).

The isolation of several additional clock genes has helped to refine this mechanism (for review, see Young and Kay 2001; Hardin 2006). For example, some of the more recently identified genes regulate the phosphorylation or dephosphorylation of PER and TIM, affecting PER/TIM subcellular localization and stability (Harms et al. 2003; Bae and Edery 2006).

S2 CELLS AND NUCLEAR TRANSLOCATION OF PER AND TIM

To better understand the regulation of PER/TIM interactions and their nuclear translocation, we began a new phase of work employing cultured *Drosophila* cells (Saez and Young 1996). Subsequently, this cell line (S2; Schneider's line 2), has become an important tool for investigating the function of *Drosophila*'s circadian clock using transcription assays (Darlington et al. 1998), immunoprecipitation assays (Ceriani et al. 1999), degradation assays (Naidoo et al. 1999), and assays of phosphorylation and dephosphorylation (cf. Ko et al. 2002; Sathyanarayanan et al. 2004).

S2 cells were originally derived from dissociated embryos 35 years ago (Schneider 1972) and do not have a functional circadian clock. They express *doubletime* (*dbt*), *casein kinase 2* (*CK2*), and *cycle* (*cyc*) (Nawathean et al. 2005; L. Saez, unpubl.) but lack expression of other known clock genes such as *Clock* (*Clk*) (Darlington et al. 1998), *per*, and *tim* (see Fig. 1) (Saez and Young 1996). Ectopic expression of *Clock* induces the expression of *tim*, and sometimes *per* in individual S2 cell lines (see Fig. 1) (McDonald and Rosbash 2001; Lim et al. 2007).

Initially, we established immunocytochemically in fixed S2 cells that PER and TIM must be coexpressed to achieve efficient nuclear localization of either protein. Nuclear accumulation of both proteins was found to be complete approximately 6 hours after their initial synthesis (Saez and Young 1996). Expression of PER alone led to its accumulation in the cytoplasm with a half-life of approximately 10 hours as determined by pulse-chase experiments (see Fig. 1). TIM

shuttles between the nucleus and cytoplasm with most cells showing cytoplasmic rather than nuclear accumulation (Ashmore et al. 2003; Meyer et al. 2006). Amino acid sequences that mediate PER/TIM interaction and cytoplasmic localization of PER and TIM (CLD; cytoplasmic localization domains), were also identified (Saez and Young 1996). Although these early studies reproduced elements of the temporal delay seen in nuclear translocation in vivo, and the inter-dependence of PER and TIM to achieve the delay, they gave little information about the kinetics of the PER/TIM interaction and did not provide the necessary resolution to establish a mechanism for regulated nuclear entry.

PER AND TIM INTERACTION DOES NOT INDUCE NUCLEAR TRANSLOCATION

To individually trace PER and TIM movements in the cytoplasm and to assess the role of PER/TIM interaction in nuclear translocation, we developed a single-cell, live imaging assay in S2 cells (Meyer et al. 2006). We individually or jointly expressed in a single cell, using a heat shock promoter, PER and TIM tagged at their carboxyl termini with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively.

Initially, we followed PER-CFP for approximately 10 hours, when independently expressed in S2 cells. PER-CFP remained in the cytoplasm for the duration of the assay. Independent expression of TIM-YFP resulted in mainly cytoplasmic accumulation, with about 10% of the cells showing nuclear YFP (Meyer et al. 2006). These results are consistent with our previous observations using fixed S2 cells. We also confirmed that leptomycin-B abolishes nuclear export of TIM in S2 cells (Ashmore et al. 2003). However, our findings with PER differed from certain studies of subcellular localization that coexpressed *Clk* and *per* in S2 cells (Chang and Reppert 2003; Nawathean and Rosbash 2004). As previously discussed (Meyer and Young 2007), it seems likely that endogenous TIM that is produced in response to coexpressed *Clk* would influence PER nuclear accumulation in such studies.

We next measured the onset of nuclear accumulation of PER and TIM when coexpressed in the same cell. Figure 2 shows time-lapse images of PER-CFP and TIM-YFP that were collected over several hours following heat shock induction of both proteins. The images show that PER and TIM were evenly distributed in the cytoplasm for a period of about 4 hours, followed by a gradual shift of both proteins to discrete cytoplasmic foci surrounding the nucleus. The onset of nuclear accumulation of PER and TIM occurred abruptly (e.g., ~340 minutes after heat shock induction for TIM in this cell), with the phase of nuclear accumulation lasting less than 1 hour (Fig. 2) (Meyer et al. 2006). Surprisingly, the onset of nuclear translocation proved to be largely independent of PER and TIM levels in this system (Meyer et al. 2006; Meyer and Young 2007). Even in the presence of leptomycin-B, TIM, which otherwise would accumulate in the nucleus, remained for approximately 5.5 hours in the cytoplasm in PER-TIM-expressing cells, presumably due to a rapid association with PER that retains TIM in the cytoplasm. The treatment with leptomycin-B did not affect the onset

Figure 1. Response of endogenous clock genes in S2 cells to transient expression of *Clock*. (A) S2 cells were transfected with a plasmid containing a heat shock (*HS*) promoter fused to *Clock* cDNA; 48 hours after transfection, cells were heat-shocked for 30 minutes at 37°C, and an equal amount of the culture was collected at the times (hr) indicated. *HS-tim* and *HS-per* are controls from S2 cells expressing heat-shock-induced PER or TIM. Following protein extraction and SDS-PAGE, proteins were transferred to a membrane and probed with anti-PER (top) or anti-TIM antibodies (bottom). (B) Half-life of PER in S2 cells. S2 cells transfected with *HS-per* were incubated in Schneider M3 media (Schneider 1972) without methionine and cysteine for 1 hour. Cells were heat-shocked for 30 minutes at 37°C, allowed to recover for 30 minutes at 25°C, and resuspended in media containing [³⁵S]methionine-cysteine for 30 minutes. Cells were collected by centrifugation and resuspended in complete M3 media (Sigma-Aldrich), and equal amounts of the growing culture were taken every 2 hours thereafter. ³⁵S-labeled PER was immunoprecipitated from each sample and subjected to PAGE. The amount of ³⁵S-labeled PER recovered from each sample was expressed as the percentage remaining from the initial time point. The graph represents the average values obtained from four experiments.

Figure 2. Nuclear accumulation profiles of PER-CFP and TIM-YFP in a single S2 cell. (*Top two panels*) Time-lapse images of a cell expressing PER-CFP and TIM-YFP. Starting at the top left, images were taken every 20 minutes, with the first image collected 180 minutes after heat shock. The last image (*bottom right*) was collected 400 minutes after heat shock. Arrows indicate the cytoplasmic foci whose formation preceded nuclear entry. The graph below these images shows the nuclear accumulation profiles (fluorescence measurements) of PER-CFP (*thick line*) and TIM-YFP (*thin line*). In this example, the onset of nuclear accumulation of PER was advanced compared to TIM by about 25 minutes. (Redrawn, with permission, from Meyer et al. 2006 [© AAAS].)

of nuclear entry in PER + TIM experiments (Meyer et al. 2006). These results indicate that nuclear translocation is not simply initiated by PER/TIM interaction, as corroborated by FRET analysis (see below).

PER AND TIM NUCLEAR ACCUMULATION PROFILES ARE DIFFERENT

Another intriguing observation came from a study of the individual patterns of nuclear accumulation of PER and TIM. A detailed analysis showed that nuclear accumulation profiles can be separated into three categories: In about 30% of the cells, PER and TIM have similar onsets and profiles of nuclear accumulation; in about 40% of the cells, PER and TIM had the same onsets of nuclear entry, but PER was transferred to nuclei more rapidly than TIM; in the remaining (~30%) cells, profiles of PER and TIM nuclear accumulation were similar, but the PER-CFP onset was advanced with respect to TIM (cf. Fig. 2). Thus, in approximately 70% of the cells analyzed, PER accumulated in the nucleus ahead of TIM (Meyer et al. 2006). This condition is similar to that previously observed in lateral neurons (pacemaker cells) of larval and adult brains, in which PER was detected in the nucleus before TIM (Shafer et al. 2002, 2004; Ashmore et al. 2003). These observations indicate that PER and TIM enter the nucleus independently.

FRET ANALYSIS OF PER AND TIM INTERACTION

Tagging PER and TIM with derivatives of GFP allowed us to develop a fluorescence resonant energy transfer (FRET) assay to measure PER/TIM interactions

in a single cell. FRET is an indication of close proximity of two proteins, as intermolecular distances of less than 10 nm are needed for resonant energy transfer from one fluorophore to another (Forster 1948). In our S2 cell experiments, maximum levels of FRET were observed without a measurable delay following PER-CFP and TIM-YFP induction (see Fig. 3), suggesting rapid formation of PER/TIM complexes. Levels of FRET remained constant until the onset of PER and TIM nuclear translocation. A rapid decline in FRET was observed as the proteins moved to the nucleus. The interval over which FRET declined matched that of the independently measured nuclear translocation of PER and TIM in the same cell. Together with the divergent profiles of nuclear accumulation of PER and TIM, the loss of FRET suggests that following a prolonged physical association in the cytoplasm, PER and TIM dissociate and enter the nucleus independently.

PER^L MUTATION DELAYS NUCLEAR ENTRY WITHOUT AFFECTING THE PROFILE OF PER/TIM BINDING

As the molecular behaviors of PER and TIM in S2 cells retained many properties previously observed *in vivo*, we sought a further test of this correlation by examining the behavior of proteins produced by two clock mutants, *per^L* and *tim^{UL}*, that generate long-period behavioral rhythms by different mechanisms. The mutation in *per^L* is a single-amino-acid change (V242A) within the PAS (PER-ARNT-SM) domain (Baylies et al. 1987) that delays

Figure 3. Profiles of FRET and nuclear translocation associated with PER⁺/TIM and PER^L/TIM expression. (*Top and bottom panels*) Whole-cell FRET (*thin lines*) and nuclear accumulation (*thick lines*) profiles of PER-CFP and PER^L-CFP, respectively, in five (*top*) and four (*bottom*) S2 cells expressing TIM-YFP. The decline in FRET levels coincides with the time of nuclear entry at approximately 330 minutes for PER-CFP (PER⁺) and about 500 minutes for PER^L-CFP (PER^L). The bottom panel also shows the level of whole-cell FRET beginning 30 minutes after induction. For each panel, *thick and thin lines* of the same color represent measurements of the same cell. (Redrawn, with permission, from Meyer et al. 2006 [© AAAS].)

nuclear translocation of PER and TIM by 4 hours in pacemaker cells of the brain (Curtin et al. 1995), causing a 28-hour behavioral rhythm (Konopka and Benzer 1971). *tim^{UL}* is a single-amino-acid substitution that delays the turnover of PER and TIM in the nucleus, giving a 33-hour circadian period without affecting the timing of nuclear translocation (Rothenfluh et al. 2000).

The onset of nuclear entry for PER/TIM^{UL} was quite similar to the onset observed for PER/TIM, indicating that *tim^{UL}* does not affect nuclear entry in vivo or in S2 cells (Meyer et al. 2006). The *per^L* mutation, on the other hand, could faithfully reproduce in S2 cells the increased delay observed in vivo, shifting the onset of nuclear accumulation of PER and TIM to about 8.5 hours (Fig. 3) (Meyer et al. 2006). Thus, a single-amino-acid change in PER appears to affect the function of an interval timer that regulates cytoplasmic retention of PER and TIM (Meyer et al. 2006). FRET, in cells expressing the wild-type and the mutant proteins, showed comparable rates of emergence and decay, indicating that association and dissociation kinetics of the PER/TIM complex are not substantially affected in the mutants. Because dissociation of the PER/TIM complex appears to be required for nuclear entry, it is possible that PER^L alters a step needed to trigger dissociation from the PER^L/TIM complex, delaying nuclear entry of both proteins.

DBT, A NUCLEAR PROTEIN, IS RETAINED BY PER IN THE CYTOPLASM AND AFFECTS PER STABILITY

We next focused our attention on DBT, another clock protein that associates with PER and the PER/TIM complex (Kloss et al. 1998, 2001). *dbt* is expressed throughout the circadian cycle. Nevertheless, in lateral neurons and in photoreceptor cells, DBT is nuclear in *per* and *tim* null mutants and shows a circadian subcellular distribution, cycling between the cytoplasm and nucleus in a fashion that parallels the behavior of PER accumulation in wild-type fly heads (Kloss et al. 2001). In vivo, DBT physically interacts with PER but is only found associated with TIM in PER/TIM complexes (Kloss et al. 2001). DBT-dependent phosphorylation destabilizes PER, promoting PER's degradation by the ubiquitin-proteasome degradation pathway (Price et al. 1998; Ko et al. 2002). TIM promotes PER stability, apparently by suppressing PER's phosphorylation by DBT (Kloss et al. 1998; Price et al. 1998; Ko et al. 2002). Thus, DBT-dependent phosphorylation is believed to affect the timing of nuclear entry in part by regulating the stability of PER. In turn, PER influences the subcellular distribution of DBT through formation of DBT/PER and DBT/PER/TIM complexes (Kloss et al. 2001).

To study DBT in cell culture, we tagged it with YFP and induced DBT-YFP expression using a heat shock promoter. Consistent with observations made in vivo with *per⁰* mutants, DBT-YFP in S2 cells localized exclusively to the nucleus when expressed alone (Fig. 4A) or in the presence of TIM-CFP (Fig. 4B, top). Coexpression of DBT-YFP and PER-CFP immediately relocalized DBT from the nucleus to the cytoplasm, presumably due to a

Figure 4. Doubletime and PER interact in S2 cells. (A) Subcellular localization of DBT in S2 cells. DBT-YFP in S2 cells accumulates in the nucleus immediately following induction. (B) DBT interacts with PER and not with TIM. Images show cells expressing TIM-CFP and DBT-YFP (top) and PER-CFP and DBT-YFP (bottom). Approximately 3 hours after induction, TIM-CFP remains in the cytoplasm and DBT localizes to the nucleus. Coexpression of PER and DBT localized both proteins to the cytoplasm. (C) Time-lapse images of a single S2 cell expressing PER-CFP and DBT-YFP. As previously observed in vivo, in the absence of TIM, DBT promotes PER degradation. Images shown were taken every 32 minutes.

physical association with cytoplasmic PER (see Fig. 4B, bottom). Because DBT affects the stability of PER, we collected time series data from these S2 cells over several hours (Fig. 4C). PER and DBT accumulated in the cytoplasm for approximately 180 minutes following their heat shock induction. Thereafter (~30 minutes), a progressive and rapid degradation of PER was observed with a relocalization of DBT to the nucleus. Because the degradation of PER did not occur immediately after its induction, and DBT was initially confined to the cytoplasm with PER, binding and a progressive phosphorylation of PER by DBT may be required for PER degradation. Endogenous DBT, which is found at low levels in S2 cells, did not have a detectable effect on the stability of PER in our experiments: PER, when expressed alone in S2 cells, was stable for at least 10 hours after induction, and *dbt* RNA interference (RNAi) did not alter the pattern of PER stability or the kinetics of nuclear entry in PER/TIM-expressing S2 cells (data not shown). Although progressive phosphorylation and degradation of PER were observed in our studies when PER was constitutively expressed and newly formed DBT was added through an inducible promoter, it was also shown by other investigators that coexpression of PER and DBT by a constitutive promoter can give constant low levels of PER that is highly phosphorylated (Ko et al. 2002; Ko and Edery 2005). These observations indicate that DBT affects PER stability and that patterns of DBT localization can be influenced by PER in S2 cells as previously observed in vivo.

PER AND DBT ENTER THE NUCLEUS IN TIMELESS-EXPRESSING S2 CELLS

To see whether TIM will protect PER from DBT-dependent degradation, we coexpressed PER-CFP and DBT-YFP in TIM-expressing cells. Figure 5 shows time-

Figure 5. Coordinated nuclear accumulation of PER, TIM, and DBT. S2 cells expressing TIM from a heat shock promoter were cotransfected with *HS-per-cfp* and *HS-dbt-yfp*; 48 hours after transfection, cells were heat-shocked at 37°C for 30 minutes and imaged for 464 minutes. Time-lapse images of a single cell expressing PER-CFP and DBT-YFP show that both proteins enter the nucleus with an onset similar to that previously seen for PER and TIM. Arrowheads indicate the formation of cytoplasmic PER/DBT foci before nuclear accumulation.

lapse images of PER-CFP and DBT-YFP accumulation in these cells. Destabilization of PER by DBT was suppressed by TIM expression, because PER was observed to be stable in these cells for more than 8 hours, a period sufficient for complete DBT-dependent degradation of PER in the absence of TIM. As in the case of PER and TIM coexpression, DBT and PER progressively accumulated in the cytoplasm, forming discrete foci that presumably contain PER-CFP, DBT-YFP, and TIM (see small arrows in Fig. 5). The kinetics of nuclear accumulation of PER and DBT in these TIM-expressing cells has not been studied in detail but appears to be similar to that of PER and TIM, occurring at about 5–6 hours after induction. These results indicate that S2 cells recapitulate many of the *in vivo* behaviors of PER, TIM, and DBT. A summary of PER/TIM/DBT interactions and functions is indicated in the model in Figure 6. Further work will be required to determine patterns of PER degradation, whether PER and DBT independently enter nuclei, and profiles of FRET in cells coexpressing the three proteins.

THE INTERVAL TIMER

Although PER and TIM binding is not sufficient to trigger nuclear entry, physical association appears to be a prerequisite for their timed nuclear accumulation. The formation of the PER/TIM complex could, perhaps, allow subsequent posttranslational modifications or the binding of factors that determine the specific duration of its cytoplasmic phase.

The formation of cytoplasmic foci is especially intriguing as these are not observed when PER, or TIM, or DBT is expressed alone. The formation of these foci always precedes the onset of nuclear accumulation and they disappear as nuclear accumulation proceeds. The foci represent the final cytoplasmic phase of PER/TIM/DBT accumulation. Their contents may be further explored by a program of protein tagging that includes the remaining

Figure 6. Model for regulated nuclear accumulation of PER, DBT, and TIM in S2 cells. Wild-type PER and TIM proteins associate with little or no delay and thereafter remain in the cytoplasm for about 5.5 hours. During this time, PER and TIM progressively associate in distinct foci, which are lost as PER and TIM dissociate and move to the nucleus. In the nucleus, PER, DBT, and possibly TIM, associate with Clock/Cycle complexes, inhibiting transcription of *Clock/cycle*-dependent genes including *per* and *tim* (see also Kim et al. 2007; Nawatheat et al. 2007). The duration of the PER/TIM complex in the cytoplasm is affected by *PER^L* which delays the onset of nuclear accumulation by about 3 hours. A similar delay is observed *in vivo* (Curtin et al. 1995) and is thought to generate the abnormal (28 hours) period length of *per^L* flies. In addition to these interactions, DBT association leads to phosphorylation and destabilization of PER in the absence of TIM. Our studies have not determined whether DBT and PER move to the nucleus independently or as a complex.

clock proteins, as well as proteins known to be involved in either cytosolic trafficking or transport to the nucleus.

CONCLUSIONS

We have shown that a simple nonoscillating cell culture system can be used to study the molecular features of a key step in the circadian clockworks. This single-cell assay not only recapitulates many of the *in vivo* behaviors of PER and TIM and their mutants, but it has also provided a revised model of the *Drosophila* circadian clock.

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REFERENCES

- Ashmore L.J., Sathyanarayanan S., Silvestre D.W., Emerson M.M., Schotland P., and Sehgal A. 2003. Novel insights into the regulation of the timeless protein. *J. Neurosci.* **23**: 7810.
- Bae K. and Edery I. 2006. Regulating a circadian clock's period, phase and amplitude by phosphorylation: Insights from *Drosophila*. *J. Biochem.* **140**: 609.
- Bargiello T.A. and Young M.W. 1984. Molecular genetics of a biological clock in *Drosophila*. *Proc. Natl. Acad. Sci.* **81**: 2142.

- Baylies M.K., Bargiello T.A., Jackson F.R., and Young M.W. 1987. Changes in abundance or structure of the per gene product can alter periodicity of the *Drosophila* clock. *Nature* **326**: 390.
- Ceriani M.F., Darlington T.K., Staknis D., Mas P., Petti A.A., Weitz C.J., and Kay S.A. 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* **285**: 506.
- Chang D.C. and Reppert S.M. 2003. A novel C-terminal domain of *Drosophila* PERIOD inhibits dCLOCK:CYCLE-mediated transcription. *Curr. Biol.* **13**: 758.
- Citri Y., Colot H.V., Jacquier A.C., Yu Q., Hall J.C., Baltimore D., and Rosbash M.A. 1987. A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. *Nature* **326**: 42.
- Curtin K.D., Huang Z.J., and Rosbash M. 1995. Temporally regulated nuclear entry of the *Drosophila* period protein contributes to the circadian clock. *Neuron* **14**: 365.
- Darlington T.K., Wager-Smith K., Ceriani M.F., Staknis D., Gekakis N., Steeves T.D., Weitz C.J., Takahashi J.S., and Kay S.A. 1998. Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* **280**: 1599.
- Forster T. 1948. Intermolecular energy migration and fluorescence. *Ann. Physics* **2**: 55.
- Gekakis N., Saez L., Delahaye-Brown A.M., Myers M.P., Sehgal A., Young M.W., and Weitz C.J. 1995. Isolation of timeless by PER protein interaction: Defective interaction between timeless protein and long-period mutant PERL. *Science* **270**: 732.
- Hardin P.E. 2006. Essential and expendable features of the circadian timekeeping mechanism. *Curr. Opin. Neurobiol.* **16**: 686.
- 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.
- Harms E., Young M.W., and Saez L. 2003. CK1 and GSK3 in the *Drosophila* and mammalian circadian clock. *Novartis Found. Symp.* **253**: 267.
- Hunter-Ensor M., Ousley A., and Sehgal A. 1996. Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell* **84**: 677.
- Jackson F.R., Bargiello T.A., Yun S.H., and Young M.W. 1986. Product of per locus of *Drosophila* shares homology with proteoglycans. *Nature* **320**: 185.
- Kim E.Y., Ko H.W., Yu W., Hardin P.E., and Ederly I. 2007. A DOUBLETIME kinase binding domain on the *Drosophila* PERIOD protein is essential for its hyperphosphorylation, transcriptional repression, and circadian clock function. *Mol. Cell. Biol.* **27**: 5014.
- Kloss B., Rothenfluh A., Young M.W., and Saez L. 2001. Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the *Drosophila* clock. *Neuron* **30**: 699.
- 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 Iepsilon. *Cell* **94**: 97.
- Ko H.W. and Ederly I. 2005. Analyzing the degradation of PERIOD protein by the ubiquitin-proteasome pathway in cultured *Drosophila* cells. *Methods Enzymol.* **393**: 394.
- Ko H.W., Jiang J., and Ederly I. 2002. Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature* **420**: 673.
- Konopka R.J. and Benzer S. 1971. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* **68**: 2112.
- Konopka R., Wells S., and Lee T. 1983. Mosaic analysis of a *Drosophila* clock mutant. *Mol. Gen. Genet.* **190**: 284.
- Lim C., Lee J., Choi C., Doh E., and Choe J. 2007. Functional role of CREB-binding protein in the circadian clock system of *Drosophila*. *Mol. Cell. Biol.* **27**: 4876.
- McDonald M.J. and Rosbash M. 2001. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107**: 567.
- Meyer P. and Young M.W. 2007. The 2006 Pittendrigh/Aschoff Lecture: New roles for old proteins in the *Drosophila* circadian clock. *J. Biol. Rhythms* **22**: 283.
- Meyer P., Saez L., and Young M.W. 2006. PER-TIM interactions in living *Drosophila* cells: An interval timer for the circadian clock. *Science* **311**: 226.
- Myers M.P., Wager-Smith K., Rothenfluh-Hilfiker A., and Young M.W. 1996. Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* **271**: 1736.
- Naidoo N., Song W., Hunter-Ensor M., and Sehgal A. 1999. A role for the proteasome in the light response of the timeless clock protein. *Science* **285**: 1737.
- Nawathean P. and Rosbash M. 2004. The doubletime and CKII kinases collaborate to potentiate *Drosophila* PER transcriptional repressor activity. *Mol. Cell* **13**: 213.
- Nawathean P., Menet J.S., and Rosbash M. 2005. Assaying the *Drosophila* negative feedback loop with RNA interference in S2 cells. *Methods Enzymol.* **393**: 610.
- Nawathean P., Stoleru D., and Rosbash M. 2007. A small conserved domain of *Drosophila* PERIOD is important for circadian phosphorylation, nuclear localization, and transcriptional repressor activity. *Mol. Cell. Biol.* **27**: 5002.
- Price J.L., Dembinska M.E., Young M.W., and Rosbash M. 1995. Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation timeless. *EMBO J.* **14**: 4044.
- Price J.L., Blau J., Rothenfluh A., Abodeely M., Kloss B., and Young M.W. 1998. double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**: 83.
- 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.
- Rothenfluh A., Saez L., and Young M. W. 2000. A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron* **26**: 505.
- Saez L. and Young M.W. 1988. In situ localization of the per clock protein during development of *Drosophila melanogaster*. *Mol. Cell. Biol.* **8**: 5378.
- . 1996. Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. *Neuron* **17**: 911.
- Sathyanarayanan S., Zheng X., Xiao R., and Sehgal A. 2004. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell* **116**: 603.
- Schneider I. 1972. Cell lines derived from late embryo stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **27**: 353.
- Sehgal A., Price J.L., Man B., and Young M.W. 1994. Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science* **263**: 1603.
- Sehgal A., Rothenfluh-Hilfiker A., Hunter-Ensor M., Chen Y., Myers M.P., and Young M.W. 1995. Rhythmic expression of timeless: A basis for promoting circadian cycles in period gene autoregulation. *Science* **270**: 808.
- Shafer O.T., Rosbash M., and Truman J.W. 2002. Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster*. *J. Neurosci.* **22**: 5946.
- Shafer O.T., Levine J.D., Truman J.W., and Hall J.C. 2004. Flies by night: Effects of changing day length on *Drosophila*'s circadian clock. *Curr. Biol.* **14**: 424.
- Siwicki K.K., Eastman C., Petersen G., Rosbash M., and Hall J.C. 1988. Antibodies to the period gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**: 141.
- Vosshall L.B., Price J.L., Sehgal A., Saez L., and Young M.W. 1994. Block in nuclear localization of period protein by a second clock mutation, timeless. *Science* **263**: 1606.
- Young M.W. and Kay S.A. 2001. Time zones: A comparative genetics of circadian clocks. *Nat. Rev. Genet.* **2**: 702.

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