the delay observed between *per* and *tim* RNA synthesis on the one hand, and nuclear accumulation of their encoded proteins on the other: Although *per* and *tim* RNAs begin to rise at midday, TIM's light sensitivity evidently precludes substantial accumulation of TIM protein until nightfall. Circadian pacemakers usually show species-specific, intrinsic periodicities that differ from 24 hours, whereas behavioral rhythms uniformly occur with a 24-hour period in the presence of a solar day. TIM's light sensitivity suggests a mechanism for adjusting to the period of the environmental cycle.

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20. Fly head extracts were prepared as described (*12*). TIM-specific antibodies were raised in rats against affinity-purified glutathione-S-transferase fusion proteins expressing either residues 222 to 577 [antibodies (Ab) 305 and 307] or 1133 to 1389 (Ab 310) of TIM. For protein immunoblots, Ab 307 was used, and for cytology Ab 305 was used. The antibodies were prepared by HRP (Denver, PA). All blots were visualized by enhanced chemiluminescence (ECL, Amersham).
21. per^0 flies were maintained in constant darkness for 4 days. At time zero, a set of flies was pulsed with light (~8000 lux) for 1 hour, then returned to constant darkness. Control flies and light-pulsed flies were harvested at 0, 1, 2, 3, 5, 7, and 9 hours from the start of pulse. One group of flies was harvested immediately after 15 min of light exposure. Head extracts and protein immunoblots were performed as in Fig. 1A. per^+ flies were maintained for 4 days in LD12:12 and subdivided for use as controls (constant dark) or in 1-hour light-pulse experiments as for per^0. All molecular phase-resetting experiments were done at least four times (twice immunocytochemically and twice by protein immunoblotting) with similar results.
22. *cn bw* flies were entrained to an LD cycle for at least 3 days and then transferred to constant darkness. Ten-minute pulses of light (~8000 lux) were administered at the indicated times. For each time point, the average phase of the locomotor activity rhythms of 16 pulsed flies was compared to that of 16 untreated control flies. Activity rhythms were assessed continuously for several days in nonpulsed controls and after phase-resetting light pulses as described (*4*, *14*). The new phase of the activity rhythm was usually evident in the record within 24 hours of light-pulse administration. Standard errors of the mean were derived from at least three independent experiments for each time point.

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Resetting the *Drosophila* Clock by Photic Regulation of PER and a PER-TIM Complex

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Circadian clocks can be reset by light stimulation. To investigate the mechanism of this phase shifting, the effects of light pulses on the protein and messenger RNA products of the *Drosophila* clock gene *period* (*per*) were measured. Photic stimuli perturbed the timing of the PER protein and messenger RNA cycles in a manner consistent with the direction and magnitude of the phase shift. In addition, the recently identified clock protein TIM (for *timeless*) interacted with PER in vivo, and this association was rapidly decreased by light. This disruption of the PER-TIM complex in the cytoplasm was accompanied by a delay in PER phosphorylation and nuclear entry and disruption in the nucleus by an advance in PER phosphorylation and disappearance. These results suggest a mechanism for how a unidirectional environmental signal elicits a bidirectional clock response.

Circadian rhythms in biochemical, physiological, and behavioral phenomena persist in the absence of environmental cues and are governed by one, or a few, endogenous circadian oscillators or "clocks" (*1*). However, external time cues (zeitgebers), most notably light-dark cycles, can synchronize or entrain these rhythms by shifting their phases. This adaptive feature of biological clocks allows for the precise temporal coordination of the function of the organism with environmental conditions. The direction and magnitude of the phase shift is a function of the time in a daily cycle that the zeitgeber is administered: An environmental cue will elicit either a phase delay or a phase advance depending on the time of day that the stimulus is administered. Numerous studies in different model systems have shown that protein and mRNA synthesis are required for circadian clocks and resetting mechanisms (*2*), and candidate molecules and photic input pathways have been identified in animals (*3*) and plants (*4*). Nevertheless, how such clock mechanisms are perturbed by environmental regulators of circadian rhythms is not clear.

The PER protein from the *Drosophila melanogaster period* (*per*) gene (*5*) is a key clock component. In the absence of *per* activity (per^{01} nonsense mutation), there is no observable rhythmicity of eclosion or of locomotor activity (*5*). Moreover, missense mutations shorten (per^s) to 19 hours or lengthen (per^L) to 29 hours the free-running periods of both rhythms in the wild type (~24 hours) (*5*). PER is temporally regulated in the adult fly head, the anatomical location of the fruitfly circadian pacemaker (*6*): Both its abundance (*7–9*) and phosphorylation (*7*, *8*) fluctuate daily, and PER nuclear entry is temporally gated (*10*). Moreover, *per* mRNA levels oscillate by means of a feedback loop, likely negative (*8*), whereby PER activity is required for the circadian regulation of *per* transcription (*11*).

A second clock gene, *timeless* (*tim*), is also required for circadian rhythmicity in *Drosophila* (*12*). In the tim^{01} mutant (a presumptive null allele) (*13*), loss of behavioral circadian rhythms is accompanied by a loss of daily fluctuations of *per* mRNA (*12*) and a failure of several reporter PER fusion proteins to accumulate in the nucleus (*14*). Furthermore, circadian fluctuations in the

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abundance and phosphorylation of PER are suppressed in tim^{01} flies (*15*). The amount of *tim* mRNA cycles with a phase and amplitude indistinguishable from those for *per* (*16*). Daily oscillations in *tim* transcripts depend on the presence of both PER and TIM, which suggests that a shared mechanism participates in the autoregulation of *per* and *tim* (*16*). The *tim* protein (TIM) may contribute to cyclic expression of *per* and *tim* (and possibly downstream output genes) by regulating the timing of PER nuclear entry (*14*, *16*, *17*).

One prediction suggests that cycles of *per* that are integral components of the timekeeping mechanism should be perturbed in a relatively rapid manner by environmental signals that shift the phase of the clock. To determine the effects of photic stimuli on PER phosphorylation and abundance, we simultaneously entrained two identical sets of wild-type Canton-S (CS) flies with four cycles of 12 hours of light and 12 hours of dark [LD 12:12; here, zeitgeber time 12 (ZT12) is lights off and ZT24 is lights on] and subsequently maintained them under constant dark conditions (DD). Light pulses of 5 to 60 min in duration (*18*) were administered at either ZT15 or ZT21.5, because these times in a daily cycle yield the largest phase delays (approximately 3.5 to 4 hours) and phase advances (approximately 2.5 to 3 hours) in locomotor activity rhythms, respectively (*19*, *20*). Untreated control and light-pulsed flies were collected at various times before and after the environmental perturbation, and head extracts were probed for PER by immunoblotting (Fig. 1) (*21*).

Not only did the abundance of PER fluctuate (*7*–*9*) (Fig. 1A), reaching peak levels at time 20 to 22 (T20-22, the number of hours since the last dark-to-light transition at ZT0), but the mobility of PER in SDS-polyacrylamide gels oscillated on a daily basis (*7*, *8*). There was an increase in the apparent molecular weight of the largest (slowest migrating) PER isoforms between T15 and T22-24, and the smaller PER species disappeared beginning at T20-22 (Fig. 1, A and B) (compare the distance between the slowest and fastest migrating PER species and the internal size standard). These variations in the apparent molecular weight of PER are a result of daily changes in its phosphorylated state (*7*).

A light pulse at ZT15 elicited delays in both the phosphorylation of PER and its disappearance (Fig. 1, A and B) (compare the mobility of PER at T22 and T24 and its abundance at T30). Migration of the largest PER species in ZT15-treated flies is most similar to that measured in control flies 3 to 4 hours earlier (Fig. 1B). These light-induced delays in PER phosphorylation and disappearance are consistent with the direction and magnitude of the phase shift in behavior in flies pulsed with light at ZT15 (*19*, *20*) (Fig. 1C). The earliest detectable changes in the mobility of PER were at T17 (*22*). Furthermore, the delay in the PER biochemical cycles was maintained in the second day of DD after a light pulse at ZT15 (Fig. 1D) which demonstrates that light pulses evoke a stable shift in the temporal regulation of PER.

In contrast, light treatment at ZT21.5 caused the disappearance of PER approximately 2 to 4 hours earlier (Fig. 1E). PER was essentially undetectable in the light-pulsed and control flies at T26-28 and T30, respectively. Although in untreated flies PER does not undergo large increases in electrophoretic mobility after T22 (Fig. 1, B, F, and G), the average mobility of PER is greater in the light-pulsed flies than in control flies collected at the same times (Fig. 1, F and G). These changes in PER abundance and phosphorylation correlate with the ability of photic stimuli administered at ZT21.5 to cause phase advances (*19*, *20*). The first detectable changes in the mobility of PER occurred between 30 to 60 min after the beginning of the light pulse (Fig. 1, F and G) (*22*). This is almost certainly a maximum estimate of the time required for light to elicit changes in PER phosphorylation, because detectable differences in the electrophoretic mobility of a protein the size of PER (155 to 185 kD) (*7*) probably require multiple phosphorylation events (*22*). No significant changes in the PER biochemical cycles were observed in flies pulsed at T30 (Fig. 1H), a phase in the circadian cycle that does not elicit behavioral phase shifts in *Drosophila* (*19*, *20*).

To determine whether PER nuclear entry could also be modulated by photic signals, we collected control and ZT15 light-pulsed flies and probed frozen fly head sections with antibodies to PER (Fig. 2) (*23*). PER staining in the adult fly head was mainly observed in the nuclei of the photoreceptor cells of the compound eye and in pacemaker cells termed lateral neurons (LNs) (Fig. 2A) (*9*, *10*). As in the LNs of wild-type flies (*10*), cytoplasmic PER was first detected at ZT15-16 with no visible nuclear staining (Fig. 2A) (cytoplasmic PER is indicated by a relatively large staining area that lacks signal in the central portion of the LN) (*10*); within a relatively short time window (ZT18-19), PER entered the nucleus and remained there for several hours, as indicated by its smaller and more condensed staining pattern (Fig. 2A) (*10*).

The nuclear accumulation of PER in the

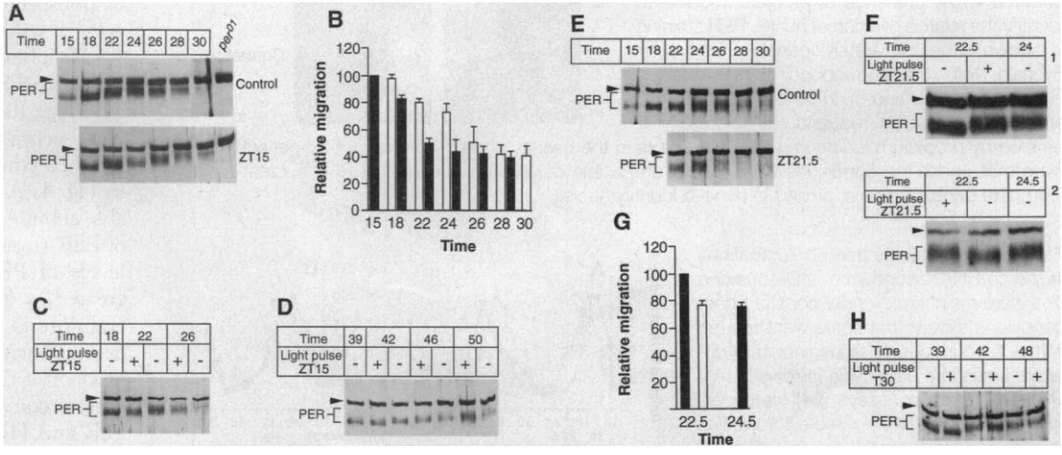

Fig. 1. Light pulses shift cycles in PER phosphorylation and disappearance. During the last dark period of LD, a group of flies was exposed to a 1-hour light pulse beginning at (i) ZT15 (**A** through **D**), (ii) ZT21.5 (**E** through **G**), and (iii) T30 (**H**); a second group served as controls. The hours since the last dark:light transition at ZT0 are shown at the top of the panels. Panels (A) and (E) show a comparison of PER amounts in untreated control flies (top) and treated flies (bottom). The arrowheads show the cross-reacting size standard; this band also reacts with preimmune sera (*22*). The lane containing extract from ZT15-pulsed flies and collected at T24 is underloaded. For each independent experiment (B and G), the distance between the largest detectable PER isoform and the middle of the size standard for untreated flies collected at either T15 (B) or T22.5 (G) was set to 100. The relative migration in (B) and (G) is indicated as a function of zeitgeber time (T) for control (closed bars) and light-pulsed (open bars) flies. The error bars show the standard deviation; $n = 3$ to 6. In (C), (D), (F), and (H), extracts from untreated (−) or light-pulsed (+) flies were analyzed side by side. In (D), flies were collected on the second day after a light pulse at ZT15. In (F), two independent experiments are shown: experiment 1 consisted of a 1-hour light pulse; experiment 2, a 30-min light pulse. Each experiment was done at least five independent times (*22*), and representative examples are shown.

LNs of flies pulsed at ZT15 is significantly delayed (Fig. 2A). In control flies, PER is found predominantly in the nucleus from T20 on; in the pulsed flies, it was present only in the cytoplasm at T20 and at T21. Nevertheless, the mechanism responsible for regulating the subcellular distribution of PER is fully functional in flies treated with light at ZT15, because robust nuclear PER staining was detected at later time points (for example, T24). A light-induced delay in the timing of PER nuclear entry is also shown in Fig. 2B. Thus, the nuclear entry time of PER in light-pulsed flies at ZT15 is delayed by 3 to 4 hours (Fig. 2) (*22*), as is the daily change in PER phosphorylation and disappearance (Fig. 1).

The observation that light perturbs PER protein cycles (Figs. 1 and 2) suggests that photic stimuli should eventually lead to changes in *per* mRNA levels by means of the transcriptional feedback loop (*11*). Instead or in addition, light may directly modulate *per* expression independent of changes in the temporal regulation of PER and feedback regulation. To determine *per* transcript levels, we performed ribonuclease protection assays (*24*) on RNA samples from control flies (Fig. 3A) and from flies light pulsed at either ZT15 or ZT21.5. Approximately 2 to 3 hours after a light pulse at ZT15, there was a clear decrease in the rate at which *per* mRNA levels declined (T17 to T24), which is consistent with the notion that PER acts as a negative repressor of *per* transcription (*8*, *25*) and that its nuclear entry time is delayed under these conditions (Fig. 2). In addition, both the accumulation and peak abundance of *per* mRNA were delayed. A smaller but reproducible decrease in the levels of *per* mRNA compared to those in the control occurred shortly after the start of a light pulse at ZT15 (Fig. 3A). In six independent experiments, the abundance of *per* between T16 and T17.5 was 10 to 30% lower than that in untreated controls (*22*). This observation suggests that in addition to light-induced changes in the ability of PER to autoregulate its own transcript levels, photic stimuli may directly cause a small, rapid decrease in the abundance of *per* mRNA.

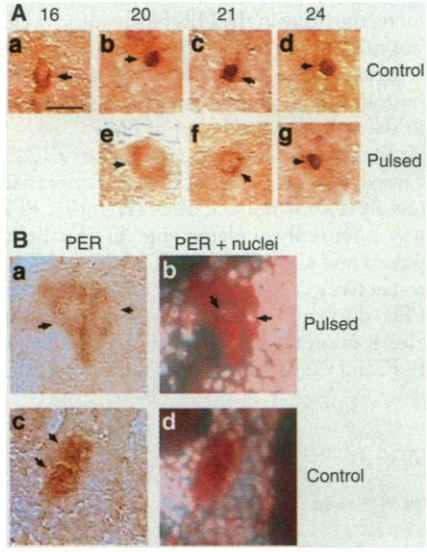

Fig. 2. Light pulses at ZT15 delay the nuclear entry of PER in key pacemaker cells. During the last dark period (day 4), one set of entrained flies received a 1-hour light pulse beginning at ZT15, followed by continued darkness. (**A**) At the indicated times (hours since the last dark:light transition at ZT0, top), heads from control (panels a through d) and light-pulsed (panels e through g) flies were isolated, and frozen head sections were incubated in the presence of antibodies to PER (*23*). Representative examples are shown of the PER staining pattern in LNs (arrows). Five independent experiments showed that in ZT15 light-pulsed flies, the timing of PER nuclear entry in the LNs is delayed (*22*). The bar in panel a in (A) represents 10 μm. (**B**) Similar to (A) except that frozen head sections from flies collected at T20 were also incubated in the presence of propidium iodide to identify the relative position of nuclei. PER staining is shown either separately (panels a and c) or overlaid with the corresponding fluorescence staining (panels b and d). In panels b and d, PER staining is shown in red and the fluorescence emitted by propidium iodide in white. The colors in the overlay panels were computer-generated (*23*). Note that under the conditions used, nuclei are visible only in the absence of PER nuclear staining (compare panels b and d; arrows in panel b identify nuclei).

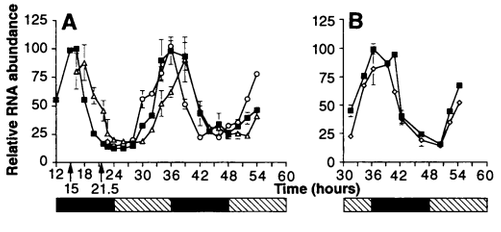

Fig. 3. Photic signals perturb fluctuations in *per* mRNA abundance. LD-entrained flies were maintained under constant dark conditions except that some were treated with a 1-hour light pulse beginning at different times in a daily cycle [arrows in (A)]. RNase protection assays (*24*) were performed on head RNA collected from flies frozen at the indicated times (hours in DD after the last dark:light transition at ZT0). Relative RNA abundance refers to *per*/RP49 values (*24*). (**A**) Untreated control flies (closed squares), ZT15 (open triangles), and ZT21.5 (open circles) light-pulsed flies. The control peak value at T16 was set to 100. (**B**) Untreated control (closed squares) and T30-pulsed (open diamonds) flies. The control peak at T36 was set to 100. Error bars indicate the standard deviation; $n = 2$ to 6. Hatched bars, subjective day; solid bars, subjective night.

In flies pulsed with light at ZT21.5, the phase of *per* mRNA cycling was advanced (Fig. 3A, open circles). The earliest detectable differences in *per* mRNA levels occurred 6 hours after the photic stimuli. Light pulses at ZT21.5 did not cause any rapid changes in the abundance of *per* transcripts (the smallest amounts of *per* mRNA were at least five- to tenfold greater than the lower limit of detection) (*22*). These observations indicate that during phase advances light-induced perturbations in *per* mRNA amounts occur downstream of changes in PER phosphorylation and abundance (Fig. 1, E through G) and are mediated by autoregulation. No differences were observed between the *per* mRNA cycling profiles in untreated and T30-pulsed flies (Fig. 3B), which is consistent with observations that light pulses at T30 neither elicit behavioral phase shifts (*19*, *20*) nor modulate PER biochemistry (Fig. 1H).

TIM and PER associate in vitro (*17*). To determine whether this interaction occurs in vivo and whether light regulates the interaction, we performed immunoprecipitation experiments (Fig. 4) (*26*) with the use of transgenic flies (*per* HA/C) whose only functional copy of PER is modified with a hemagglutinin (HA) tag at its COOH-terminus (PER-HA) (*7*). These PER-HA–producing flies manifest behavioral rhythms (*27*) and cycles in PER protein (*7*) and mRNA (*22*) similar to those of wild-type flies. PER-HA and any associated factors were recovered with an antibody against the HA epitope, and immune complexes were probed with polyclonal antibodies. Antibodies to TIM (*28*) revealed an immunoreactive band with an apparent molecular weight of ~190 kD in PER-HA–containing immune complexes (Fig. 4B), but this band was not seen in the presence of nonspecific antibodies nor in the presence of wild-type (WT) extracts (lane 6).

We obtained similar results with antibodies to PER and extracts prepared from wild-type flies (Fig. 4C, lanes 1 to 3). The band seen with the antibody to TIM peaked at ZT20 (similar to PER) (compare lanes 2 in Fig. 4, A and B) and became undetectable about 4 hours before the disappearance of PER (from ZT20 to ZT23.9, the relative levels of PER and the 190-kD band decreased by 60 and 88%, respectively). Several criteria indicate that the 190-kD band detected with the antibody to TIM is the product of the *tim* gene (*28*). We conclude that a complex or complexes containing PER and TIM are present in vivo for much of the daily cycle (ZT15 to ZT22) but that TIM does not associate with PER during the rapid decline in PER amounts (ZT02 to ZT06) (Fig. 4, A through D).

To determine the effect of light on the PER-TIM interaction, we pulsed flies at

either ZT15 or ZT21.5 (Fig. 4D). In both cases, the amount of TIM coprecipitating with PER was rapidly reduced compared to that in untreated controls. After a 15-min exposure to light, the abundance of TIM in immune complexes was reduced to approximately one-third to one-sixth of its original amount in three independent experiments. The light-induced decrease in the amount of TIM coprecipitating with PER was observed at all times of the day (*22*). Because the abundance of TIM is rapidly reduced at all times of the day from one-third to one-fifth of its original amount after a light pulse of 15 min (*28*), it is likely that the light-induced reduction in the amount of TIM associating with PER is a direct consequence of decreases in the abundance of TIM.

Light pulses perturb the molecular cycles in PER protein and mRNA in a manner that is consistent with both the magnitude and direction of the phase shifts elicited in clock-controlled overt rhythms. Light-induced perturbations in the temporal phosphorylation of PER were the earliest changes we observed that correlate with the magnitude and direction of the phase shift, which suggests that the phosphorylated state of PER contributes to specification of the clock's time coordinates (Fig. 5). However, it is likely that the phosphorylated state of PER is not the initial target of light because the abundance of TIM is rapidly decreased by photic stimuli (*28*). Although we cannot rule out the possibility that light regulates PER and TIM independently, our demonstration that the two clock proteins interact in vivo for much of the daily cycle (Fig. 4) and that this association is rapidly disrupted by light (Fig. 4D) raises the possibility that the PER-TIM partnership ensures coordinate regulation of both clock components.

The observation that phase delays lead to an inhibition in the temporal increase in PER phosphorylation, although the converse is true during phase advances, suggests that the phosphorylated state of PER can be regulated differently by the light-induced disruption of the PER-TIM complex, depending on whether PER is either mainly in the cytoplasm (ZT10 to ZT18) or nucleus (ZT19 to ZT04) (Fig. 5). Indeed, the inflection point between delays and advances occurs in the transition period when PER is translocating to the nucleus (ZT18 to ZT19) (*10*). We propose that in *Drosophila* disruption of the cytoplasmic PER-TIM complex delays PER phosphorylation and nuclear entry (thus contributing to phase delays in the early night), whereas disruption of the nuclear PER-TIM complex advances PER phosphorylation and its degradation (thus contributing to phase advances in the late night) (Fig. 5).

Consistent with this model is the observation that nuclear accumulation of PER likely requires TIM (*14*). Thus, a reduction in the amount of the cytoplasmic PER-TIM complex might cause delayed nuclear entry of PER during light-induced phase delays (Fig. 2). More speculatively, the advanced degradation of PER in ZT21.5-pulsed flies may be caused by the light-induced decrease in the abundance of the nuclear PER-TIM complex, resulting in PER becoming hyperphosphorylated earlier, targeting it for rapid proteolysis (*7*). This possibility is supported by the demonstration that TIM does not associate with PER during times in a daily cycle when PER is highly phosphorylated and its abundance begins to decline rapidly (ZT02 to ZT06).

The effects of light pulses on the timing of PER disappearance and nuclear entry are consistent with photic signals regulating *per* mRNA cycling by means of an effect on the ability of PER to directly or indirectly inhibit its own transcription (*8*, *11*, *25*). Furthermore, no changes in *per* mRNA cycles were observed in flies light pulsed at T30 (Fig. 3B), a time in the daily cycle when the abundance of PER is essentially undetectable. These results suggest that light-induced changes in *per* transcription require PER and hence are dependent on autoregulation. The observation that the PER-TIM complex is stable for several hours after PER enters the nucleus (Fig. 4) suggests that in addition to its proposed role in regulating the nuclear entry of PER (*14*), TIM might directly modulate the ability of PER to influence transcription. How the PER-TIM complex regulates transcription is not clear, as neither factor appears to have DNA-binding domains. Although the transcriptional feedback loop may help mediate the light-induced perturbations in *per* mRNA cycling, our results also suggest that, at least during phase delays, photic stimuli might directly cause modest and rapid decreases in

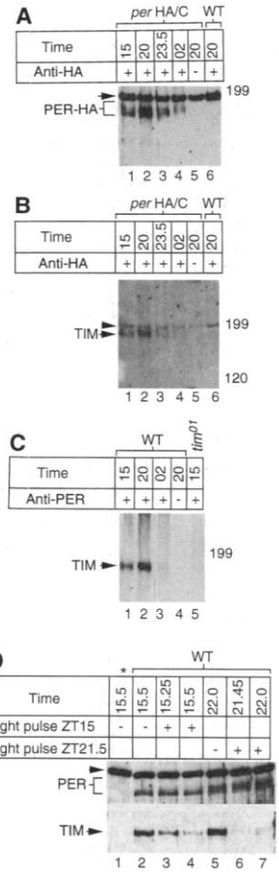

Fig. 4. TIM and PER stably interact in vivo, and their association is rapidly disrupted by light. Flies (genotypes as indicated, top) were collected at the indicated times (ZT) and head extracts incubated in the presence (+) or absence (−) of either antibody to HA (anti-HA) (**A** and **B**) or antibody to PER (anti-PER) (**C**) (*26*). (**D**) Flies were either untreated (−) or treated with a 30-min light pulse (+) beginning at either ZT15 or ZT21.5, as indicated, and head extracts incubated in the presence of antibody to PER. Recovered immune complexes were immunoblotted with either anti-PER [(A) and (D), top] or anti-TIM [(B) through (D), bottom]. In (D), immune complexes that were probed with antibody to PER were mixed with extracts prepared from per^{01} flies just before loading to detect the nonspecific internal size standard (arrowhead, left). Lane 1 shows the staining profile obtained with either per^{01} (top) or tim^{01} (bottom) flies. *per* HA/C, PER-HA producing flies; WT, wild-type flies. Size markers are shown on the right in kilodaltons.

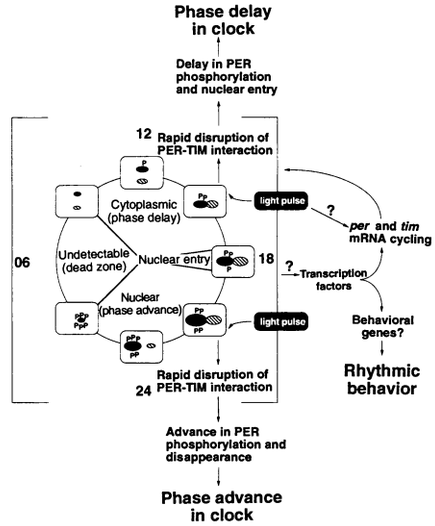

Fig. 5. How the *Drosophila* circadian timekeeping system might be reset by photic signals. PER (closed ovals) and TIM (hatched ovals) are in a 24-hour circadian clock (entrained by cycles of 12 hours of light and 12 hours of dark, where "lights off" begins at 12 and "lights on" begins at 24). The phosphorylated state of PER is indicated by "P"; the abundance of PER and TIM is indicated by the size of the oval.

the abundance of *per* transcripts.

Light pulses rapidly induce expression of the *Neurospora* clock gene *frequency* (*frq*) (*29*). Although *frq* protein was not assayed, these results suggest that the regulation of *frq* transcription is the initial clock component modulated by photic stimuli. However, in *Drosophila* the initial clock-specific photoresponsive event is likely to be the degradation of TIM (*28*) and the disruption of the PER-TIM complex. Indeed, circadian fluctuations in both the abundance of PER and behavior can be generated from a presumably noncycling *per* transcript (*30*). Together, these observations suggest that post-translational autoregulatory loops (in addition to the possible contribution of the *per* and *tim* transcriptional feedback loop) (*11*, *16*) might participate in generating the PER and TIM biochemical oscillations.

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18. Similar results were obtained for flies light pulsed (approximately 1000 lux) from 5 to 60 min (*22*). This is consistent with the observation that similar phase-response curves are obtained for wild-type CS flies exposed to light pulses from 10 to 60 min in duration (*19*, *20*). Unless otherwise indicated, our results were obtained with 1-hour light pulses because these pulses produced the most consistent results (*22*). Light pulses were administered during the last dark cycle of LD to minimize desynchronization of the fly population during free-running conditions, as the biochemical analysis of PER requires material from many flies.
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21. Preparation of total fly head extract and visualization of PER by immunoblotting were done essentially as described (*7*, *8*).
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23. For each experiment, two sets of CS flies were entrained under identical LD conditions, and on the last dark period (day 4) one set received a light pulse at ZT15, followed by continued darkness. Heads of anesthetized flies were quickly isolated and immediately incubated in 4% paraformaldehyde at 4°C in the dark. All subsequent sectioning and staining steps were as described (*8*). To detect nuclei (Fig. 2B), we incubated slides with propidium iodide as described (*10*). Frozen head sections were observed under 1000× magnification with a Leica microscope interfaced with an image analysis system (Bioquant), and images were transferred to Adobe Photoshop software for printout.
24. For each time point, total RNA was extracted from ~50 μl of fly heads and ribonuclease protection assays were performed (*11*) with modifications described by Zeng *et al.* (*8*). The protected bands were quantified with a PhosphorImager from Molecular Dynamics.
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26. Head extracts were prepared in HP solution [defined in (*7*)] and either PER-HA or PER immunoprecipitated from equal amounts of homogenate as described (*7*). Protein immunoblotting in the presence of antibody to PER or antibody to TIM was as described (*7*), except that the antibody to TIM was diluted 1:500 in blocking solution that contained 0.02% Tween-20. The antibody to TIM used in this study was raised in rats with the use of a bacterially expressed fragment of TIM (amino acids 1 to 580) fused to polyhistidine as an immunogen (*28*).
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Abnormal Centrosome Amplification in the Absence of p53

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The centrosome plays a vital role in mitotic fidelity, ensuring establishment of bipolar spindles and balanced chromosome segregation. Centrosome duplication occurs only once during the cell cycle and is therefore highly regulated. Here, it is shown that in mouse embryonic fibroblasts (MEFs) lacking the p53 tumor suppressor protein, multiple copies of functionally competent centrosomes are generated during a single cell cycle. In contrast, MEFs prepared from normal mice or mice deficient in the retinoblastoma tumor suppressor gene product do not display these abnormalities. The abnormally amplified centrosomes profoundly affect mitotic fidelity, resulting in unequal segregation of chromosomes. These observations implicate p53 in the regulation of centrosome duplication and suggest one possible mechanism by which the loss of p53 may cause genetic instability.

The centrosome is a major microtubule-organizing center in eukaryotic cells and features prominently in mitosis, where it is required for the establishment of spindle bipolarity, spindle microtubule assembly, the establishment of the cleavage furrow plane, and balanced segregation of chromosomes (*1*). In addition, during interphase it nucleates and organizes the cytoplasmic microtubules, which leads to the redistribution of cellular organelles and the establishment of cellular polarity (*1*). The centrosome duplicates only once during each cell cycle; duplication begins near the G_1-S boundary, when replication of the centriole (the core component of centrosome) commences, and is completed in G_2 (*2*).

The *p53* tumor suppressor gene is frequently mutated in human and rodent tumors (*3*, *4*), and its loss or inactivation is correlated with genetic instability (*5*). The p53 protein has been shown to associate with the centrosome during interphase, but not during mitosis (*6*). To investigate

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