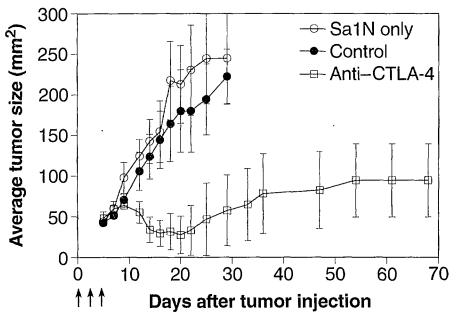

Fig. 4. Treatment with anti–CTLA-4 reduces the growth of the murine fibrosarcoma Sa1N. Groups of five mice were injected subcutaneously in the flank with a suspension of 1×10^6 Sa1N fibrosarcoma cells. Treated groups were injected intraperitoneally with 100 μg of anti–CTLA-4 or irrelevant hamster control antibody at days 0, 3, and 6 as indicated by the arrows. All control animals were killed by day 30. Two of five animals treated with anti–CTLA-4 remained tumor-free at day 55.

able, rapidly growing tumors within 7 days, whereas only two mice treated with anti–CTLA-4 had tumors by day 30, and one additional mouse developed a tumor around day 40 after injection. The remaining mice were still tumor-free 70 days after injection. In another experiment, control mice injected with 4×10^5 Sa1N tumor cells also developed rapidly growing tumors, whereas 7 of 10 mice treated with anti–CTLA-4 were tumor-free by day 25 after injection (*25*).

Our results indicate that removing inhibitory signals in the costimulatory pathway can enhance antitumor immunity. Although it has been shown that anti–CTLA-4 interferes with signals that normally down-regulate T cell responses in vivo (*17*, *18*), the exact mechanisms of antitumor immunity elicited by CTLA-4 blockade are not clear. In the case of B7-negative tumors, antigens are most likely transferred to and presented by host APCs (*27*), where CTLA-4 blockade might effect T cell responses in two nonexclusive ways. First, removal of inhibitory signals may lower the overall threshold of T cell activation and allow normally unreactive T cells to become activated. Alternatively, CTLA-4 blockade might sustain proliferation of activated T cells by removing inhibitory signals that would normally terminate the response, thus allowing for greater expansion of tumor-specific T cells.

Regardless of the mechanism, it is clear that CTLA-4 blockade enhances antitumor responses. Most importantly, we have observed these effects against unmanipulated, wild-type tumors. Current methods of enhancing antitumor immunity generally require the engineering of tumor cells (*8*). Some of these methods, such as the induction of B7 expression, rely on enhancing the costimulatory activity of the tumor cells themselves. Others, such as engineering tumor cells to express MHC class II molecules (*26*, *28*, *29*) or to produce granulocyte-macrophage colony-stimulating factor (*27*, *30*, *31*) or pulsing dendritic cells with tumor antigen ex vivo (*32*, *33*), seek to enhance antigen presentation, antigen transfer, or both. Thus, CTLA-4 blockade, by removing potentially competing inhibitory signals, may be a particularly useful adjunct to other therapeutic approaches involving the costimulatory pathway.

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Light-Induced Degradation of TIMELESS and Entrainment of the *Drosophila* Circadian Clock

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Two genes, *period* (*per*) and *timeless* (*tim*), are required for production of circadian rhythms in *Drosophila*. The proteins encoded by these genes (PER and TIM) physically interact, and the timing of their association and nuclear localization is believed to promote cycles of *per* and *tim* transcription through an autoregulatory feedback loop. Here it is shown that TIM protein may also couple this molecular pacemaker to the environment, because TIM is rapidly degraded after exposure to light. TIM accumulated rhythmically in nuclei of eyes and in pacemaker cells of the brain. The phase of these rhythms was differentially advanced or delayed by light pulses delivered at different times of day, corresponding with phase shifts induced in the behavioral rhythms.

Circadian rhythms, found in most eukaryotes and some prokaryotes (*1*), are ~24-hour rhythms governed by an internal clock that functions autonomously but can be entrained by environmental cycles of light or temperature. Circadian rhythms produced in constant darkness can also be reset by pulses of light. Such light pulses will shift the phase of the clock in different directions (advance or delay) and to a varying extent in a manner that depends on the time of light exposure (*2*).

In the fruit fly *Drosophila melanogaster*, two genes, *period* (*3*) and *timeless* (*4*), are

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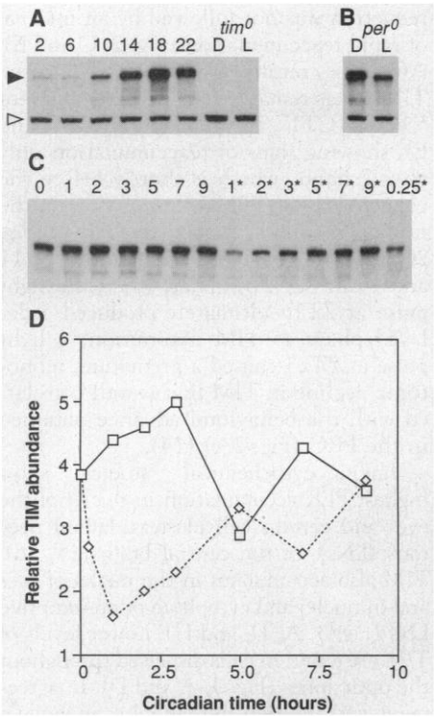

Fig. 1. Protein immunoblot analysis of TIM protein in wild-type, *tim*^{0}, and *per*^{0} flies. Equivalent amounts of total protein from fly heads isolated at various times were separated by SDS–polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with antibodies to TIM (*20*). (**A**) Levels of TIM protein at 4-hour intervals in a LD cycle (12 hours light:12 hours dark). TIM, filled arrowhead; nonspecific band, open arrowhead. Lanes marked (D) (dark) and (L) (light) are extracts from *tim*^{0} fly heads isolated from flies at ZT19 and ZT7, respectively. (**B**) Levels of TIM protein in *per*^{0} fly heads under dark and light conditions. The *per*^{0} dark sample was prepared from dark-reared flies, and the light sample from ZT7 of a LD cycle. (**C**) Levels of TIM protein in extracts from control (*per*^{0} in constant dark) and light-treated *per*^{0} flies previously reared in constant darkness (*21*). Lane numbering corresponds to time (in hours) from the start of a 1-hour light pulse. (*) Light-treated flies. (**D**) All TIM bands shown in (C) were quantified by densitometry with reference to a constitutively produced, nonspecific protein that cross-reacts with the antibody to TIM [see (A) and (B)]. Dashed profile is light-pulsed data.

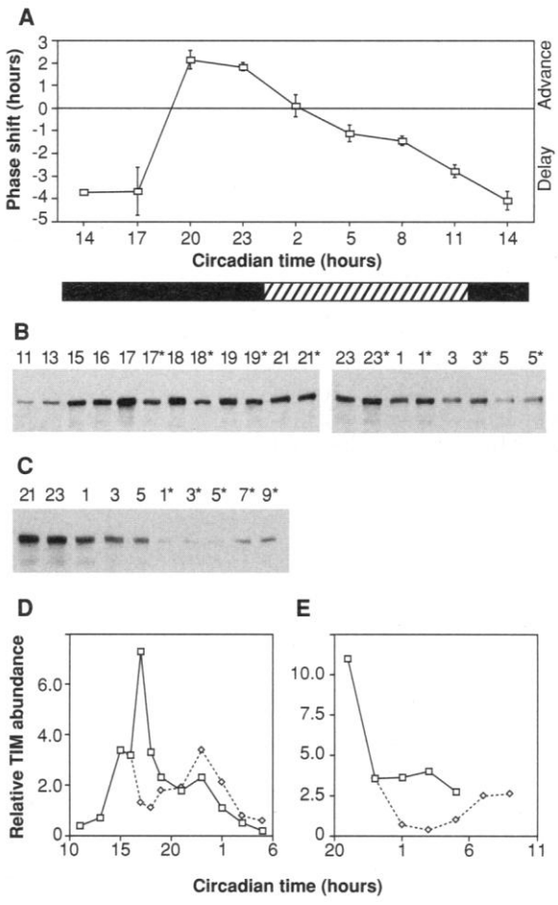

Fig. 2. Effects of light pulses on the phase of locomotor activity and TIM protein rhythms. (**A**) PRC of wild-type flies (*22*). The graph depicts the phase change of the locomotor activity rhythm after a 10-min light pulse as a function of circadian time. Subjective day is indicated by the hatched bar. (**B**) Response of TIM protein levels to a light pulse administered at ZT16 followed by transfer to constant darkness (*21*). (*) Light-pulsed flies. (**C**) Response of TIM protein levels to a light pulse administered at ZT23. Treatment as in (B), except that light exposure occurred at ZT23 instead of ZT16. Except as noted, methods were as described for light-pulsed *per*^{0} flies (*21*). (**D**) TIM bands shown in (B) were quantified by densitometry with reference to Hsp70. (**E**) Because Hsp70 migrated past the region blotted, quantitation of TIM in (C) was performed against a nonspecific, cross-reacting protein as described (Fig. 1). Dashed profiles are light-pulsed data.

essential components of the circadian clock. Mutations in either of these genes can produce arrhythmicity or change the period of the rhythm by several hours (*3–5*). Molecular studies (*6–9*) have shown that *per* and *tim* are transcribed with indistinguishable circadian rhythms that are influenced by an interaction between the TIM and PER proteins (*4*, *10*). A physical association of the two proteins appears to be required for accumulation and nuclear localization of PER (*4*, *10*, *11*). It is likely that nuclear localization results in suppression of *per* and *tim* transcription (*8*, *9*). Cycles of gene expression are thought to be sustained by ~6-hour differences in the phases of RNA and protein accumulation. The observed delays in PER accumulation may result, in part, from a requirement for TIM to stabilize PER by transport to nuclei (*4*, *9*, *11*).

To directly characterize TIM from *Drosophila* heads, we raised antibodies against several recombinant TIM proteins expressed in bacteria (Fig. 1). TIM, like PER, accumulates rhythmically in LD 12:12 (cycles of 12 hours light:12 hours dark) (Fig. 1A). The time of peak TIM accumulation in wild-type fly heads occurred at about ZT18 (ZT, zeitgeber time; ZT0 = lights on, ZT12 = lights off) and thus corresponds to the time of peak PER accumulation (*11*, *12*). These rhythms were also observed in constant darkness (*13*). Although TIM has an apparent molecular size of ~180 to 190 kD, there was an increasingly upward shift in TIM mobility late at night (compare ZT14 to ZT22). On shorter exposures, the TIM signal at ZT22 was resolved into at least two closely migrating bands (*13*). This behavior is similar to that of PER (*11*, *12*). The antisera detects bona fide TIM protein: Extracts prepared from *tim*^{0} fly heads lacked the TIM band (Fig. 1A), and antisera raised against a different region of TIM protein detected the same band seen in Fig. 1 (*13*).

Although PER protein levels are reduced in a *tim*^{0} genetic background (*4*, *11*), TIM was expressed at fairly high levels in *per*^{0} flies reared in constant darkness (Fig. 1B). Exposure to light also appeared to reduce the amount of TIM (presumably by degradation), because a sample from *per*^{0} flies maintained in LD 12:12 and harvested at ZT7 showed much less TIM than a sample from dark-reared flies (Fig. 1B).

We therefore exposed *per*^{0} flies (previously in constant darkness for 4 days) to a 1-hour pulse of light, followed by a recovery period in constant darkness. The amount of TIM protein decreased rapidly after the light treatment and began to rise within the first hour after return to darkness (Fig. 1, C and D). A return to the pre-light exposure level occurred by ~5 hours (Fig. 1, C and D). The rapidity of this response was further indicated by measurement of TIM protein amounts immediately after a 15-min pulse of light. A substantial loss of TIM was again observed (Fig. 1C, 0.25*, and Fig. 1D). Taken together, these data indicate that, unlike PER in a *tim*^{0} background, TIM protein is stable in *per*^{0} flies. Moreover, light induces loss of

TIM protein without requiring PER and is therefore clock-independent.

Exposure of wild-type flies to constant light produces a tim^0 phenocopy, including behavioral arrhythmia, suppression of PER accumulation, and reduced PER phosphorylation (*11*). Because constant light treatment of tim^0 flies produced no further change in the PER protein, it was suggested that light's effects on PER in wild-type flies might be mediated by an activity associated with *tim* (*11*).

Our data point to a mechanism for these effects of constant light and suggest a role for TIM in light-dependent entrainment of the *Drosophila* clock. The influence of light delivered at different times of day on the phase of circadian behavioral rhythms is quantitatively expressed by a phase-response curve (PRC) (Fig. 2A). Maximum phase delays of 4 to 5 hours are produced by light pulses delivered between CT14 to 16 (CT, circadian time; CT0, subjective dawn), whereas maximum phase advances, ~1 to 2 hours, are produced when light pulses of the same duration and intensity are provided between CT20 to 23 (Fig. 2A) (*14*). If adjustment of TIM levels by premature light exposure (relative to the phase of the existing rhythm) mediates light-resetting of the clock, we would expect TIM levels to respond in a manner predicted by the *Drosophila* PRC. Therefore, we exposed wild-type flies to 1-hour light pulses beginning at either ZT16 or ZT23, returned them to constant darkness, and collected fly heads at various times for protein immunoblot analysis.

Consistent with the phase delay in behavioral rhythms, light exposure at ZT16 produced a reduction in TIM levels, followed by a rapid reaccumulation of TIM (Fig. 2, B and D). The level of TIM was substantially reduced by the end of the 1-hour light treatment (compare CT17 to CT17* in Fig. 2, B and D) and began to rise again within 2 hours after the transfer to darkness (compare CT19* to CT18* in Fig. 2, B and D). As the TIM level in control flies progressively decreased from a maximum at CT17, the TIM level in light-pulsed flies entered a reaccumulation phase with a new peak at CT23. Starting at CT23*, the level of TIM in the light-pulsed samples was always higher than that of control flies sampled at the same circadian time (Fig. 2, B and D). The amount of TIM at CT23* was most similar to the amount accumulated in control flies at CT18, whereas CT1* most resembled CT19 to 23 (Fig. 2, B and D). The results demonstrate that the light pulse delays the molecular rhythm of TIM by 2 to 6 hours. This result corresponds well with the ~4-hour delay in the behavioral rhythm calculated from the PRC (Fig. 2A) (*14*).

A light pulse administered at ZT23, which produces 1- to 2-hour phase advances by PRC analysis (Fig. 2A) (*14*), reduced TIM like the ZT16 pulse, but the reduction was not followed by an interval of rapid reaccumulation (Fig. 2, C and E). After the return to constant darkness, TIM levels remained barely detectable for 5 hours (CT1*, 3*, and 5* in Fig. 2, C and E), showing signs of reaccumulation only after 7 hours, which is shortly before the expected rise in TIM accumulation on the next subjective day (compare CT9* in Fig. 2C with the control accumulation at 11 and 13 in Fig. 2B). Thus, whereas a light pulse at ZT16 ultimately produced a delayed phase of TIM diminution, a light pulse at ZT23 caused a premature, monotonic decline in TIM that is well correlated with the behavioral advance obtained in the PRC (Fig. 2A) (*14*).

Immunocytochemical studies show highest PER accumulation in nuclei of the eyes and certain cell clusters, lateral neurons (LNs), of the central brain (*15, 16*). TIM also accumulates in the nuclei of eyes and in nuclei and cytoplasm of presumptive LNs (Fig. 3, A, D, and H). Lower levels of TIM are found in cells dispersed throughout the optic lobes (Fig. 3, A and D). In agreement with protein immunoblot analysis, a rhythm of TIM staining was observed immunocytochemically in wild-type flies (Fig. 3, A to E).

TIM is produced at high levels in heads from per^0 flies at night (Fig 1B). Immunocytochemical analysis revealed that high amounts of TIM accumulate in per^0 photoreceptor cells, but the protein is not associ-

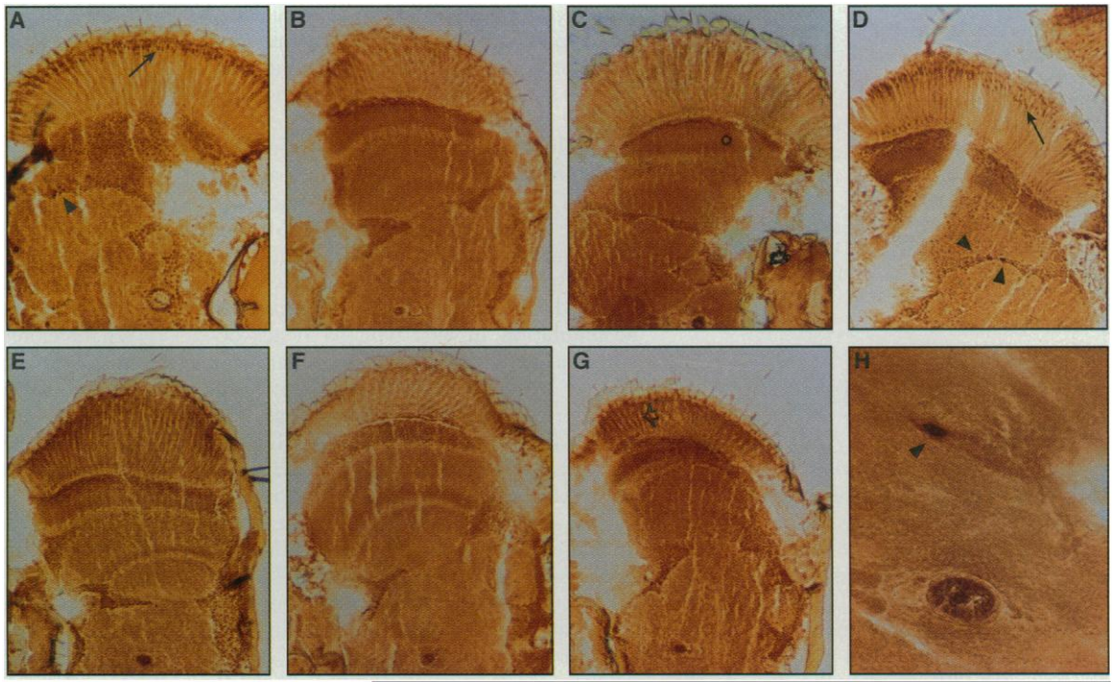

Fig. 3. Localization of TIM proteins in the *Drosophila* head. (**A** to **D**) Cycles of TIM immunostaining observed in LD 12:12. Frontal sections of wild-type heads show high levels of eye and brain staining at night (ZT22 and ZT20.5) (A and D, respectively) and low levels during the day (ZT1 and ZT7) (B and C). (**E**) TIM staining at CT6 (middle of subjective day in constant dark). (**F**) Pattern of staining in control, tim^0 head sections reared in DD. (**G**) TIM staining in cytoplasm, but not nuclei, of eyes in per^0 flies reared in DD. (**H**) Higher magnification showing cytoplasmic and nuclear TIM staining in putative lateral neurons (LNs) in wild type (ZT17). Circular structure is esophagus. Arrows in (A) and (D) indicate labeling of photoreceptor nuclei. Filled arrowheads (A, D, and H) show staining of putative LNs. Open arrowhead (G), cytoplasmic staining of per^0 photoreceptors. For "dark" time points, flies were collected and frozen under a safelight (15 W bulb with Kodak GBX-2 filter). Sectioning, and immunostaining of heads, were done as described (*16*). Except as noted, light pulses were administered as described (*22*).

ated with nuclei. Rather, TIM accumulates in the cytoplasm (compare Fig. 3, F and G). TIM is required for both accumulation and nuclear localization of PER (*4*, *11*). We conclude that TIM nuclear localization depends on PER, whereas TIM accumulation does not.

Blotting of proteins from light-treated flies indicated rapid loss of TIM (Fig. 1, B to D, and Fig. 2, B to E), but did not reveal the affected cell types. We monitored the effects of light over an extended time course in wild-type flies. A 10-min light pulse given at ZT16 reduced the TIM staining in photoreceptors within 1 hour (CT17; Fig. 4D). Staining in putative LNs was also clearly reduced, but only after a delay of 2 hours (Fig. 4, D to F). The observed delay in light-dependent diminution of TIM in LNs may be related to the abundance of the protein in these pacemaker cells, because their staining was often stronger than that of individual photoreceptor nuclei (compare Fig. 4, A, B, and C). Alternatively, the delay may reflect some dependence of the LNs on the eyes for TIM turnover.

Our data suggest that TIM couples intracellular circadian cycles to light stimulation. Progression through the molecular cycle can be reset by light-induced elimination of TIM. If a light pulse is given at a time of night when a behavioral phase delay is induced (for example, ZT16) (Fig. 2, B and D), TIM diminution is immediately followed by reaccumulation. The molecular cycle of TIM levels is now reset to an earlier time point, and the magnitude of the resulting molecular phase delay corresponds well with that of the behavioral phase delay. When a light pulse is given at a time of night that is associated with a small advance in the phase of the behavioral rhythm (ZT23; Fig. 2, C and E), TIM is prematurely lost, and recovery is not seen until the next day's cycle of accumulation. As indicated in Fig. 5, the different responses should be influenced by the different amounts of *tim* mRNA available for new protein synthesis at different times of night: Highest levels of *tim* mRNA occur in the early evening, and lowest levels occur near dawn (*9*). Although we have not examined the response to light pulses during the subjective day, extremely low levels of TIM may be responsible for a less sensitive "dead zone" in the PRC (Fig. 5) (*2*). These conclusions are also supported by the kinetics of recovery of TIM after light pulses of per^0 flies. Because per^0 flies have no measurable circadian pacemaker and produce *tim* RNA at high, constitutive levels (*9*, *17*), the amount of RNA is probably the only factor influencing the rate of TIM reaccumulation in the mutant. The biochemical process mediating TIM's light sensitivity in per^0 and per^+ flies is unknown.

The mechanism underlying entrainment of the *Drosophila* pacemaker appears to differ from that described for *Neurospora*, in which light pulses at any time of day uniformly stimulate transcription of the clock gene *frq* (*18*). Because this light responsiveness fails to cycle in *Neurospora*, key factors mediating entrainment in that species should not include products of clock genes, or state variables (*18*). Although light-induced loss of TIM should produce secondary changes in *tim* and *per* transcription (Fig. 5), we have not observed acute effects of light on *per* and *tim* transcription (*9*, *19*).

In a natural environment, the light: dark cycle must make a contribution to

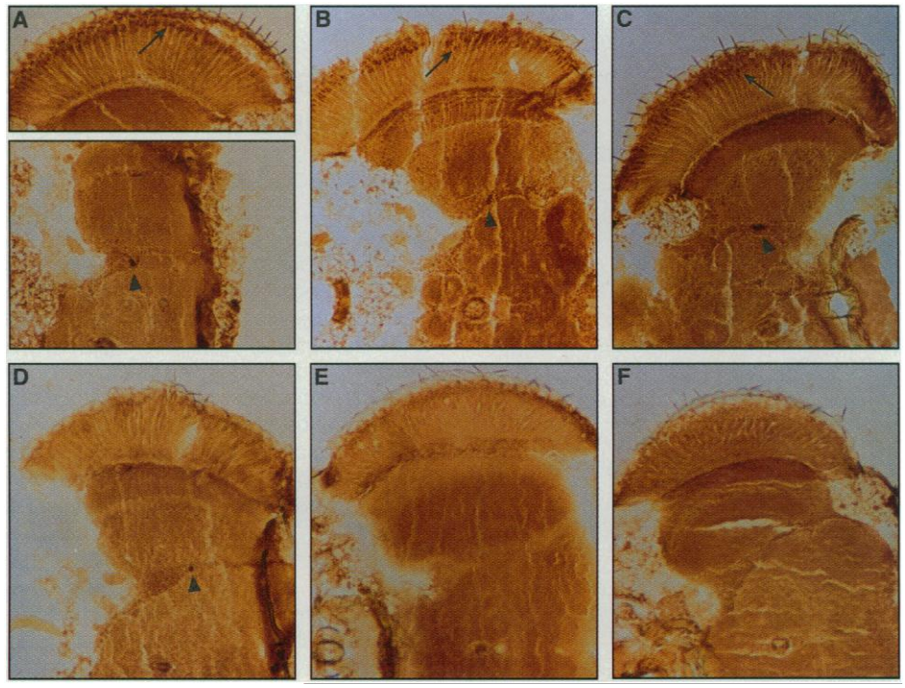

Fig. 4. Time course of light-dependent loss of TIM in wild-type photoreceptors and putative LNs. Flies entrained to LD12:12 and exposed to a 10-min light pulse (*21*, *22*) delivered at ZT16 were collected at CT17 (**D**), CT18 (**E**), and CT21 (**F**). Control flies (not light-pulsed) collected at CT17 (**A**), CT19 (**B**), and CT21 (**C**). Examples of TIM staining in photoreceptor nuclei (arrows in A to C) and putative LNs (arrowheads in A to D) are indicated.

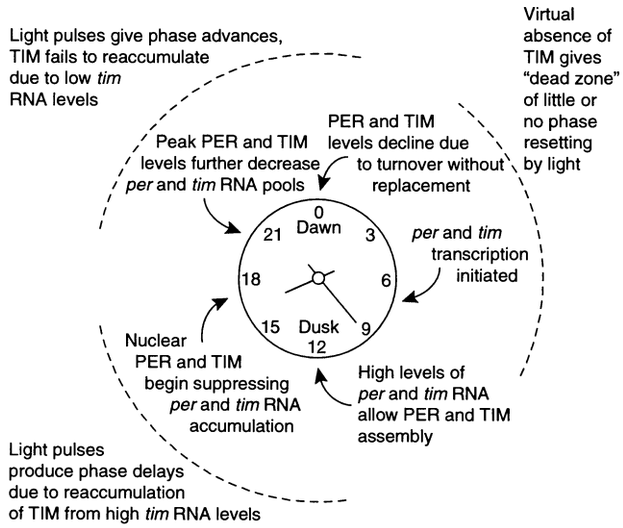

Fig. 5. Model for entrainment of the circadian pacemaker. Because TIM protein appears to be an essential component of the *Drosophila* clock (*9*) and is rapidly degraded by light, the phase of molecular and behavioral rhythms should be altered by light at times of day when TIM proteins are present. *tim* RNA is most abundant in the early evening, when light pulses only transiently eliminate TIM proteins (because they can be resynthesized from existing RNA pools). If TIM suppresses accumulation of *tim* and *per* RNA (*9*), the extended interval of TIM accumulation should delay subsequent rounds of RNA synthesis. Lowest amounts of *tim* RNA are observed near dawn (*9*). Light pulses at that time prematurely eliminate TIM, which cannot be replaced until new *tim* RNA synthesis ensues with the next day's cycle. Premature elimination of TIM by light should result in advanced synthesis of *tim* and *per* RNA.

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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