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JETLAG Resets the *Drosophila* Circadian Clock by Promoting Light-Induced Degradation of TIMELESS

Kyunghee Koh, Xiangzhong Zheng, and Amita Sehgal*

Howard Hughes Medical Institute, Department of Neuro-science, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

Abstract

Organisms ranging from bacteria to humans synchronize their internal clocks to daily cycles of light and dark. Photoc entrainment of the *Drosophila* clock is mediated by proteasomal degradation of the clock protein TIMELESS (TIM). We have identified mutations in *jetlag*—a gene coding for an F-box protein with leucine-rich repeats—that result in reduced light sensitivity of the circadian clock. Mutant flies show rhythmic behavior in constant light, reduced phase shifts in response to light pulses, and reduced light-dependent degradation of TIM. Expression of JET along with the circadian photoreceptor cryptochrome (CRY) in cultured S2R+ cells confers light-dependent degradation onto TIM, thereby reconstituting the acute response of the circadian clock to light in a cell culture system. Our results suggest that JET is essential for resetting the clock by transmitting light signals from CRY to TIM.

Travel across time zones often produces jet lag because it takes some time to re-synchronize internal circadian clocks to the new day and night cycle. Although the molecular mechanisms for generating circadian rhythms through interlocking transcriptional feedback loops and posttranslational modifications have been characterized in some detail (1), few components of the light entrainment pathway are known (2). Photoc entrainment in *Drosophila* can be mediated by the visual system and by CRY, a circadian blue-light photoreceptor expressed in clock cells (3). When the fly is exposed to light, CRY binds a core clock protein, TIM, which leads to subsequent ubiquitination and degradation of TIM by the proteasome pathway (4–8). Rapid, light-dependent degradation of TIM underlies the fly's ability to reset the circadian phase to reflect environmental fluctuations in light levels (9,10). However, the specific signals that drive the TIM response to light are not known.

In the course of characterizing rest:activity rhythms of various fly strains, we discovered a strain with anomalous activity patterns in constant light (LL). Whereas wild-type flies became arrhythmic after a day or two in LL, the mutant flies were rhythmic for more than a week (Fig. 1A and Table 1). Although the mutants could be entrained to light:dark (LD) cycles, they took longer to be re-entrained to a new schedule than wild-type flies (Fig. 1B), and so we named the mutation *jetlag* (*jet*). The behavior of *jet* flies in LD and in constant darkness (DD) conditions was normal (Fig. 1 and Table 1). These phenotypes are reminiscent of those of *cry* mutants (11) and suggest a defect in circadian photoreception.

Using meiotic recombination and deficiency mapping strategies, we mapped the mutation to a small region containing 18 genes on the left arm of the second chromosome. One of these genes, CG8873 (Flybase), encodes an F-box protein with leucine-rich repeats (LRRs), a

*To whom correspondence should be addressed. amita@mail.med.upenn.edu.

Supporting Online Material www.sciencemag.org/cgi/content/full/312/5781/1809/DC1 Materials and Methods Figs. S1 to S3 References

putative component of a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex. We sequenced the coding region of the gene in 13 strains, including some wild-type strains, the original mutant strain, and several other strains that did not complement the original mutation for the LL phenotype. In six of the seven mutant strains, we found a phenylalanine-to-isoleucine substitution in a conserved LRR domain. In the remaining mutant strain, there was a serine-to-leucine substitution in an adjacent LRR domain (Fig. 1, C and D). The two mutations will be referred to as *common* and *rare* (*c* and *r*), respectively. The two alleles did not complement each other, nor did they complement chromosomal deletions that remove the *jet* locus (Table 1).

The JET protein contains an N-terminal F-box domain thought to be involved in binding the Skp1 component of the SCF complex, as well as seven LRRs constituting a protein-protein interaction domain thought to be involved in target recognition (12) (Fig. 1C). Functions of the mammalian F-box proteins with highest similarity to JET (F-box and LRR protein 15) have yet to be determined.

Almost all (>96%) of the *jet^r* and *jet^c* flies had rhythmic behavior in LL, whereas very few of the wild-type control flies did (Table 1). In contrast, the mutants' behavior was indistinguishable from wild-type behavior in DD, which suggests that the mutants have a largely intact circadian system with a specific defect in the light input pathway. Consistent with its limited role in free-running rhythms, the *jet* mRNA does not cycle in a circadian fashion (13) (fig. S1). The reduced light sensitivity of *jet* mutants is similar to that of *cry* mutants; however, unlike *cry* mutants (11), *jet* mutants showed rhythmic activity of a luciferase reporter for a clock gene, *period* (*per*) (14), in DD, which suggests that their peripheral clocks function normally (Fig. 1E). Because luciferase is assayed in whole flies and therefore reports the activity of multiple peripheral clocks, rhythmic luciferase activity in *jet* mutants also indicates synchrony among these clocks. Peripheral clocks can be entrained to an LD cycle via CRY-independent pathways (15), which may account for the synchrony of peripheral clocks in *jet* mutants. Loss of *per*-luciferase cycling in *cry* mutants most likely occurs because CRY, in addition to its role as a circadian photoreceptor, has a role in the regulation of core clock components in the periphery (16).

To characterize the behavioral light sensitivity of *jet* mutants in more detail, we measured phase shifts in response to brief light pulses at night. *jet* mutants had significantly reduced phase shifts relative to wild-type control flies (Fig. 2A). Expression of wild-type JET from a *UAS-jet* transgene under the control of a *cry*- or *tim-Gal4* driver partially rescued the mutant phenotype (Fig. 2B). The increase in phase shifts was greater with *tim-Gal4* than with *cry-Gal4*, probably because the former is a stronger driver. Together with the sequence data described above, the rescue results provide strong evidence that the mutations in the *jet* locus are responsible for the observed mutant phenotypes.

To determine the molecular correlates of the behavioral defects, we examined the changes in TIM levels in central clock neurons after brief light pulses. Light-dependent degradation of TIM was substantially reduced in *jet* mutants (Fig. 2C) and was restored in rescued flies expressing the *UAS-jet* transgene (Fig. 2D), which suggests that the behavioral defects in the mutants are mediated by defects in TIM degradation. Light-dependent TIM degradation was also reduced in head extracts of mutants (fig. S2), implying that JET facilitates TIM degradation in the peripheral clock in the eye as well.

To further explore the role of JET in TIM degradation, we next turned to an S2R+ *Drosophila* embryonic cell line. Unlike in the fly, in S2R+ cells, TIM does not degrade in response to light (7) (Fig. 3A). To test whether JET is the crucial component missing in these cells, we expressed JET with the use of a constitutive promoter. The JET protein had little

effect on TIM levels in the dark, but it rapidly reduced TIM levels upon light exposure (Fig. 3A and fig. S3A). This light-induced degradation of TIM required coexpression of CRY and was blocked by a proteasome inhibitor, MG132. JET did not promote degradation of another core clock protein, PER (Fig. 3B), demonstrating specificity for its target selection. In addition, light-dependent degradation of CRY did not require JET (Fig. 3A and fig. S3B), although it was facilitated by JET in the presence of TIM (Fig. 3, A and B). The JET protein itself was also not affected by light in S2R+ cells (Fig. 3A).

One of the mutant versions of the protein (the *r* allele) was significantly less effective than wild-type JET at promoting TIM degradation (Fig. 3C and fig. S3C). The *r* mutation reduced the stability of the JET protein (Fig. 3C and fig. S3D), which may explain its mutant phenotype. The other mutant allele (*c*) was also less effective than the wild-type allele, although the difference was not statistically significant. The *r* mutation is in a residue conserved among insects and mammals, but the *c* mutation is in a residue conserved only among insects (Fig. 1D), which may explain the stronger effects of the *r* allele in both behavioral and molecular assays.

Wild-type JET protein also promoted ubiquitination of TIM in cultured cells. In the presence of wild-type JET and CRY, a significant increase in TIM ubiquitination was observed after only 10 min of exposure to light (Fig. 3D and fig. S3E). Mutant proteins, especially the *r* allele, were less effective at ubiquitination of TIM (17). Consistent with its role as a component of an SCF complex, JET interacts with SkpA, one of several Skp1 homologs in *Drosophila* (Fig. 3E). In addition, JET physically associates with TIM, and the association is stronger in light than in dark (Fig. 3F and fig. S3F).

Flies share many of the core clock components with mammals (18), but their mechanism for light-induced phase resetting appears to differ. Circadian photoreception in mammals relies on adenosine 3',5'-monophosphate response element-binding protein (CREB)-mediated induction of mPer-1 transcription (19,20) and does not appear to involve CRY (21). Fly circadian photoreception resembles that of plants, where CRY functions as a circadian photoreceptor, although the mechanism is somewhat different from that of *Drosophila* CRY (4,22). Notably, the plant F-box protein ZEITLUPE mediates dark-dependent degradation of the clock protein TIMING OF CAB EXPRESSION 1 (23).

jet mutants did not show any detectable defects in the free-running rhythm in constant darkness. We propose that the *Drosophila* circadian system uses two separate mechanisms for controlling TIM levels: a clock-controlled one for maintaining rhythm in the dark, and a light-dependent one for entraining the clock to the photic environment. Both mechanisms use SCF complexes but with distinct F-box proteins: SLIMB for the clock-controlled mechanism (24,25) and JET for the light-dependent mechanism.

We have identified a component of the *Drosophila* light entrainment pathway that is critical for light-induced degradation of TIM. Single amino acid substitutions in JET lead to molecular and behavioral defects in light entrainment. Our results, together with those of previous studies, suggest the following model of how light resets the clock in *Drosophila*. Upon light exposure, CRY undergoes conformational change, allowing it to bind TIM (4–8). TIM is then modified by phosphorylation (7), which allows JET to target TIM for ubiquitination and rapid degradation by the proteasome pathway.

Supplementary Material

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Acknowledgments

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Fig. 1.

Mutant phenotypes and mapping of the jet mutations. **(A)** Activity records of representative wild-type (*y w*) and mutant flies in LL and DD. The gray and black bars at the top indicate the LD cycle. **(B)** Activity records showing average activity of wild-type and mutant flies ($n = 13$ and 15, respectively) in a “jet lag” experiment. The flies were transferred from one LD schedule (indicated by the bars at the top) to another with an 8-hour delay (indicated by the bars at the bottom) during the third day. Red dots indicate evening onsets, and the blue arrowhead indicates a startle response at lights-off after the transfer to a new LD schedule. **(C)** Schematic representation of the JET protein. Arrows indicate the Phe → Ile (*c*) and Ser → Leu (*r*) substitutions in the fourth and fifth LRRs, respectively. **(D)** Alignment of the fourth and fifth

LRRs of insect, mouse, and human F-box proteins with highest similarity to JET. See supporting online material for GenBank accession numbers. (E) Normal circadian expression of *per* in peripheral clocks of *jet^c* mutants. Flies (21 control, 22 mutant) carrying a *per*-luciferase (*BG-luc*) transgene were entrained to a 12 hour:12 hour LD cycle and were individually monitored in DD for ~5 days; data are mean numbers of counts per second.

Fig. 2.

Reduced responses to light pulses in *jet* mutant flies. **(A)** Behavioral response to phase-delaying and phase-advancing light pulses. All differences between control and mutant flies for both alleles and both zeitgeber times (ZTs) were significant [$P < 0.0001$ by analysis of variance (ANOVA)]. In this and subsequent figures, error bars denote SEM. **(B)** Rescue of reduced light sensitivity of *jet* mutants with a *UAS-jet* transgene. Phase delays in response to light pulses at ZT 16 in *jet^c* flies with a *UAS-jet* transgene and either a *cry-* or *tim-Gal4* driver were assayed as in (A). Two independent *UAS-jet* lines, A and B, produced similar results. $^xP = 0.06$, $^*P < 0.01$, $^{**}P < 0.001$ by ANOVA. **(C)** Reduced light-dependent degradation of TIM in clock neurons of *jet^c* mutants. Representative TIM staining in small ventral lateral neurons is shown 1 hour after a 2-min light pulse (LP) at ZT 16. TIM staining without LP is shown for comparison; 8 to 10 fly brains were examined per condition. **(D)** Rescue of the TIM response to light in clock neurons of *jet^c* mutants with a *UAS-jet* transgene. *jet^c* mutants with a *tim-Gal4* driver alone or with a *UAS-jet* transgene and a *tim-Gal4* driver were assayed as in (C).

Fig. 3.

Light-dependent degradation of TIM in S2R+ cells. (A) Light-induced degradation of TIM is mediated by CRY, TIM, and the proteasome pathway. S2R+ cells were transiently transfected with pAc-*tim* along with the pIZ vector or FLAG epitope-tagged pIZ-*jet* (pIZ-FLAG-*jet*), with or without MYC epitope-tagged pIZ-*cry* (pIZ-MYC-*cry*). MYC-CRY and FLAG-JET protein levels were determined with antibodies to MYC and JET, respectively. (B) JET does not promote degradation of PER. Hemagglutinin (HA)-tagged pAct-*per* and pIZ-MYC-*cry* were transfected with or without pIZ-FLAG-*jet*. (C) Mutant JET proteins are less effective at promoting TIM degradation than wild-type JET, and the *r* version of mutant JET is less stable than wild-type JET. Cells were transfected with pIZ or pIZ-FLAG-*jet* (wild-type or mutant) at one-fifth the concentration used in (A) along with pIZ-*tim* and pIZ-MYC-*cry*. (D) JET promotes light-dependent ubiquitination of TIM. S2R+ cells were transfected with HA-tagged ubiquitin (UB) under the control of a heat shock promoter along with pAc-*tim* and wild-type or mutant pIZ-FLAG-*jet*, with or without pIZ-MYC-*cry*. Transfected cells were kept at 25°C. Cell extracts were immunoprecipitated with antibody to TIM, and the relative amounts of ubiquitinated TIM were measured with antibodies to HA and TIM. (E) JET interacts with SkpA in vitro. Purified JET protein fused to maltose binding protein (MBP) was incubated with either glutathione S-transferase (GST) or GST-SkpA fusion protein. MBP-JET was detected with an

antibody to JET (top), and GST and GST-SkpA were visualized by Coomassie staining (bottom). **(F)** JET physically associates with TIM in S2R+ cells in a light-dependent manner. Extracts from cells transfected with pAc-*tim*, pIZ-MYC-*cry*, and pIZ or pIZ-FLAG-*jet* were immunoprecipitated with antibody to FLAG, and TIM and JET levels were measured. Experiments in each panel were repeated three to five times with similar results. Data are quantified in fig. S3.

Table 1

Activity rhythms of *jet* mutants. For each fly, the free-running period was determined with the use of χ^2 periodogram analysis. FFT, determined by fast Fourier transform analysis, is a measure of rhythm strength.

Genotype	DD			LL		
	% Rhythmic (n)	Period $\pm$ SEM (hours)	FFT $\pm$ SEM	% Rhythmic (n)	Period $\pm$ SEM (hours)	FFT $\pm$ SEM
Control- <i>r</i>	97.7 (44)	23.75 $\pm$ 0.08	0.141 $\pm$ 0.008	5.6 (18)	23.5	0.045
<i>jet</i> ^r	95.7 (69)	23.65 $\pm$ 0.07	0.148 $\pm$ 0.008	96.9 (32)	24.29 $\pm$ 0.15	0.168 $\pm$ 0.012
Control- <i>c</i>	100 (46)	24.00 $\pm$ 0.09	0.165 $\pm$ 0.008	0 (16)		
<i>jet</i> ^c	96.2 (42)	24.20 $\pm$ 0.07	0.143 $\pm$ 0.008	100 (15)	23.57 $\pm$ 0.13	0.200 $\pm$ 0.018
<i>jet</i> ^r / <i>jet</i> ^c	100 (22)	23.91 $\pm$ 0.09	0.166 $\pm$ 0.014	100 (16)	24.34 $\pm$ 0.11	0.171 $\pm$ 0.013
<i>Df</i> ⁺	89.5 (19)	23.71 $\pm$ 0.12	0.206 $\pm$ 0.013	4.3 (23)	24.5	0.079
<i>Df</i> / <i>jet</i> [*]	100 (44)	23.74 $\pm$ 0.09	0.172 $\pm$ 0.010	93.8 (16)	23.93 $\pm$ 0.15	0.228 $\pm$ 0.017
<i>Df</i> 1/ <i>jet</i> [†]	100 (15)	23.70 $\pm$ 0.11	0.150 $\pm$ 0.016	100 (22)	23.31 $\pm$ 0.19	0.148 $\pm$ 0.009
<i>Df</i> 2/ <i>jet</i> [†]	100 (17)	23.74 $\pm$ 0.11	0.161 $\pm$ 0.014	100 (25)	22.74 $\pm$ 0.15	0.178 $\pm$ 0.012

* Comparable results were obtained with two overlapping deficiencies [*Df*(2*L*)/*Exel*8012 and *Df*(2*L*)/*Exel*7021] that remove the *jet* locus; pooled data are presented.

† One of the deficiencies [*Df*2: *Df*(2*L*)/*Exel*7021] over *jet*^c produced a shorter period in LL than the other [*Df*1: *Df*(2*L*)/*Exel*8012], and data are presented separately.