Morning and Evening oscillators cooperate to reset circadian behavior in response to light input

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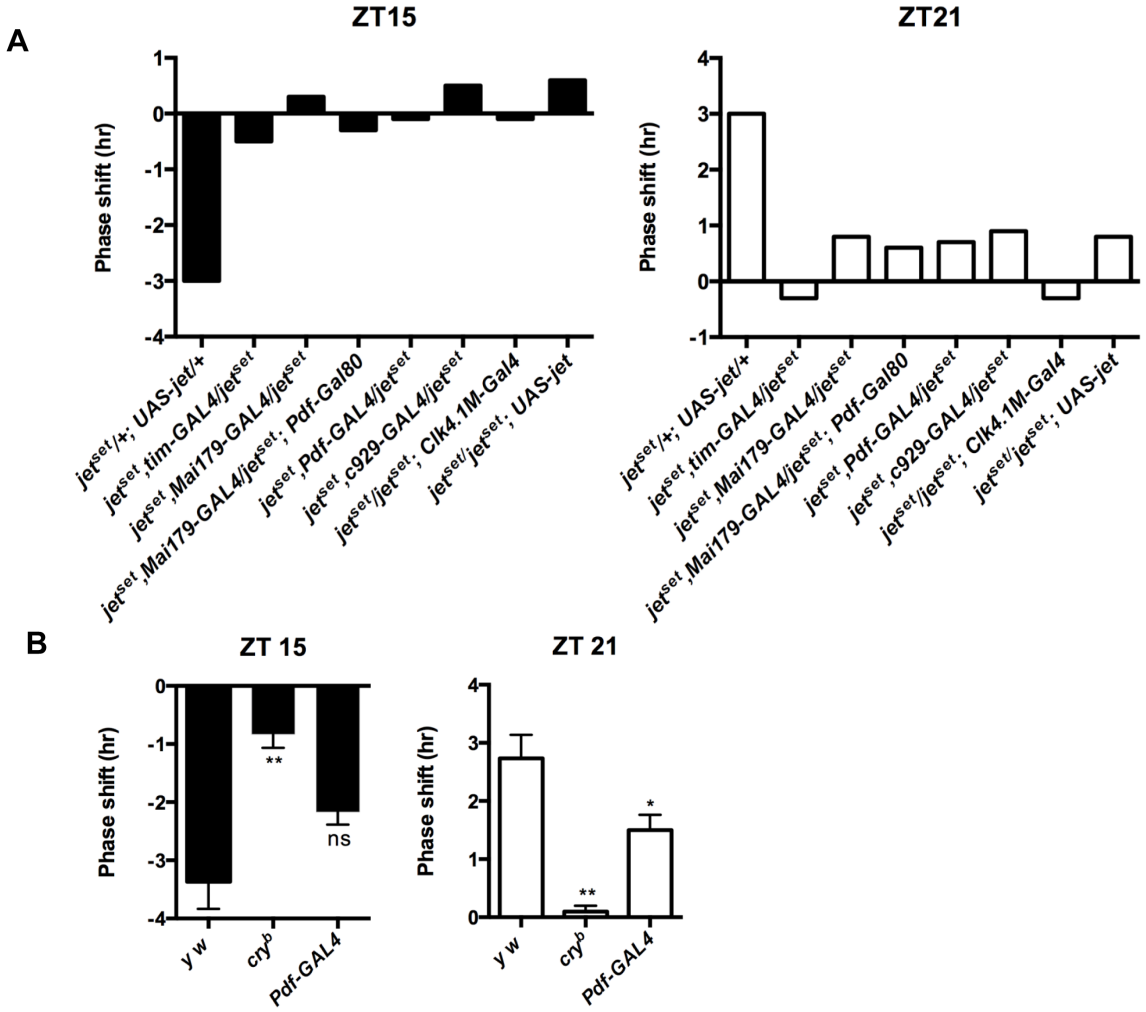


Figure S1: Behavioral phase shifts in control flies, Related to Figure 2.

A. Left panel shows behavioral phase shifts in response to a light pulse at ZT15 and the right panel shows the response to a light pulse at ZT21. y-axis indicates the amplitude of the phase shift in hours and the x-axis indicates the genotypes. Neither the *GAL4* driver lines without UAS-*jet* nor the UAS-*jet* transgene without a *GAL4* driver could correct the phase shifting defects of jet^{set} flies. As a positive control jet^{set}/+; UAS-*jet* flies were also included. As expected, these flies can phase delay and advance their behavior since they are heterozygous for the recessive jet^{set} mutation.

B. Behavioral phase-shifts of cry^{b} flies with CRY expression limited to the M-oscillators. In a previous study, CRY expression limited to the M-oscillators was found to fully rescue phase shifts in cry^{b} mutants at ZT21, and partially at ZT15 (Emery et al., 2000). While we obtained similar results at ZT15 when rescuing jet^{set} mutants, we observed no rescue at ZT21 (figure 2A). Thus, we measured phase shifts in cry^{b} flies in which CRY expression is rescued with *Pdf-GAL4* exactly the same way as we did for jet^{set} rescues. Left and the right panels correspond to phase shifts observed after light pulses at ZT15 and ZT21, respectively. Phase delays and phase advances are partially rescued when CRY expression was driven by *Pdf-GAL4*. This is in line with our JET rescue data (figure 2A): the M-oscillators alone are not sufficient. We note that CRY rescue is

stronger than JET rescue, probably because CRY overexpression increases sensitivity to light (Emery et al., 1998; Emery et al., 2000; Klarsfeld et al., 2004; Tang et al., 2010). Also, although the rescue at ZT15 appears clearly partial, the difference with wild-type closely missed statistical significance, because of higher than usual variability with the *y w* control. We are confident that our interpretation that this is a partial rescue is correct, since very similar results were obtained in a previous report (Emery et al., 2000). Moreover, we also observe partial rescue with JET (figure 2A). **, $P < 0.01$, *, $P < 0.05$, n.s., not significant at the 0.05 level as determined by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons, $F(2, 6) = 15.06$, with P value = 0.0046 for phase delays. The phase advance was also analyzed similarly by ANOVA, $F(2, 6) = 21.30$ with P value = 0.0019.

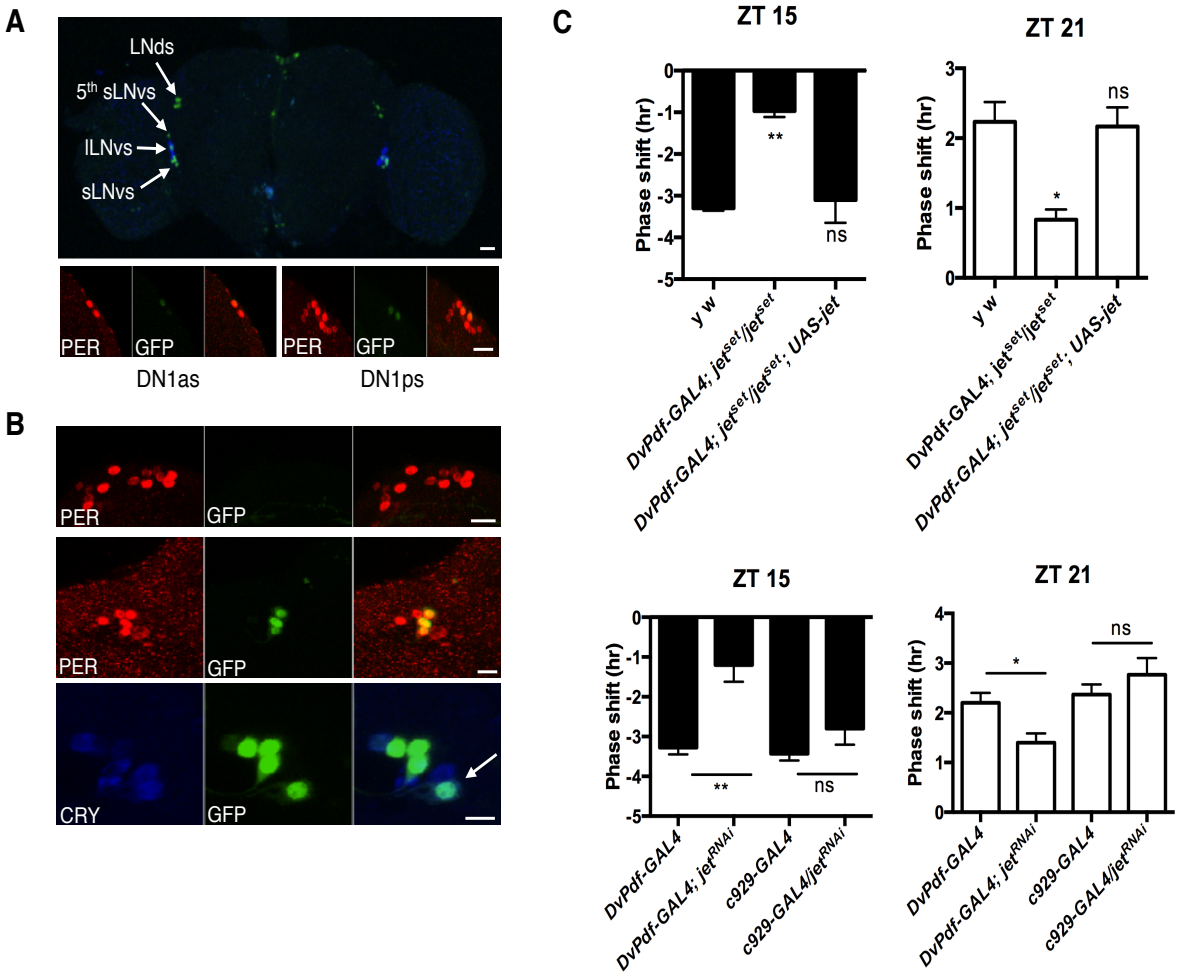


Figure S2: DN1s and l-LNvs are not required for phase shifts, Related to Figure 2.
A. Expression pattern of the *Mai179-GAL4* enhancer trap line. The brains of flies expressing GFP under the control of *Mai179-GAL4* were dissected and stained for anti-GFP (green), anti-PDF (blue) and anti-PER (red). Upper panel shows the whole brain, and bottom panel shows a very weak expression of GFP when driven by *Mai179-GAL4*

in DN1as (left) and two DN1ps (right). This pattern of expression is very similar to that described previously (Cusumano et al., 2009).

B. Expression pattern of the *DvPdf-GAL4* enhancer trap line. Upper panel shows the dorsal region, where there is no GFP expression in the DN1s. Middle panel shows the expression in the LNds. *DvPdf-GAL4* is expressed in four LNds. Bottom panel shows that one of these *DvPdf-GAL4* positive LNds (green, pointed by an arrow) expresses CRY (blue). The CRY-positive and *Mai179-GAL4* positive LNds are the same neurons (Yoshii et al., 2008). Thus, *Mai179-GAL4* and *DvPdf-GAL4* expression overlap in one LNd in addition to the 5th sLNv (Bahn et al., 2009). All images are Z-stacks. Scale bars indicate 10 μm.

C. The DN1s and l-LNvs are not required for behavioral phase shifts. Upper panel shows that rescue of JET expression using *DvPdf-GAL4* restores the phase shifting defects of jet^{set} mutants at both ZT15 and 21, indicating that JET expression is not required in the DN1s for circadian behavioral photoresponses. 16 flies per genotype were used for all the behavioral analysis and each experiment was repeated three times. Error bars represent S.E.M. **, $P < 0.01$, n.s., not significant at the 0.05 level as determined by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons, $F(2, 6) = 15.31$ and $P = 0.0044$ for phase delay, and $F(2, 6) = 10.59$ and $P = 0.0108$ for phase advance. Lower panel shows *jet* downregulation using *DvPdf-GAL4* and *c929-GAL4*. The jet^{RNAi} flies were compared to their *GAL4* control. Downregulating JET expression in the l-LNvs using *c929-GAL4* has no effect on phase shifts, indicating that these cells are not required for JET dependent photoresponses. Error bars represent S.E.M. **, $P < 0.01$; *, $P < 0.05$ tested using student's t-test. n.s., not significant at the 0.05 level tested using student's t-test.

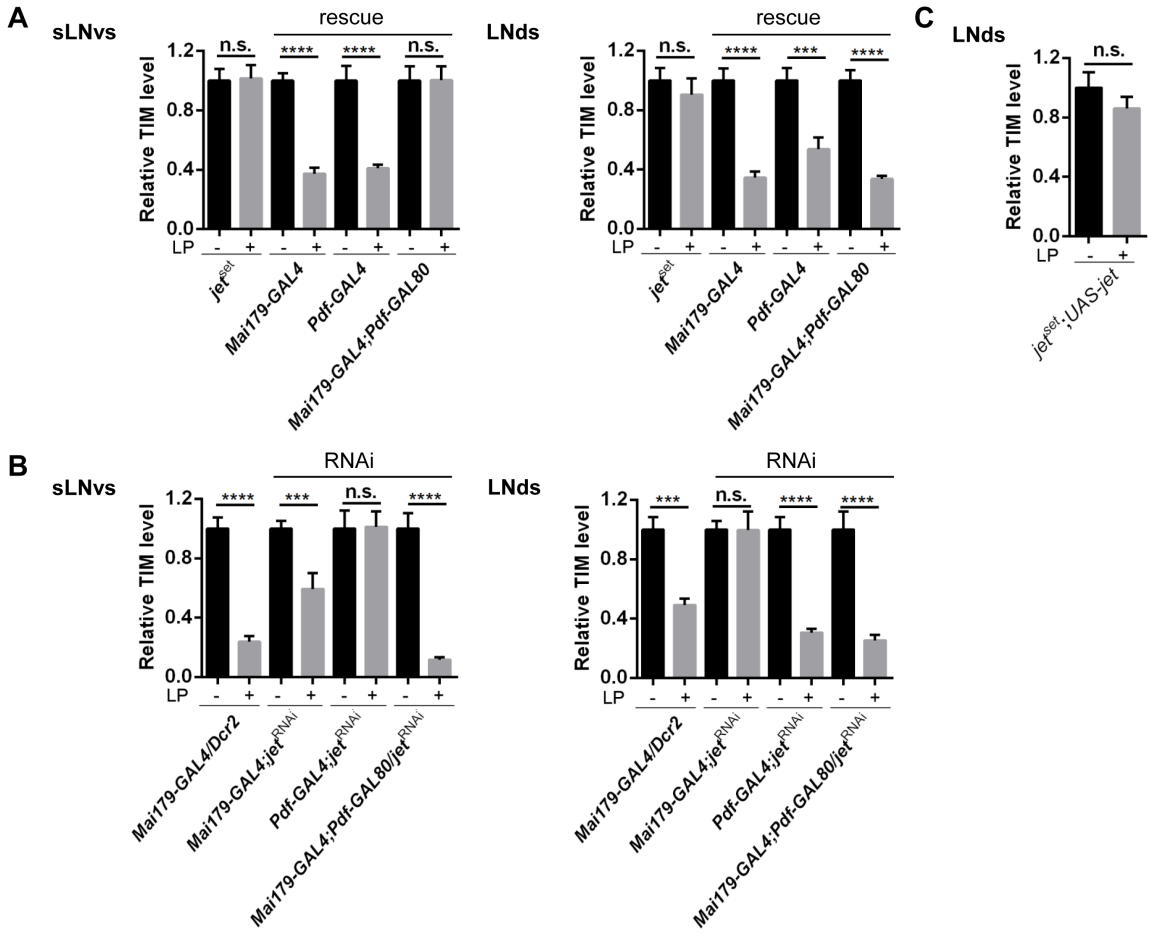
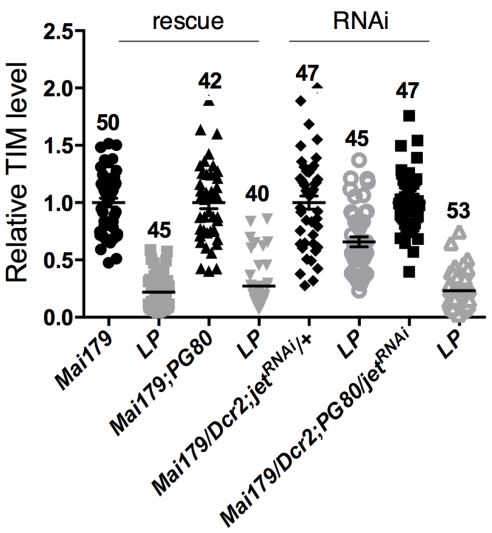


Figure S3: TIM degradation in the M- and E- oscillators after a ZT15 light pulse, Related to Figure 3 and 4.

A. Quantification of TIM levels in the M-oscillators (left) and E-oscillators (right) in neuron-specific rescued jet^{set}. y-axis shows relative TIM levels normalized to no light pulse controls for each genotype. Error bars correspond to S.E.M. n.s. - no significance, ****, $P < 0.0001$; ***, $P < 0.001$ as determined by t-test. Abbreviations of the genotypes are the same as in Fig 3A.

B. TIM levels in M-oscillators (left) and E-oscillators (on right) when *jet* is knocked down using RNAi. Relative TIM levels normalized to no light pulsed control are plotted on the y-axis. Statistics are the same as in Fig S3A. Abbreviations of the genotypes are the same as in Fig 3C.

C. TIM levels in the LNds of jet^{set} flies carrying *UAS-jet* but no *GAL4 driver*. *UAS-jet* alone does not rescue the TIM degradation jet^{set} phenotype. Thus there is no leaky expression of JET in LNds. LP was given at ZT21.



Genotype	% Overlap between light pulsed and non-pulsed LNds
jet^{set}, Mai179-GAL4; UAS-jet	12
jet^{set}, Mai179-GAL4; Pdf-GAL80/UAS-jet	22
Mai179-GAL4/UAS-Dcr2; jetRNAi	98
Mai179-GAL4/UAS-Dcr2; jetRNAi/Pdf-GAL80	14

Figure S4: Distribution of TIM signals in individual LNds with or without light pulses at ZT21, Related to Figure 4.

Left: Each spot represents the relative TIM signal in an individual LNd. Note that most LNds appear to behave similarly within a genotype (and within a brain), which shows that TIM degradation in *Mai179-GAL4* negative LNds is triggered by non-autonomous signals. Error bars correspond to S.E.M. The fly genotypes are 1) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/+, 2) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/*Pdf-GAL80,* 3) *Mai179-Gal4/UAS-Dcr2*; *jet^{RNAi}*/+, 4) *Mai179-Gal4/UAS-Dcr2*; *jet^{RNAi}*/*Pdf-GAL80*. LP is abbreviated for light pulse. Number of neurons quantified are indicated.

Right: Percentage overlap of TIM staining intensity between light-pulsed and non-pulsed LNds. If *Mai179*-positive and –negative LNds behaved as separate groups, overlap should be 50%, since 3 of the 6 LNds are *Mai179*-positive. Indeed, only the rescued cells should show TIM degradation, or only the LNds that do not express *jet* dsRNAs. This is clearly not the case. In each case the percentage was clearly less or more than 50% suggesting that the most LNds behaved as a single population. Chi-square test with Yate's correction confirms our interpretation that the LNds do not behave as two equally divided populations: $p < 0.0001$ for all four genotypes.

Table S1: Circadian locomotor behavior under constant light and constant darkness, Related to Figure 1.

Genotype	Number of flies (n)	Percent rhythmic	Period average (±SEM)	Power average* (±SEM)
Constant light				
w^{1118}	40	0	NA	NA
y w	32	3	20.5	11.5
y w;jet^{set}	30	100	24.36 ± 0.11	73.15 ± 4.23
y w;cry^{b}	32	91	23.9 ± 0.11	63.2 ± 4.49
y w;jet^{set}/*jet*^{c}	16	100	24.3 ± 0.09	101.8 ± 4.56
y w;jet^{set}/*jet*^{r}	14	88	24.9 ± 0.12	104.4 ± 6.78
Constant darkness				
y w	58	76	23.7 $\pm$ 0.06	56.8 $\pm$ 3.59
y w;jet^{set}	51	84	24.1 $\pm$ 0.06	64.3 $\pm$ 3.36

*****Power is a measure of rhythm amplitude and corresponds to the height of the periodogram peak above the significance line (Ewer et al., 1992).

Table S2: TIM degradation in M- and E- oscillators and behavioral phase shifts after light pulses, Related to Figure 2, 3 and 4.

Genotype	JET expression	TIM degradation after light pulse ZT15/ZT21		Phase shift
		s-LNvs (M-oscillator)	LNds (E-oscillator)	ZT15/ZT21
jet^{set}	No JET expression	-/-	-/-	-/-
jet^{set}, Mai179-GAL4;UAS-jet	JET expression in both M and E oscillators	++/++	++/++	++/++
jet^{set}, Pdf-GAL4;UAS-jet	JET expression only in M oscillator	++/++	+/+	+/-
jet^{set}, Mai179-GAL4;UAS-jet/Pdf-GAL80	JET expression only in E oscillator	-/-	++/++	-/-
Mai179-GAL4/UAS-Dcr2	JET expression in both M and E oscillators	++/++	++/++	++/++
Mai179-GAL4/UAS-Dcr2; jetRNAi	JET expression knocked down in M and E oscillators	+/+	+/+	-/+
Pdf-GAL4/UAS-Dcr2; jetRNAi	JET expression knocked down in M oscillator	-/-	++/++	+/+
Mai179-GAL4/UAS-2; jetRNAi/Pdf-GAL80	JET expression knocked down in E oscillator	++/++	++/++	++/++

"++" represents full TIM degradation or phase shift, "+": partial, "-": none

Supplemental Experimental Procedures

Fly stocks

All the flies were raised on cornmeal/agar medium at 25ºC under a light: dark (LD) cycle. The following *Drosophila* strains were used in this study: *y w; jet*^{set} (identified in an ethyl methanesulfonate (EMS) screen), *jet*^{c}, *jet*^{r} and *UAS-jet* (Koh et al., 2006), *y w*, *cry*^{b} (Stanewsky et al., 1998), *tim-GAL4* (Kaneko et al., 2000), *Pdf-GAL4* (Renn et al., 1999), *c929-GAL4* (Hewes et al., 2003), *Clk4.1M-GAL4* (Zhang et al., 2010), *Mai179-GAL4* (Grima et al., 2004), *DvPdf-GAL4* (Bahn et al., 2009), *Pdf-GAL80* (Stoleru et al., 2004), *jet* RNAi (TRiP.JF01506, Bloomington Drosophila Stock Center), *UAS-myc-cry; cry*^{b} (Busza et al., 2004), *Pdf-GAL4; cry*^{b} (Emery et al., 2000) *UAS-jet* and the *GAL4* lines were genetically recombined with *y w; jet*^{set}. *UAS-Dcr2* was combined with the *jet* RNAi lines to increase the efficiency of knockdown. The presence of both *GAL4* and *jet*^{set} in the recombinants was confirmed by PCR and behavior analysis. The presence of the *s-tim* allele in *jet*^{set} mutants and recombinants was determined by PCR and sequencing. All the flies used for *jet*^{set} rescues are homozygotes for the *s-tim* allele. In the RNAi experiments, most flies are *s-tim/ls-tim* heterozygotes, with the exception of the flies expressing *jet* RNAi with *tim-GAL4*, and their *tim-GAL4/UAS-Dcr2* control, which are homozygous for the *ls-tim* allele.

Protein extracts and Western blotting

The *y w; jet*^{set} and *y w* flies were entrained in a 12:12 LD cycle for three days and fly heads were collected on the fourth day at six Zeitgeber times – 1, 5, 9, 13, 17 and 21. For the acute response of light pulse on TIM levels, one group of flies was exposed to a 10 minutes light pulse (1500 lux) at ZT21 and then kept in dark for 1 hour. Protein extraction from the heads of the pulsed and the no light pulsed flies was performed as described previously (Emery et al., 1998). The samples were then run on a 6% SDS-polyacrylamide gels and transferred to nitrocellulose membrane using a semi-dry electroblotting apparatus. Quality of protein transfer was verified by Ponceau red staining. The membranes were probed with 1:1000 dilution of guinea pig anti-TIM. The signal on the film was digitalized using IR-LAS-1000 Lit V2.12 (Fujifilm) and quantified using Image J software. TIM levels were normalized to α-Spectrin.

Behavioral monitoring and analysis

For constant light behavior (LL), locomotor activity of single adult male fly (2-5 days old) was measured with Trikinetics Activity Monitors (Waltham, MA) for 3 days in a 12 hour light: 12 hour dark (LD) cycle at 25°C followed by six days in constant light at an intensity of ca. 200 lux. Data was analyzed using the FaasX software (courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). Rhythmicity was defined by the criteria – power $\geq$10, width $\geq$2 using the χ2 periodogram analysis. Group activity actograms were generated by signal-processing toolbox (Levine *et al.,* 2002) for MATLAB (MathWorks).

For phase shift experiments, flies were entrained to a 12:12 LD cycle for 5 days and were exposed to a 5-minute pulse of a white fluorescent light (1500 lux) at ZT15 and

ZT21 on the 5th day. A separate control group of flies was not light-pulsed. Following the light pulse, flies were released in DD and their locomotor activity was monitored for six days. We found the mid-point of the off-set of subjective evening activity to be the most reliable phase marker across genotypes. It is defined as the time at which the activity of each group of flies (averaged between day 2-6 after the light pulse) reaches 50% of peak value. For the statistical analysis of the phase shifting behavioral experiments, in rescue situation, we used one way analysis of variance (ANOVA) coupled to Tukey's post hoc test for comparison amongst the genotypes. For the *jet* RNAi experiments; we compared each genotype and its *GAL4* driver control using student's t-test.

Whole Mount Immunocytochemistry

For TIM staining, adult flies (2-5 day old) were entrained for 3 days in a 12:12 LD cycle and were subjected to a 5 minutes light pulse of 1500 lux at ZT15 and ZT21 and returned to darkness for an hour before dissection. Flies were then anesthetized and fixed in 4% paraformaldehyde for 45 minutes in darkness. The fixed fly heads were then removed and dissected in PBS. The whole brains were then rinsed and washed with PBT (PBS + 0.1% Triton) three times for 10 mins. For CRY staining, flies were entrained for three days and kept in constant darkness for three days and dissected on the third day at ZT23. Whole-mount immunohistochemistry for fly brains were then done as previously described (Zhang et al., 2010). The brains were incubated with 10% normal goat serum diluted in PBT for 40 mins at room temperature and then incubated with primary antibodies at 4 ºC overnight. Primary antibodies used were: mouse anti-PDF (1:400, from

the Developmental Studies Hybridoma Bank), rabbit anti-PER (1:1500, generous gift from Dr. R. Stanewsky), an affinity purified guinea pig anti-TIM (1:100 (Rakshit et al., 2012)), rabbit anti-CRY (1:200, generous gift from Dr. C. Helfrich-Forster) and mouse anti-GFP (1:200). Brains were incubated with the relevant secondary antibody at 4ºC overnight followed by another round of six washes with PBT. All samples were viewed on a Zeiss LSM5 Pascal confocal microscope. Up to eight fly brains for each genotype were dissected for imaging. Representative images are shown. ImageJ software (NIH) was used for TIM quantification in 18-24 LNds, 12-18 sLNvs and 10-12 DN1as from at least five brains. For quantification, signal intensity in each neuron was measured and then the average signals in three neighboring non-circadian neurons were subtracted. For each genotype, the light pulsed group was compared to its no light pulsed group using a student's t-test. TIM degradation was measured in two independent experiments for both ZT15 and 21 and for all genotypes. Very similar results were obtained. Figures show one of these two independent experiments.

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