Supplementary Information

Methods

Behavioral analysis

Rhythmic flies were defined by $X^2$ periodogram analysis with the following criteria (filter on): power ≥ 20, width ≥ 2 h, with no selection on period value. Power and width are the height and width of the periodogram peak, respectively, and give the significance of the calculated period. Mean daily activity (number of events per 0.5 h) is calculated over the whole period in LD or DD conditions.

Immunolabelings

Fluorescence signals were analyzed with a Zeiss Z1 epifluorescence microscope equipped with a AxioCam MRm digital camera. A Zeiss apotome confocal module was used for some experiments (indicated in legends). For apotome images, stacks of 5-15 confocal sections were used generate a maximum intensity projection. Fluorescence intensity was quantified from digital images with the ImageJ software. We calculated the fluorescence intensity by applying the formula $I = 100n(S - B)/N*B$, which gives the fluorescence percentage above background (where n is the number of labeled cells among the N cells of the considered group (PDF-expressing sLNvs: 4, larval DN1s: 2), S is fluorescence intensity and B is average intensity of the region adjacent to the positive cell).

Primers for quantitative RT-PCR

TCCTTGTCGCTGTGAAACA (5') and CCGAACGAGTGGAAGATGAG (3') for tubulin (464 bp), CCAATAATCCACTGAAAACAT (5') and GAGAGGCACCATTTTCGGAGTAC (3') for clk (367 bp), ACCCGCATCCTTCGCTTTTCTACA (5') and GGGCAGGAGTGGTGACCGAGTGGA
Degradation assays

dsRNA constructs were generated as described previously (Hung et al., 2007), using primers that contained a 5’ T7 RNA-polymerase binding site followed by gene specific sequences for \( egfp \) (Hung et al., 2007), for exon 6 of \( ctrip \) in OBS 66948: TAATACGACTCACTATAGGGACTCAGCATGGTAGTGGATCG (5’) and TAATACGACTCACTATAGGGCATGTTAGGCAGCAATTAGCC (3’), and for exon 3 of \( ctrip \) in OBS 71876: TAATACGACTCACTATAGGGATCACACCAGACTTGCTACTGG (5’) and TAATACGACTCACTATAGGGGAGCGGTAGAAATCAACTTAGG (3’). DNA fragments of the coding region of \( egfp \) and \( ctrip \) target genes were generated by PCR, \textit{in vitro} transcribed using the T7 RiboMAX Express Large Scale RNA Production System (Promega) and RNA products were purified using the SV Total RNA Isolation System (Promega). Purified dsRNAs were annealed by incubation at 65°C for 15 min and slow cooling to room temperature. To analyze for effects of dsRNAs on CLK degradation kinetics, 1 ml of \textit{Drosophila} S2 cells (3 \( \times \) 10⁶ cells/ml) were incubated in serum-free Schneider’s insect medium (Sigma) with 15 µg of dsRNA for 2 hours prior to the addition of 1 ml of Schneider’s insect medium containing 20 % FBS. Cells were incubated for 48 hours prior to the transfection of 3 mg of CLK-expression plasmid (pAc-Clk) with 10 ml of Lipofectin (Invitrogen) as described previously (Hung et al., 2009), with the difference that another 15 µg of dsRNA were added 45 min prior to the addition of 20 % FBS. Two days after
transfection, cells were split into 24 well plates and incubated for 24 hours. Subsequently, new protein synthesis was inhibited by the addition of cycloheximide at a final concentration of 0.58 mM. Cells were harvested over a time-course as indicated in the figure after the addition of cycloheximide and CLK protein levels were determined by western blot analysis, using the anti-CLK antibody described in Hung et al., (2007). CLK protein levels were quantified for different time-points by densitometry and normalized towards an unspecific control band. Amounts of CLK proteins are shown relative to the amount of CLK prior to the addition of cycloheximide (time-point 0 set to 1).
Legends to the Supplementary Figures

Fig S1: *ctrip* cDNA and predicted protein sequences. The cDNA sequence of the longest isolated head transcript is shown (GenBank JF775582). Exons 1, 3, 5, 7, 9 and 11 are in black, exons 2, 4, 6, 8, 10 and 12 are in blue, with exon 4 split in two parts, defined by a splicing acceptor site that was found in some transcripts (GenBank JF775584). The first 30 nucleotides (in red) represent a 5' extension (exon 1) of the FlyBase sequence that we identified by 5' RACE analysis. Polymorphic nucleotides changes (compared to the Flybase sequence) are shown in red (14 sites). The CAG triplet at the E4-E5 junction (indicated in green) was present in half of the E5-containing cDNAs, reflecting the use of two different splicing acceptor sites. Two protein sequences are shown, which correspond to the longest cDNA (first) and the most abundant (second, lacking exons E4-E5, Genbank JF775583) predicted proteins. The 12 polymorphic nucleotides of the coding region generate three amino-acid changes (C197G, Q852H, V1069M) indicated in red, in addition to the optional A913 that is shown in green (numbering according to the longest protein sequence). The Armadillo, WWE and HECT domains are shown on the sequence of the most abundant protein as shaded in yellow, green and grey, respectively.

Fig S2 *ctrip* transcripts do not cycle in the head. Quantitative RT-PCR for three different *ctrip* cDNA regions in LD: E3-containing transcripts (all transcripts) (blue), E3-E4b -containing transcripts (red) and E3-E4a -containing transcripts (green). mRNA abundance values of E3-E4a and E3-E4b were multiplied by 10 for easier comparison. Black bars represent s.e.m., which is calculated for time point values averaged from three independent experiments. Other values are averaged from two independent experiments. Primers: E3 (377 bp): 5': TCGAATTCAAGAATCAACTTTAGTGCGAC, 3':
Fig S3 Immunofluorescence triple-labelings of larval (top) and adult (bottom) sLNvs at CT0. CT0 Images are shown to illustrate the labelings that were used for the quantifications shown in Figure 2. Only one focal plan is shown that contains either two or four neurons. CLK is green, PER is red and TIM is blue. Although GP47 recognizes non CLK specific epitopes in non clock cells (Houl et al., 2006, 2008), no GP47 signal could be observed in the clock neurons of clkjrk larval and adult brains (not shown). Scale bar represents 10μm.

Fig S4: ctrip but not Hus1-like affect CLK and PER levels. Quantification of CLK and PER immunolabelings in the larval DN1s (A), larval sLNvs (B) and adult sLNvs (C) at CT0. Images were taken with an apotome module. w flies are used as controls. Df6142 is for the Df(3R)Exel6142 deficiency, which deletes the 82D2-82D6 region of chromosome 3R chromosome and thus includes ctrip (Parks et al., 2004). Df6243 is for Df(3R)Exel6143, which deletes the 82E4-82E7 adjacent region that does not include ctrip. Error bars indicate the s.e.m. (n=20-25 brain hemispheres).

Fig S5: Clock protein oscillations in flies expressing ctrip RNAi under pdf-gal4 control

CLK, PER and TIM immunofluorescence in the adult sLNvs. Flies were entrained in 12:12 LD cycles and transferred to DD, at 25°C. Brains were dissected every four hours during the first and second days of DD. Labelings and quantifications of the signal were done as
described in the Methods section. Error bars indicate the s.e.m. (n=20-25 brain hemispheres). Grey and black bars represent subjective day and night, respectively. Circadian Time is indicated in hours.

**Fig S6 CLK levels in head extracts of ctrip RNAi flies and controls.** Quantification of anti-CLK western blots in LD and DD1 (as illustrated in Fig. 3A). Autoradiograms of anti-CLK and anti-PER (after stripping the membrane and reprobing with anti-PER antibody) western blots were scanned. Files were analyzed with ImageJ software to quantify the CLK band and a PER unspecific band that was used as a loading control (see Fig. 3A). A CLK/control ratio was calculated and values were normalized to the *tim-gal4/+* ZT0 or CT0 value for each gel. Curves represent mean values from two (top) or three (bottom) independent gels. Top: Triangles (*tim-gal4/+*) and squares (*tim-gal4/ctrip RNAi*) bracketing each mean value represent the two independent values that were used to calculate the mean. Bottom: bars represent the s.e.m.

**Fig S7 PER and TIM oscillations in head extract of ctrip RNAi flies.** Western blot analysis of PER and TIM protein in head extracts during LD and the first day of DD. Flies were collected every 1.5h during day or subjective day and every 3h during night or subjective night. Control: *w;tim-gal4/+, ctrip RNAi: w;tim-gal4/+;UAS-ctrip RNAi/+.*
References


Supplementary Table 1: Activity rhythms of ctrip RNAi rescued flies in DD.

R: Rhythmic flies; Tau: period value in hours; s.e.m: standard error of the mean; 14R3 and 17R3 are UAS-ctrip RNAi transgenes targeting ctrip exons 2 and 6, respectively. 17R2 (RNAi line 17735R2) and 17R3 are different insertions of the same CG17735 RNAi transgene targeting the ctrip E6 exon. tim-gal4 -driven UAS-ctrip induces a 4 fold increase of ctrip mRNA levels in heads, which were reduced by two folds in the presence of ctrip RNAi (not shown). UAS-gfp is added to mimic putative titration of the GAL4 protein by UAS sequences that may occur when adding UAS-ctrip to ctrip RNAi flies. As opposed to UAS-ctrip, UAS-gfp does not change the circadian period of the ctrip RNAi flies.

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<th>Power (s.e.m)</th>
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<td>100</td>
<td>24.3 (0.1)</td>
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Figure S1

ctrip longest cDNA (E1-E12, 10 587 bp)

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Longest putative CTRIP protein (3140 aa, encoded by E2-E12)

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Most abundant putative CTRIP protein (2707 aa, lacking E4-E5)

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Figure S3
Figure S4
Figure S5

Mean intensity

CLK

PER

TIM

;pdf-gal4;17R3,14R3

;pdf-gal4
Figure S6
Figure S7