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The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in *Drosophila*

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In the *Drosophila* circadian clock, the CLOCK/CYCLE complex activates the *period* and *timeless* genes that negatively feedback on CLOCK/CYCLE activity. The 24-h pace of this cycle depends on the stability of the clock proteins. RING-domain E3 ubiquitin ligases have been shown to destabilize PERIOD or TIMELESS. Here we identify a clock function for the *circadian trip* (*ctrip*) gene, which encodes a HECT-domain E3 ubiquitin ligase. *ctrip* expression in the brain is mostly restricted to clock neurons and its downregulation leads to long-period activity rhythms in constant darkness. This altered behaviour is associated with high CLOCK levels and persistence of phosphorylated PERIOD during the subjective day. The control of CLOCK protein levels does not require PERIOD. Thus, CTRIP seems to regulate the pace of the oscillator by controlling the stability of both the activator and the repressor of the feedback loop.

Keywords: circadian; CLOCK; PERIOD; ubiquitination; UFD pathway

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INTRODUCTION

Circadian clocks rely on transcriptional negative feedback loops. In *Drosophila*, the basic helix–loop–helix PAS domain proteins CLOCK (CLK) and CYCLE (CYC) activate the transcription of *period* (*per*) and *timeless* (*tim*) in the evening (Weber, 2009; Allada & Chung, 2010). PER and TIM progressively accumulate and enter the nucleus, in which they repress CLK/CYC-dependent transcription late in the night. PER and TIM are subject to complex post-translational controls that involve several kinases such as DOUBLE TIME CK1e, CK2 and SHAGGY GSK3, as well as the PP1 and PP2A phosphatases. CLK phosphorylation cycles in a similar manner, with mildly phosphorylated CLK activating transcription in the evening (Kim & Edery, 2006; Yu *et al*, 2006). PER represses CLK activity by triggering its hyperphosphorylation and release from DNA (Kim & Edery, 2006; Yu *et al*, 2006, 2009; Menet *et al*, 2010). However, CLK protein turnover remains unclear as the contributions of protein-level cycling and phosphorylation cycling are still debated (Houl *et al*, 2006; Yu *et al*, 2006; Hung *et al*, 2009).

The stability of phosphorylated PER relies on a proteasome-dependent pathway that requires the SUPERNUMERARY LIMBS E3 ubiquitin ligase (Chiu *et al*, 2008). SUPERNUMERARY LIMBS is part of a CULLIN 1-based SCF complex that belongs to the RING family of E3 ubiquitin ligases (Nakayama & Nakayama, 2005). Another family of E3 ubiquitin ligases has been characterized, whose members contain a HECT (homologous to E6AP C-terminus) domain (Rotin & Kumar, 2009). The mammalian protein TRIP12 (thyroid hormone receptor-interacting protein 12; Lee *et al*, 1995) is one of them and has been proposed to be involved in amino-terminal ubiquitination (Park *et al*, 2009; Chen *et al*, 2010).

In an enhancer trap screen, two *Gal4*-encoding P-elements secreting the pigment-dispersing factor (PDF) neuropeptide were found that are strongly expressed in the neurons which are key pacemaker cells for the control of *Drosophila* rest–activity rhythms (Dubruille & Emery, 2008). Here, we show that the target gene is the orthologue of *trip12*, and we named the *Drosophila* gene *circadian trip* (*ctrip*) because its downregulation lengthens the period of behavioural rhythms. Molecular analysis indicates that CTRIP destabilizes CLK protein in a PER-independent manner and helps degradation of phosphorylated PER and TIM in the morning.

RESULTS AND DISCUSSION

ctrip is strongly expressed in the PDF clock neurons

The expression of the gal1118 *Gal4* enhancer trap has been described as essentially restricted to the PDF-expressing ventral lateral neurons of the *Drosophila* brain, with weaker expression in other clock cells as well as a few non-clock cells (Blanchardon *et al*, 2001). Another insertion from the same collection of P-element *Gal4* lines (Boquet *et al*, 1999), gal1501, showed a similar expression pattern in the adult (Fig 1A) and larval (Fig 1B)

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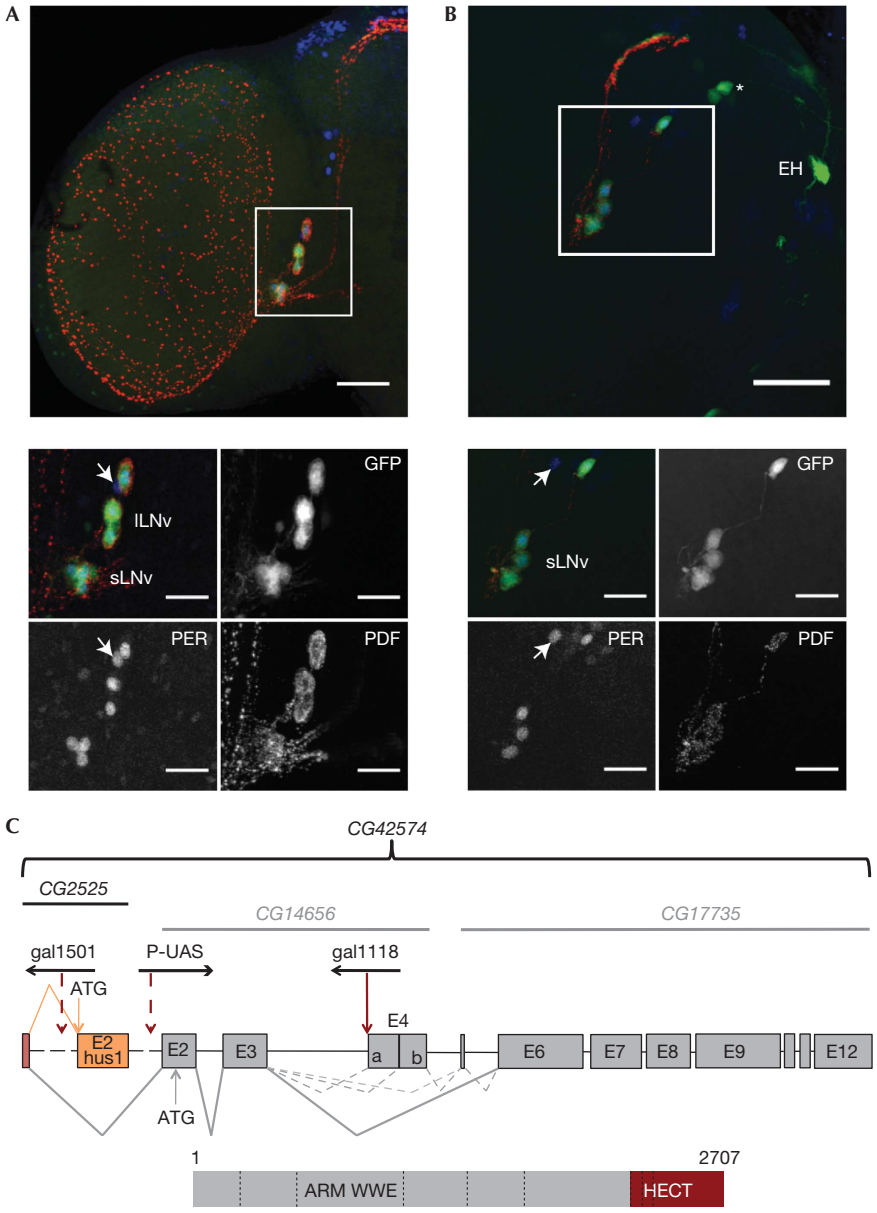

Fig 1 | Brain expression and genomic organization of *ctrip*. (**A, B**) GFP labelling of adult (**A**) or larval (**B**) brain of *w; UAS-gfp; gal1501/+* flies at zeitgeber time (ZT) 0 in light–dark conditions (ZT 0 is lights-ON). gal1501-driven GFP fluorescence is combined with PER and PDF antibodies. All images are projections of apotome stacks. The white squares on the half-brain images correspond to the small images. Colours in the merge pictures are green for GFP, blue for PER and red for PDF. Arrow points to the fifth PDF-negative SLNV. GFP can be seen in at least three PER-negative cells in each dorsal hemisphere of the larval brain: the EH-synthesizing neurons and a pair of dorsal neurons (asterisk) that seem to be the prothoracic-gland-innervating neurons of the lateral protocerebrum (PG–LP; Blanchardon *et al*, 2001). Weak expression in other clock neuron subsets can be detected in flies homozygous for the gal1501 and UAS-*gfp* transgenes (not shown). Scale bars represent 50 (half brains) or 25 (small images) μm. (**C**) Schematic representation of the *ctrip* gene. Top: current (black) and previous (grey) Flybase nomenclature. Middle: exon/intron structure with common (red), *Hus1-like*-specific (orange) and *ctrip*-specific (grey) exons as boxes. Dotted lines represent out-of-scale regions (see http://flybase.org/). Splicing events are shown as grey lines for the most abundant transcripts and dotted grey lines for the less abundant ones. E4a/b boundary is defined by a splicing acceptor site located 630 bp downstream of the beginning of E4. Putative translation start sites (ATG) are indicated for both *Hus1-like* and *ctrip*. P-element insertions are shown with the transcription orientation of the *gal4* gene (gal1118 and gal1501) or UAS sequence (P-UAS). gal1501 is inserted 3208 bp upstream from *Hus1-like* coding exon and gal1118 is inserted 78 bp upstream from E4. P-UAS refers to P-UAS 11430 that is inserted 1673 bp upstream from E2. Bottom: predicted protein encoded by the most abundant transcript. Exon boundaries are represented by dotted lines. ARM and WWE domains are encoded by E6, whereas the HECT domain is encoded by E11–12. ARM, Armadillo; *ctrip*, *circadian trip*; EH, eclosion hormone; GFP, green fluorescent protein; HECT, homologous to E6AP C-terminus; lLNv, large ventral lateral neuron; PDF, pigment-dispersing factor; PER, PERIOD; P-UAS, P-element-carrying Upstream Activating Sequence; RNAi, RNA interference; sLNv, small ventral lateral neuron.

Table 1|Locomotor activity rhythms in dark–dark conditions

Genotype	Number of flies	Rhythmic flies (%)	Tau (s.e.m.)	Power (s.e.m.)
w	26	96	23.4 (0.1)	108 (9)
w;pdf-gal4/+	8	100	24.0 (0.1)	68 (2)
w;tim-gal4/+	29	97	24.1 (0.1)	129 (4)
w;;17R3/TM6	8	100	23.7 (0.1)	135 (6)
w;;17R3	7	100	24.0 (0.2)	117 (9)
w;;14R3/TM6	16	88	24.0 (0.0)	53 (3)
w;;14R3	16	94	24.2 (0.2)	80 (10)
w;;Y4	20	90	23.7 (0.1)	86 (9)
w;;hus1-like^{37}	30	100	24.3 (0.1)	104 (4)
w;;19F1	17	94	24.2 (0.1)	121 (4)
w;pdf-gal4/+;17R3/+	32	81	25.3 (0.1)	56 (4)
w;pdf-gal4/+;14R3/+	46	89	25.1 (0.1)	66 (2)
w;pdf-gal4/+;17R3,14R3/+	16	100	26.2 (0.1)	59 (3)
w;pdf-gal4;17R3,14R3/+	26	96	27.0 (0.2)	61 (4)
w;pdf-gal4;17R3	29	83	27.8 (0.2)	84 (7)
w;pdf-gal4;14R3	34	91	26.0 (0.1)	84 (7)
w;pdf-gal4;17R3,14R3	16	87	28.2 (0.6)	71 (8)
w;tim-gal4/+;17R3/+	52	94	26.2 (0.1)	125 (6)
w;tim-gal4/+;14R3/+	75	88	25.3 (0.1)	88 (6)
w;tim-gal4/+;17R3,14R3/+	30	100	26.4 (0.1)	146 (4)

14R3 and *17R3* are *UAS-ctrip RNAi* transgenes targeting *ctrip* exons 2 and 6, respectively.
DD, dark–dark conditions; Tau, period in hours; s.e.m., standard error of the mean.

brain, with an even higher specificity for the PDF neurons. The two P-elements are located in a region that includes the DNA repair gene *Hus1-like* (*CG2525*; Abdu *et al*, 2007) and a large gene (*CG42574*; http://flybase.org/) that is homologous to the human *trip12* gene (Fig 1C). *CG42574* encodes a putative E3 ubiquitin ligase that contains Armadillo repeats, a WWE protein–protein interaction domain (Aravind, 2001) and a carboxy-terminal HECT domain.

The localization of gal1118 supported the idea that *CG42574* is responsible for the brain expression pattern and *CG42574* was named *ctrip*. Reverse transcription (RT)–PCR and sequence analysis of head transcripts indicated that *Hus1-like* and *ctrip* messenger RNAs (mRNAs) share a non-coding first exon, and that *ctrip* contained 12 exons with alternative splicing in the E3–E6 region (Fig 1C; supplementary Fig S1 online). In the adult head, *ctrip* mRNA levels were approximately tenfold more than *per* mRNA levels (not shown), suggesting a broad expression outside the brain. The most abundant *ctrip* head transcript contained 10 exons (lacking E4–E5) and encoded a 2707-amino-acid putative protein, but transcripts including E4–E5 or E4b–E5 or only E5 could be detected at levels 10–15 times lower (Fig 1C; supplementary Figs S1,2 online). None of the *ctrip* mRNAs showed oscillations of its abundance in the head in light–dark cycles (supplementary Fig S2 online).

The *CG17735* transcript was recently identified as 5–15-fold enriched in the PDF neurons compared with all brain neurons (Kula-Eversole *et al*, 2010), validating the expression pattern of the two *ctrip* P-*gal4* insertions. In contrast to *ctrip* transcripts in whole-head extracts, *CG17735* transcripts cycled in the PDF cells, reaching peak levels in the first half of the night (Kula-Eversole *et al*, 2010). The strong expression of *ctrip* in the head suggests that it includes both clock cells and non-clock cells, which could mask oscillations in specific subsets.

Loss of *ctrip* lengthens the clock period in larval neurons

Deletions were generated by P-element excision. The Y4 deletion removed the *Hus1-like* coding exon and induced female sterility, similarly to the *Hus1-like^{37}*-null mutant (Abdu *et al*, 2007). Homozygous Y4 or *Hus1-like^{37}* flies showed normal activity rhythms, indicating that *Hus1-like* is not involved in the behavioural clock (Table 1). The 19F1 deletion removed the *ctrip* alternative exons E4–E5, leading to an mRNA species that is identical to the most abundant wild-type transcript. 19F1 homozygous flies were viable and behaved similarly to wild-type (Table 1), indicating that the glutamine-rich region encoded by E4–E5 was not required for clock function. The 32.3 deletion removed both *Hus1-like* and exons 1–5 of *ctrip*, and was homozygously lethal at the pupal stage. *ctrip* larvae were analysed for clock protein oscillations in the lateral neurons (Fig 2A; supplementary Fig S3 online). High-amplitude PER and TIM oscillations were observed in the 32.3 mutant, with an increasing delay over 2.5 days in dark–dark conditions, revealing a long

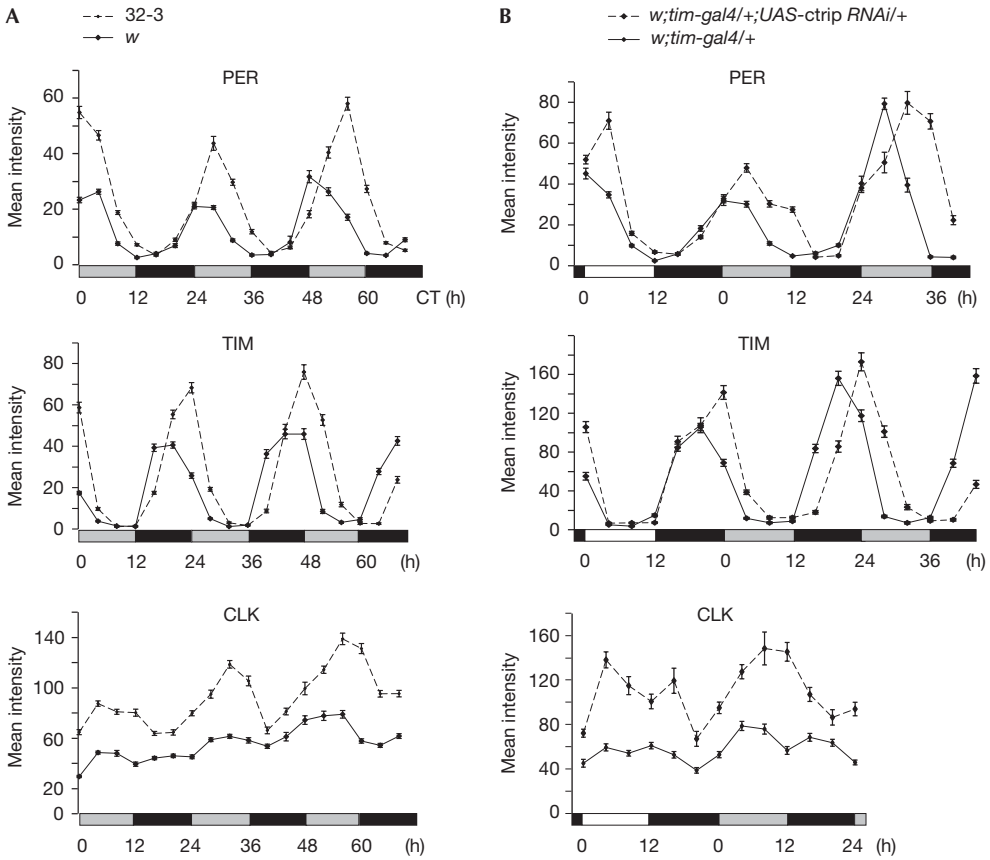

Fig 2 | Clock-protein cycling in *ctrip* mutant larvae and *ctrip* RNAi adults. CLK, PER and TIM immunofluorescence in the larval (**A**) or adult small ventral lateral neurons (**B**). Error bars indicate the s.e.m. ($n = 20–25$ brain hemispheres). White, grey and black bars represent day, subjective day and night, respectively. Time is indicated in hours. (**A**) Larvae were grown in 12:12 LD cycles and transferred to DD conditions. Experiments were done at 20 °C to slow development, so that larval brains could be dissected up to 3 days after transfer to DD conditions. Larvae were collected every 4 h from the first day in DD conditions for brain dissection. (**B**) Flies were entrained in 12:12 LD cycles and transferred to DD conditions, at 25 °C. Brains were dissected every 4 h on the last day of LD conditions, followed by 1 or 2 days in DD conditions. CLK, CLOCK; CT, circadian time (CT0 is the beginning of the first day in DD); *ctrip*, *circadian trip*; DD, dark–dark; LD, light–dark; PER, PERIOD; RNAi, RNA interference; TIM, TIMELESS.

period. As reported previously (Hung *et al*, 2009), the wild-type larvae displayed low-amplitude CLK oscillations, but higher CLK levels and higher cycling amplitude were observed in 32.3 larvae (Fig 2A; supplementary Fig S3 online). A similar CLK and PER increase was observed in the dorsal neurons, indicating that *ctrip* was also acting in PDF-negative cells (supplementary Fig S4 online). To verify that the phenotype was due to *ctrip* loss, we analysed larvae carrying 32.3 over a deficiency that did or did not encompass *ctrip* region. At the beginning of the first day in dark–dark conditions, CLK and PER levels were increased only in larvae homozygous for the loss of the *ctrip* locus (supplementary Fig S4 online). In agreement with their wild-type behaviour, *Hus1-like*^{37} adults had normal CLK and PER levels (supplementary Fig S4 online). The absence of *ctrip* thus increases CLK levels and slows clock-protein cycling in the clock neurons.

ctrip RNA interference slows the behavioural clock

RNA interference (RNAi) constructs directed against *ctrip* exons 2 (14R3) or 6 (17R3) were expressed in either the PDF-expressing cells or all clock cells (*tim*-expressing cells), and the transgenic flies were analysed for locomotor activity rhythms in dark–dark conditions. A lengthening of the period was observed with both *pdf-gal4* and *tim-gal4* drivers (Table 1), indicating that *ctrip* is required for 24-h behavioural rhythms. A P-UAS insertion (see Fig 1B) was used to overexpress *ctrip* under *tim-gal4* control, but this did not alter behaviour (supplementary Table S1 online), possibly because CTRIP acts with a partner that is present in limiting amounts. However, 24-h behavioural rhythms were rescued in flies co-expressing *ctrip* RNAi and the P-UAS-induced transcript, further supporting the idea that *ctrip* downregulation is responsible for the behavioural defect found in *ctrip* RNAi flies (supplementary Table S1 online). A large-scale RNAi screen has revealed that flies downregulated for *CG17735* (see Fig 1) had long-period behavioural rhythms (Sathyanarayanan *et al*, 2008). Although we could not detect the reported inhibition of light-induced CRY degradation with our *ctrip* RNAi flies (Peschel *et al*, 2009), it provides an independent confirmation of our behavioural results.

Clock-protein oscillations were analysed in the small ventral lateral neurons of *w;tim-gal4/+;17R3,14R3/+* flies, hereafter

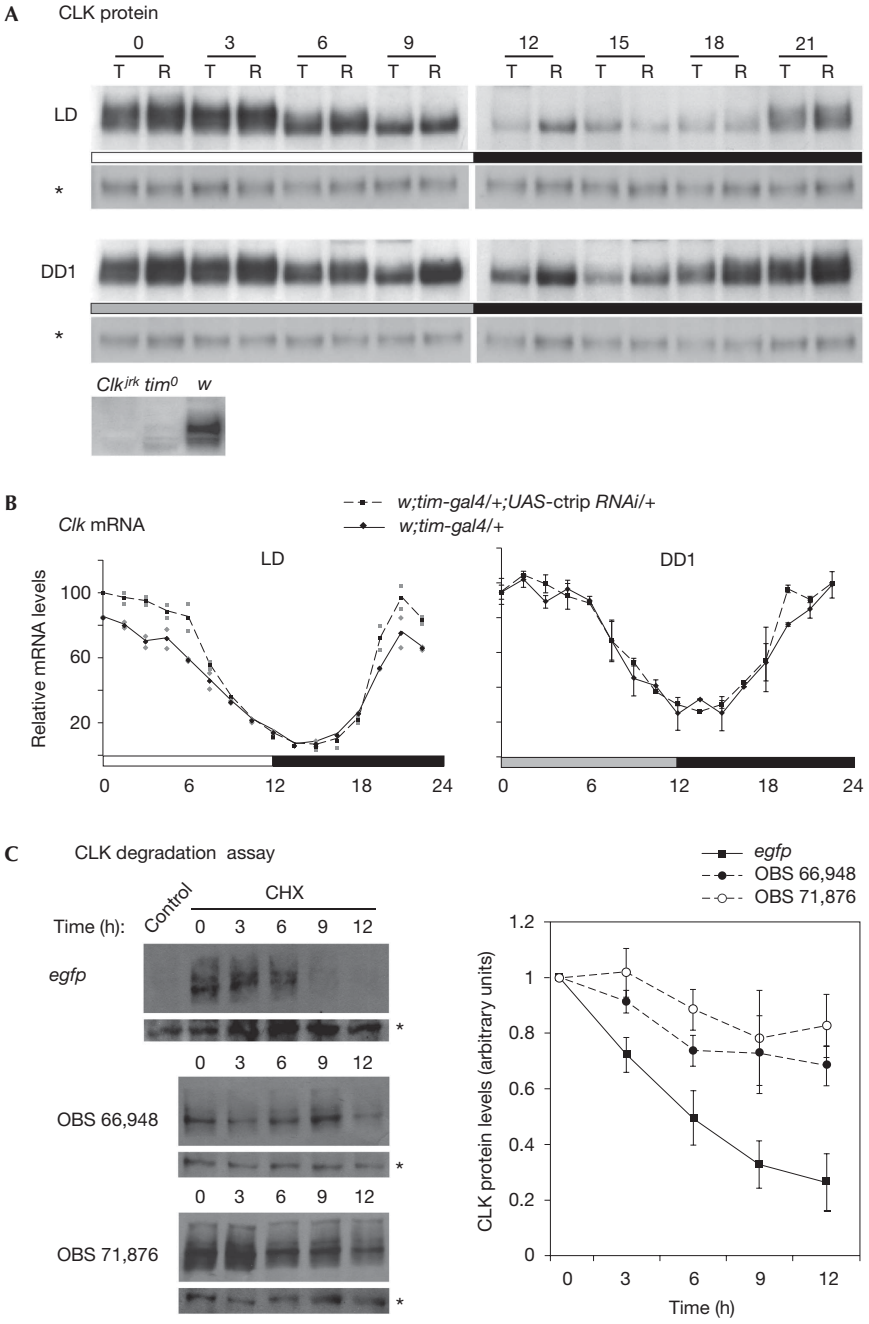

Fig 3 | CTRIP inhibition stabilizes CLK protein. Flies were entrained in 12:12 LD cycles and transferred to DD conditions, at 25 °C. Head extracts were prepared from time points collected every 3 h (**A**) or 1.5 h (**B**) during the last LD day and the first DD day. (**A**) Western blot analysis of CLK protein. T, *w;tim-gal4/+* controls; R, *w;tim-gal4;UAS-ctrip RNAi*. Asterisks indicate a PER antibody unspecific band on the same gel that was used as a loading control. Antibody specificity is shown by the absence of signal (CT3) in Clk^{irk} extracts and the very weak signal in tim^{0} extracts under the same conditions. (**B**) Quantitative RT–PCR of *Clk* mRNA. The results are the average of two (LD) or three (DD) independent experiments. Grey dots indicate the maximum and minimum values. Error bars indicate the s.e.m. ($n = 3$ experiments). (**C**) RNAi-mediated inhibition of CTRIP reduces CLK degradation in *Drosophila* S2 cells. Degradation kinetics of CLK were analysed after blocking new protein synthesis by the addition of cycloheximide in the presence of dsRNA targeting *ctrip* exons 3 (OBS 71874) or 6 (OBS 66948) or *egfp*. Left panel: anti-CLK western blots of S2 cell lysates harvested at 0, 3, 6, 9, and 12 h after cycloheximide treatment. The *egfp-dsRNA* control is from untransfected and untreated cells. The CLK antibody used in this experiment was described in Hung *et al* (2007) and recognizes an unspecific band (asterisk) that was used as a loading control. Right panel: average degradation kinetics of CLK protein from five cycloheximide chase experiments (as in the left panel), with the amount of CLK protein prior to the addition of cycloheximide (time 0) set to 1. Error bars indicate the s.e.m. CHX, cycloheximide; CLK, CLOCK; *ctrip*, *circadian trip*; DD, dark–dark; dsRNA, double-stranded RNA; LD, light–dark; mRNA, messenger RNA; OBS, Open BioSystem; PER, PERIOD; RNAi, RNA interference; *tim*, *timeless*.

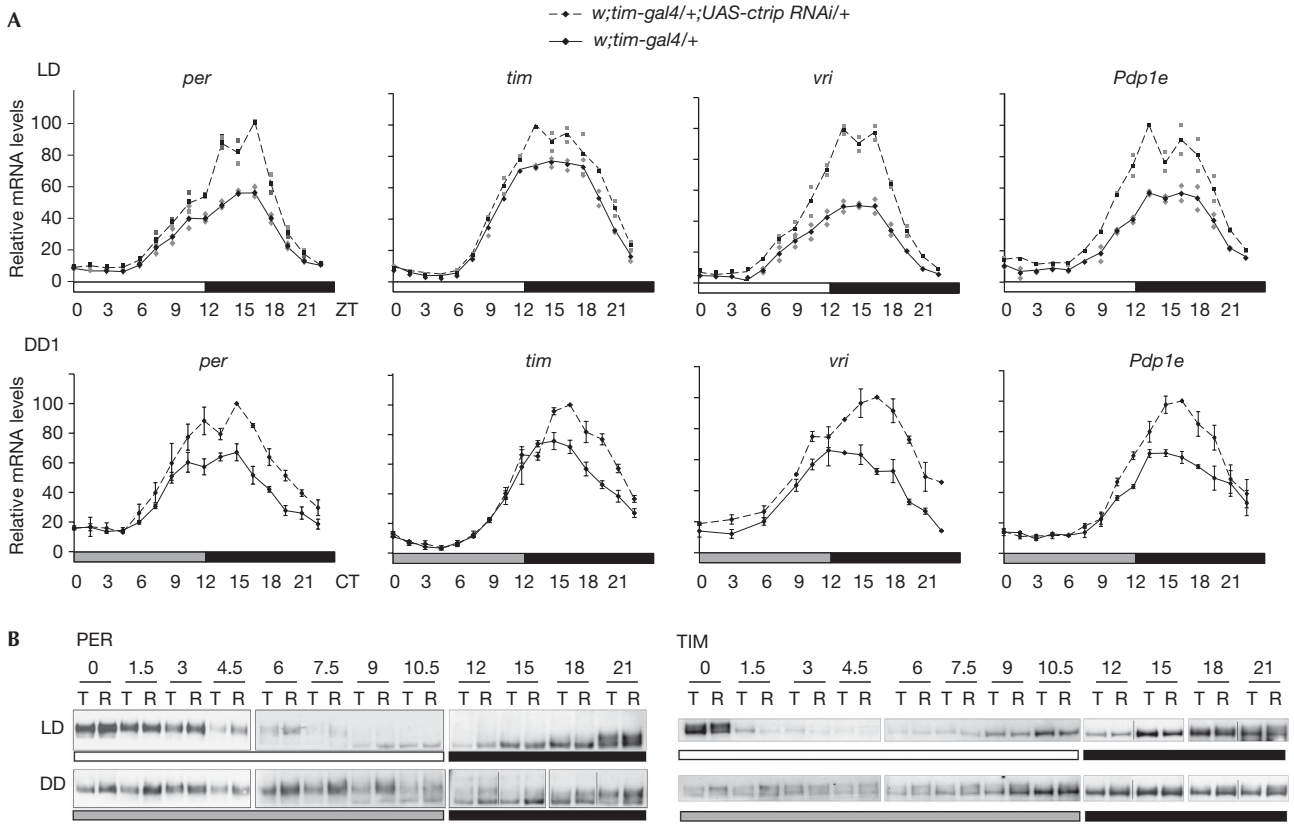

Fig 4 | Levels of *per* and *tim* RNA and PER and TIM protein in *ctrip* RNAi flies. (**A**) Relative mRNA levels of *per*, *tim*, *vri* and *Pdp1e* in head extracts were determined by quantitative RT–PCR in LD conditions and during the first day of DD conditions. The results are the average of two (LD) or three (DD) independent experiments. Grey dots indicate the maximum and minimum values. Error bars indicate the s.e.m. (**B**) Western blot analysis of PER and TIM protein in head extracts during LD conditions and the first day of DD conditions. Flies were collected every 1.5 h during day or subjective day and every 3 h during night or subjective night. T, *w;tim-gal4/+*; R, *w;tim-gal4/+;UAS-ctrip RNAi/+*. Boxes are different gels and vertical lines indicate that the lanes were not adjacent on the gel. *ctrip*, *circadian trip*; CT, circadian time; DD, dark–dark; LD, light–dark; mRNA, messenger RNA; *Pdp1e*, *Par domain protein1*; *per*, *period*; RNAi, RNA interference; RT, reverse transcription; *tim*, *timeless*; *vri*, *vrille*; ZT, zeitgeber time.

referred to as *tim-gal4 UAS-ctrip RNAi* (Fig 2B; supplementary Figs S3,5 online). A progressive delay of clock-protein oscillations was observed in dark–dark conditions, in agreement with the behavioural results. The increase at night of PER and TIM was not delayed in light–dark conditions (Fig 2B) and no difference in the nuclear entry of the proteins was observed (not shown). By contrast, the decrease of protein levels was shifted during the first subjective day of dark–dark conditions, suggesting that lengthening of the period is due to delayed protein degradation. As seen in the lateral neurons of the 32.3 mutant larvae, high CLK levels were observed at all time points. However, a stronger effect seemed to occur late in the day, suggesting that CTRIP levels (see also Kula-Eversole *et al*, 2010) and/or function might be regulated. Clock-protein oscillations were also analysed in the small ventral lateral neurons of flies expressing *ctrip* RNAi under *pdf-gal4* control, with similar results (supplementary Fig S5 online).

CTRIP controls CLK protein stability

CLK protein and *Clk* mRNA levels were analysed in *tim-gal4 UAS-ctrip RNAi* head extracts. Slightly higher CLK levels were observed in light–dark conditions, and a stronger effect was observed in dark–dark conditions (Fig 3A; supplementary Fig S6 online). The increase of CLK in *ctrip* RNAi flies was less important in head extracts than in clock neurons (see Fig 2), suggesting that RNAi expression in the eye was weaker. Although a slight increase in *Clk* mRNA peak levels was observed in light–dark conditions, *ctrip* downregulation did not affect *Clk* mRNA levels in dark–dark conditions (Fig 3B). This indicated that the higher protein levels were a consequence of post-transcriptional control. As *ctrip* encodes a ubiquitin ligase, the simplest interpretation is that *ctrip* induces CLK protein degradation.

CLK protein stability was thus tested in S2 cell culture. Cycloheximide chase experiments showed reduced CLK degradation kinetics in the presence of either of two *ctrip* double-stranded RNAs directed against exons 3 and 6 of the gene, compared with an unspecific *egfp* control double-stranded RNA (Fig 3C). This indicated that CTRIP destabilizes CLK in S2 cells, supporting the idea of such a function in the clock neurons.

ctrip downregulation decreases PER/TIM degradation

Transcripts of CLK direct targets (*per*, *tim*, *vri* and *Pdp1e*) were quantified in *tim-gal4 UAS-ctrip RNAi* flies (Fig 4A). In light–dark

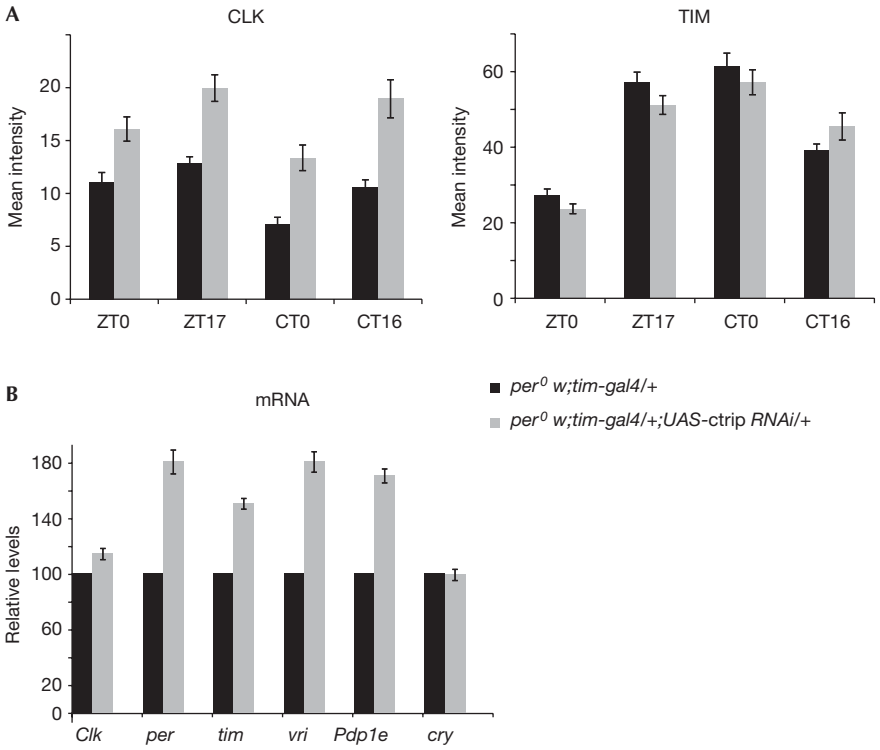

Fig 5 | Effect of *ctrip RNAi* in *per*^{0} background. (**A**) Immunofluorescence quantification of CLK and TIM protein levels in the PDF-expressing small ventral lateral neurons of adult flies in LD (ZT) and DD (CT). Error bars indicate the s.e.m. ($n = 20–25$ brain hemispheres). (**B**) Quantitative RT–PCR analysis of *Clk*, *cry* and CLK direct targets *per*, *tim*, *vri* and *Pdp1e* in LD conditions (ZT14). Each experiment has been normalized to the control value (black bar). Error bars indicate the s.e.m. ($n = 3$ experiments). CLK, clock; *ctrip*, *circadian trip*; CT, circadian time; DD, dark–dark; LD, light–dark; *Pdp1e*, *Par domain protein1*; *per*, *period*; RNAi, RNA interference; *tim*, *timeless*; *vri*, *vrille*; ZT, zeitgeber time.

conditions, peak levels of the four transcripts were approximately 1.5-fold higher in the RNAi flies, but decreased to basal levels at the end of the night. On the first day of dark–dark conditions, the RNAi flies did not show a delayed start of mRNA increase but rather a longer increase of mRNA levels, resulting in a higher peak with a slight delay. This indicates higher CLK transcriptional activity of the RNAi flies in the middle of the night. PER and TIM protein cycling was tested by western blots of head extracts (Fig 4; supplementary Fig S7 online). In light–dark conditions, only slightly more phosphorylated PER was detected in the morning in *ctrip* RNAi flies. In dark–dark conditions, phosphorylated PER was more abundant throughout the subjective day, supporting the idea of deficient protein degradation in RNAi flies in the absence of light. A similar but milder effect was observed for the TIM protein.

Transgenic flies with increased CLK levels have been shown to display either no period change (Kim *et al*, 2002) or a mild shortening of the period, which might result from a premature nuclear entry of PER (Kadener *et al*, 2008). By contrast, the persistence of phosphorylated PER and TIM during the subjective day has been associated with a long behavioural period in various mutants (Rothenfluh *et al*, 2000; Suri *et al*, 2000). It therefore seems likely that the long-period phenotype of *ctrip* RNAi flies is a consequence of long-lasting PER and TIM during the day, in the absence of light-induced degradation.

The higher peak levels of CLK target transcripts in the RNAi flies indicates that CLK-dependent transcription is more active when *ctrip* is downregulated. However, PER and TIM proteins do not seem to accumulate faster, but show a persistence of their phosphorylated forms during the subjective day. By contrast, *per* and *tim* mRNAs reach trough levels early in the day, as in the wild type. Increased PER and TIM levels in the morning are thus not a consequence of elevated *per* and *tim* mRNA levels, but seem to be due to a post-translational action of CTRIP on PER and TIM. By destabilizing both the CLK activator and the PER repressor, CTRIP might have a buffering role in the transcriptional feedback loop.

Although PER and TIM persist during the subjective day, the evening accumulation of their mRNAs is not delayed in *ctrip* RNAi flies. Higher hypophosphorylated CLK levels probably counteract the repressing effect of higher PER levels. In the evening, the normal kinetics of mRNA accumulation might thus be the consequence of elevated CLK and PER levels. In the middle of the night, PER and TIM levels do not seem to be higher and CLK target transcripts continue to increase for a longer time, indicating that the repression phase is delayed in RNAi flies. A simple interpretation of this is that elevated CLK levels are responsible for extending the time of active transcription. Alternatively, down-regulation of *ctrip* might delay repression by destabilizing the PER/CLK complex or reducing its repressing function.

CTRIP-mediated CLK regulation is PER-independent

We asked whether increased CLK levels might be a consequence of abnormal PER degradation in the *ctrip* RNAi flies. *per^0; tim-gal4 UAS-ctrip RNAi* flies were thus analysed for CLK and TIM levels in the PDF neurons (Fig 5A). In the absence of PER, TIM levels were not affected, but *ctrip* RNAi induced a 1.5-fold increase of CLK levels. In agreement with their increased CLK levels, *per^0; tim-gal4 UAS-ctrip RNAi* flies displayed higher transcript levels for the CLK target genes, whereas *Clk* and *cry* were not affected (Fig 5B). This indicates that the persistence of phosphorylated TIM in the morning requires PER, whereas the control of CLK levels by CTRIP is a PER-independent mechanism.

CLK and PER thus seem to be the main targets of CTRIP. The ubiquitin ligase might act independently on CLK and PER, with the two proteins possibly competing for CTRIP binding. Alternatively, a CTRIP-mediated effect on CLK could affect PER stability. Such a mechanism might provide an efficient way to counterbalance changes in CLK levels. For example, it could help to keep the pace of the oscillator more resistant to variations in CLK levels, which might be induced by physiological stress or environmental changes.

In mammals, TRIP12 has recently been shown to be part of the ubiquitin fusion degradation (UFD) pathway, in which poly-ubiquitin is added to the N-terminus of the target protein as a degradation signal (Ciechanover & Ben-Saadon, 2004; Park *et al*, 2009; Chen *et al*, 2010). Putative UFD pathway components are present in *Drosophila* (Lundgren *et al*, 2005), but no role for N-terminal ubiquitination has been shown. Our results raise the possibility that the UFD pathway is involved in tuning the speed of the circadian oscillator by controlling the stability of both CLK and PER.

METHODS

Fly strains and behavioural analysis. *pdf-gal4* (Park *et al*, 2000), *tim-gal4* (Kaneko, 1998), gal1118 (Blanchardon *et al*, 2001) and *hus1-like*[37] (Abdu *et al*, 2007) have been previously reported. The P-UAS 11430 line is described at http://gsdb.biol.metro-u.ac.jp/~dclust/index.html. The *ctrip* RNAi lines 14656R3 (named 14R3 here) and 17735R3 (named 17R3 here) are described at http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp, and target E2 and E6 *ctrip* exons, respectively. A recombinant chromosome carrying both 14R3 and 17R3 was used for all molecular analyses. The Y4 and 32–3 deletions were obtained by imprecise excision of the gal1501 insertion and 19F1 deletion by imprecise excision of gal1118, through standard P-element excision procedures. The gal1501, gal1118, 19F1 and 32.3 stocks were outcrossed for five generations against a *w* control stock (Canton S genetic background) before further analyses. Behavioural assays were carried out with 1–7-day-old males at 25 °C in *Drosophila* activity monitors (TriKinetics), as described previously (Klarsfeld *et al*, 2003). For dark–dark conditions analysis, flies were first entrained in 12 h:12 h light–dark conditions cycles during 2–4 days before transfer to dark–dark conditions, and data were analysed for 6 days from the second day in dark–dark conditions. Data analysis was done with the FaasX software, which is derived from the Brandeis Rhythm Package and is available on request. All behavioural experiments were performed two or three times with similar results.

Immunolabellings. *In situ* immunolabellings were done on whole-mounted brains of larvae or adult males, as described previously (Blanchardon *et al*, 2001). For quantifications, 20–25 brain hemispheres were used for each time point. Primary antibodies used were rabbit PER (Stanewsky *et al*, 1997) at 1:15,000 dilution, rat TIM (Grima *et al*, 2002) at 1:10,000, guinea-pig CLK GP47 (Houl *et al*, 2006) at 1:10,000 and mouse PDF (Developmental Studies Hybridoma Bank) at 1:50,000. Secondary goat antibodies (Invitrogen) were FP546-conjugated rabbit (1:2,000), Alexa 647-conjugated rat (1:5,000), Alexa 594-conjugated mouse (1:2,000) and Alexa 488-conjugated guinea-pig (1:2,000).

Western blots. Protein extracts were made from 30–40 male heads for each time point. Frozen heads were homogenized in 150 µl ice-cold RBS buffer (Yu *et al*, 2006) plus protease inhibitors (complete mini Roche), phosphatase inhibitors cocktails 1 (0.5%) and 2 (1%; Sigma), and 2-mM β-glycerophosphate. For SDS–PAGE, 50 µg of protein extracts were separated on 3–8% Tris–acetate gels (Invitrogen) and transferred to PVDF membranes. Primary antibodies used were rabbit PER (Stanewsky *et al*, 1997; 1:10,000), rat TIM (Grima *et al*, 2002; 1:2,000) and goat CLK sc-27070 (Santa Cruz Biotechnology; 1:1,000). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) used were rabbit goat (1:10,000), rat goat (1:15,000) and goat donkey (1:20,000). All western blots were reproduced two or three times with similar results. Degradation assays were performed as described in Hung *et al* (2009).

Genomic DNA and complementary DNA analysis. Genomic DNA surrounding the gal1118 and gal1501 insertions was isolated by standard cloning procedures to localize the P-elements. RT–PCR and 5′-rapid amplification of cloned ends (RACE) analysis of *ctrip* head complementary DNAs (cDNAs) were used to characterize the different *ctrip* transcripts from a *w* stock (Canton S background). For quantitative RT–PCR, cDNAs were synthesized from 1 µg of male head total RNA (Promega SV total RNA isolation system), and PCR was performed with a LightCycler (Roche) using the SYBR green detection protocol, as described previously (Grima *et al*, 2002). mRNA levels were normalized to the levels of tubulin mRNA and expressed as a percentage of the maximum value set to 100. The results were then averaged from two or three independent experiments.

Additional methods are available as supplementary information online.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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