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twenty-four defines a critical translational step in the *Drosophila* clock

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Abstract

Daily oscillations of gene expression underlie circadian behaviours in multicellular organisms1. While attention has been focused on transcriptional and posttranslational mechanisms1–3, other posttranscriptional modes have been less clearly delineated. Here we report mutants of a novel *Drosophila* gene *twenty-four* (*tyf*) that display weak behavioural rhythms. Weak rhythms are accompanied by dramatic reductions in the levels of the clock protein PERIOD (PER) as well as more modest effects on TIMELESS (TIM). Nonetheless, PER induction in pacemaker neurons can rescue *tyf* mutant rhythms. TYF associates with a 5′-cap binding complex, poly(A)-binding protein (PABP) as well as *per* and *tim* transcripts. Furthermore, TYF activates reporter expression when tethered to reporter mRNA even in vitro. Taken together, these data suggest that TYF potently activates PER translation in pacemaker neurons to sustain robust rhythms, revealing a novel and important role for translational control in the *Drosophila* circadian clock.

Transcriptional feedback loops are critical for setting time of eukaryotic circadian clocks. In *Drosophila*, the *Clock* (*Clk*)/*cycle* (*cyc*) dimer activates the transcription of *period* (*per*), *timeless* (*tim*), *vrille* (*vri*), *Par domain protein 1* (*Pdp1*), and *clockwork orange* (*cwo*) genes,

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Author Contributions R.A. and J.C. conceived the study; R.A., C.L., and J.C. designed the experiments; C.L. (under supervision of R.A.) and J.L (under supervision of J.C.) jointly completed Figs. 1 and 2, Supplementary Figs. 1, 4, 8, and 14, Supplementary Tables 2 and 3; J.L., S.M.P. and S.K.J. performed and analyzed the experiments in Supplementary Fig. 13; J.L., C.C., and J.K. performed the genome-wide behavioural screen; C.C. performed GST pull-down studies in Supplementary Fig. 12a; V.L.K. performed PDF quantification analysis in Supplementary Fig. 5b; C.L. performed and analyzed experiments in all remaining Figs, Supplementary Figs and Tables; C.L. and R.A. wrote the manuscript.

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which in turn feed back to inhibit CLK-activated transcription or regulate *Clk* transcription2. These components are also modified posttranslationally to alter core clock timing2–3. Regulation at multiple levels is thought to impose temporal delays in feedback allowing sustained oscillations on a circadian time scale.

To discover novel clock components, we performed a genome-wide behavioural screen. Using the KAIST-GenExel *Drosophila* library, we identified ~4000 EP lines containing P elements bearing the Upstream Activating Sequence (UAS) for the yeast GAL4 transcription factor inserted near transcription start sites. These flies were crossed with transgenic flies expressing GAL4 under the control of the *tim* promoter (*tim-GAL4*) to drive downstream gene expression in clock cells4. One EP line identified by a long-period rhythm was the *G10872* line that contains an insertion 893 bp upstream of the *CG4857* transcription start site (Supplementary Fig. 1a). Sequence analyses of the predicted amino acid sequence for *CG4857* did not reveal any apparent functional domains or obvious vertebrate homologues but do reveal conservation with genes from different *Drosophila* species and other insects (Supplementary Fig. 2). We termed this novel gene *twenty-four* (*tyf*).

To characterize the phenotype in flies bearing *tyf* loss-of-function mutations, we generated a ~2.5 kb deletion by imprecise P element excision (Supplementary Fig. 1a, $tyf^{\Delta G14151}$; tyf^{Δ}), deleting amino acids 79–449 of *tyf* and resulting in a frame-shift and premature termination. In addition, we identified a *piggyBac* insertion line that shows dramatically reduced levels of *tyf* transcript (tyf^{e00614}; tyf^{e}) without affecting the transcript levels of adjacent genes (Supplementary Fig. 1b). Wild-type flies display morning and evening peaks under 12 h light- 12 h dark (LD) cycles, anticipate the transitions between light-on and light-off by gradually increasing their activity, and maintain their locomotor rhythm in subsequent constant dark (DD). In *tyf* mutants, morning anticipation of lights-on was reduced and their rhythm was immediately less robust, resulting in weak but long periods in DD (Fig. 1 and Supplementary Table 1). Precise *piggyBac* excision in tyf^{e} restored wild-type circadian behaviour (Supplementary Table 1), indicating that the *tyf* gene disruption is responsible for its circadian phenotype. Analyses in trans-heterozygous females show that *tyf* alleles are recessive and not complemented by deletions of the *CG4857* locus (Supplementary Fig. 3).

While circadian clocks are evident in multiple tissues, brain clocks are largely responsible for circadian behaviours4. Neuroanatomical studies have established two oscillator models in which distinct groups of clock cells control morning and evening locomotor activity and behavioural rhythms5,6. The neuropeptide gene, *Pigment-dispersing factor* (*Pdf*), expressed in ventral lateral neurons (LN_vs) has been implicated in driving morning anticipation and resetting evening clocks in the dorsal LNs (LN_ds)/dorsal neurons (DNs)7–9. To map the neurons important for *tyf* effects, we generated *tyf*-GAL4 lines containing the *tyf* promoter region (from −3.0 kb to +0.5 kb), and visualized its expression using a UAS-GFP reporter. *tyf*-GAL4 expression was relatively restricted to a subset of neurons in the adult brain (Supplementary Fig. 4a–f). Anti-PER antibody staining revealed that it is strongly expressed in PDF+ LN_vs and weakly in LN_ds assessed in independent lines (data not shown). In contrast, *tyf*-GAL4 expression was not detectable in the DNs. Consistent with the idea that *tyf*-GAL4 reflects endogenous *tyf* expression, *tyf*-GAL4 along with a UAS-*tyf* transgene fully rescues the behavioural phenotypes in *tyf* mutants (data not shown).

To map the loci of *tyf* function, we restricted TYF overexpression to the PDF+ LN_vs using *Pdf*-GAL47. This results in a long period similar to the *tim*-GAL4 driver, while GAL4 inhibition in PDF+ cells by a *Pdf*-GAL80 transgene5 suppressed the long period phenotype (Supplementary Table 2). Independent UAS-*tyf* insertions confirmed these results. TYF expression restricted to PDF+ cells was also sufficient to rescue free-running locomotor rhythms in mutants (Supplementary Table 3). In addition, RNAi-mediated knockdown of *tyf* expression in PDF+ cells phenocopied circadian behaviours in *tyf* hypomorphic mutants (Supplementary Table 3). These data indicate that *tyf* expression in the PDF+ pacemaker neurons is necessary and sufficient for robust behavioural rhythms.

To determine *tyf* effects on the core clock, we analyzed molecular rhythms from head extracts, which largely reflect eye clocks10. We found that cycling expression of PER, TIM, and PDP1 proteins in *tyf* mutants is comparable to wild type (data not shown). *tyf* transcript levels were relatively constant in LD and not affected in clock mutants (Supplementary Fig. 4g,h). We then focused on the behaviourally relevant pacemaker neurons. Anti-PDF immunofluorescence revealed no overt defects in the neural projections from PDF+ LN_vs of *tyf* mutants (Supplementary Fig. 5a). Adult-specific TYF expression using a drug-inducible GAL4 was sufficient for behavioural rescue in *tyf* mutants (Supplementary Table 4 and Supplementary Fig. 5c), further reducing the likelihood that *tyf* phenotypes are due to developmental defects.

Strikingly, we found that PER protein was barely detectable in LN clock cells of *tyf* mutants (Fig. 2a). PER cycling was dampened but not absent (Fig. 2b and Supplementary Fig. 6). *tyf* mutant effects were less severe in the DNs with PER at ~50% of wild-type peak levels. TYF expression in PDF+ neurons rescued PER cycling only in PDF+ clock cells of *tyf* mutants (Supplementary Fig. 7). Consistent with dramatic PER reductions, PDF levels increased in dorsal projections from the small LN_vs of *tyf* mutants (Supplementary Fig. 5b), as observed in per^{01} flies11. TIM levels were also reduced in *tyf* mutants, but to a lesser extent than PER, with peak levels in *tyf* mutants reduced to ~50% of wild-type (Supplementary Fig. 8a). Such effects may be indirect through PER as we found that TIM reductions were also observed in per^{01} flies and there was little effect of loss of *tyf* on TIM in per^{01} mutants.

In contrast to strong effects on PER, *tyf* mutants normally expressed PDP1ε, CWO, and CLK proteins (Fig. 2a and Supplementary Fig. 8b). The oscillating expression of PDP1ε protein as well as *tim* and *Pdp1ε* transcripts in mutant flies was comparable to wild-type (Supplementary Fig. 8c,d). We reason that LD cycles, the clock neural network and/or multiple feedback loops may buffer the molecular clock against loss of *tyf* function.

Given the robust reductions in PER may be responsible for the arrhythmic behaviour, we hypothesized that PER expression via the GAL4/UAS system could rescue *tyf* locomotor rhythms12. Indeed, we find that PER, but not TIM or CLK, overexpression specifically in PDF+ cells of *tyf* mutants restored wild-type levels of rhythmicity (Fig. 3, Supplementary Fig. 9, and Supplementary Table 5). These rescue data indicate that neither posttranslational regulation of PER protein by TIM3 nor transcriptional activation of *per* gene expression by CLK2 would be limiting for normal circadian behaviour in *tyf* mutants. Moreover, these behavioural data suggest that PER is a major target of TYF in PDF neurons.

We next examined at what regulatory step PER expression in *tyf* mutants is compromised. We observed that PER protein, but not *per* RNA, levels were reduced in brain extracts (data not shown). While consistent with posttranscriptional regulation, we cannot exclude the possibility that this result could arise from the masking effect of low level non-cycling *per* RNA in non-circadian tissues. *tyf* effects were not evident on a CLK-activated *per* promoter-GAL4 transgene (Supplementary Fig. 10) but were evident on a *per* transgene lacking its natural promoter13 (Supplementary Fig. 11a), indicating that the *per* promoter is not necessary nor sufficient for *tyf* effects. *tyf* effects were also still observed on a *per*(Δ)-HAHis transgene that lacks the DOUBLETIME (DBT)-binding domain, thus reducing DBT-mediated PER degradation14. We then examined PER in flies in which we rescued *tyf* by PER overexpression. Interestingly, neither PER levels driven by constitutive *Pdf*-GAL4 nor oscillations were affected in *tyf* mutants (Supplementary Fig. 11b), indicating that *tyf* is not required for posttranslational regulation of PER. The lack of most *per* 5′ and 3′ UTRs in the UAS-*per* transgene15 and/or *per* overexpression itself may compensate for loss of *tyf*. Taken together, our observations suggest that *tyf* posttranscriptionally regulates *per* expression.

We next asked whether TYF associates with posttranscriptional regulatory factors, circadian clock components and/or specific RNA targets using immunoprecipitation of an epitope-tagged TYF (TYF-V5). We confirmed that GAL4/UAS-driven TYF-V5 rescues behavioural rhythms in *tyf* mutants (Fig. 3 and Supplementary Table 5) and that TYF-V5 expression is cytoplasmic at all times of day (Supplementary Fig. 4i). TYF-V5 driven by *tim*-GAL4 was immunoprecipitated from head extracts and then TYF-associating proteins were probed with different antibodies. TYF did not associate with either PER or TIM (Fig. 4a). However, poly(A)-binding protein (PABP) was specifically co-immunoprecipitated with TYF at two times-of-day in a RNase-insensitive manner (Fig. 4a, data not shown). In vitro assays demonstrated that PABP interacts with the N-terminal region of TYF (Supplementary Fig. 12a; TYF-N, amino acids 1–1167).

It has been proposed that PABP stimulates translation in part by binding to both 5′ cap-associating translation initiation factor eIF4G and the poly(A) tail in mRNAs, thereby facilitating mRNA circularization16. Therefore, we also examined a possible association of TYF with a cap-binding complex using 5′ cap (7-methylguanosine, m^7-GTP) affinity beads and S2 cell extracts expressing epitope-tagged TYF. We found that eIF4E, a translation initiation factor directly recognizing the 5′ cap structure, PABP, and TYF were efficiently and specifically pulled down by m^7-GTP affinity beads in a RNase-insensitive manner (Fig. 4b and Supplementary Fig. 12b). Addition of soluble m^7-GTP but not GTP inhibited their cap association, validating the specificity. Interestingly, the N-terminal portion of TYF, capable of in vitro PABP binding (Supplementary Fig. 12a), exhibits strongly reduced affinity for the 5′ cap, suggesting that PABP may not solely mediate TYF association with the 5′ cap. Our results link TYF to RNA-binding proteins involved in translation.

We further checked whether TYF associates with clock gene transcripts. RNA in TYF-V5 immunoprecipitation was analyzed by real-time RT-PCR (Fig. 4d). To quantify TYF-specific transcript enrichment, we normalized RNA levels in TYF immunoprecipitation to input RNA (enrichment fold of TYF IP) and then subtracted the signal from a background control immunoprecipitation (PDFR-V5) also normalized to input. We find that TYF pulls

down significant amounts of *per* and *tim* RNAs relative to the amounts of *Pdp1* at the times of their peak expression levels (ZT15, $p<0.029$). Moreover, *per* RNA levels in TYF immunoprecipitation were higher at their peak times than trough times (i.e., ZT15 v. ZT3) even after normalizing for input levels ($p<0.026$). As *Pdp1* has comparable input levels to *per* and *tim* (data not shown), the low *Pdp1* signal is unlikely to be explained by low input levels. However, differential anatomic distributions could contribute to these results17. We also did not detect significant TYF-specific signal for *Clk* and *cyc* RNAs in immunoprecipitated RNA, although low input levels could explain the lack of detectable association (data not shown). We could not identify any RNA recognition motif in TYF, suggesting that RNA binding intermediaries, such as eIF4E and PABP, may mediate its association with RNA.

To address whether TYF is incorporated into translating ribonucleocomplexes, we fractionated S2 cell extracts expressing TYF-3xHA by sucrose density gradient in the absence or presence of EDTA to dissociate ribosomes (Supplementary Fig. 13). Sedimentation profiles demonstrated that ribosomal protein P0 and PABP were present in polysomal fractions in an EDTA-sensitive manner. By contrast, a minor fraction of TYF co-sedimented with polysomes and exhibited a modest shift by EDTA. This pattern is similar to that of eIF4E consistent with their association (Fig. 4b,c). Taken together, the association of TYF with eIF4E, PABP, and *per* RNA suggests a direct role in PER protein synthesis, possibly at a translation initiation step.

To investigate *tyf* effects on its associating RNAs, we performed a RNA-tethering assay in cultured S2 cells18. TYF fused to the RNA-binding bacteriophage MS2 coat protein is tethered to a luciferase reporter RNA containing MS2-binding sites (Fig. 5a). TYF activity is monitored by assaying luciferase activity. TYF-MS2, compared to MS2 alone, enhanced luciferase expression in a MS2-binding sites-dependent manner (Fig. 5b). The C-terminal region of TYF (TYF-C, amino acids 1161–1911) was necessary and sufficient for the activation (Fig. 5b and Supplementary Fig. 14a–c). TYF-MS2 activation was augmented if the *per* or *tim* 3′ UTR is fused to the reporter gene but not the *cyc* 3′ UTR (Fig. 5b and Supplementary Fig. 14d). TYF without the MS2 domain could not activate reporter containing both *per* 5′ and 3′ UTRs consistent with a requirement for other RNA binding proteins (Supplementary Fig. 14h). Notably, we found that a transgenic fusion between the *per* coding region and luciferase lacking the *per* 3′ UTR (XLG-luc)19 was also reduced in *tyf* mutants indicating that the *per* 3′ UTR is not necessary for *tyf* effects in vivo (data not shown). In contrast to reporter activity, reporter RNA levels and its nuclear/cytoplasmic distribution were comparable between MS2 and TYF-MS2 transfected cells (Supplementary Fig. 14e,f). Moreover, analytical centrifugation through a sucrose cushion revealed that more reporter transcripts associate with high-density ribosomes in the presence of TYF-MS2 (Supplementary Fig. 14g, $p<0.027$), further supporting a role in translational control.

To more directly test the translational activation function of TYF, we reconstituted this tethering system in in vitro translation assays. A C-terminal TYF region (TYFc3, amino acids 1373–1911) fused to MS2, which robustly activated MS2 reporter expression in transfected cells (data not shown), was bacterially expressed, purified and incubated with in vitro transcribed MS2 reporter RNAs and translation-competent S2 cell extracts. TYFc3-

MS2 activated translation from a m^7-G capped and poly(A)-tailed reporter RNA modestly (Fig. 5c, $p<0.001$) and a non-polyadenylated RNA even more strongly (2.9×, $p<0.001$). TYFc3-MS2 effects are evident without affecting reporter RNA levels (data not shown). Moreover, this TYF region is not sufficient to bind PABP in vitro (Supplementary Fig. 12a), suggesting that these effects are not mediated by PABP recruitment. These data clearly demonstrate a translation activation function of TYF in vitro, supporting a possible role in translation of poorly adenylated transcripts.

Relative to studies of transcriptional and posttranslational regulation, little is known about other posttranscriptional/translational mechanisms of core clock regulation in different organisms3,20. While a number of RNA-binding proteins are either rhythmically expressed1 or important for behavioural rhythms21–24, direct links between specific transacting factors, specific clock gene transcripts, and in vivo core clock function have yet to be clearly established, especially in metazoans. Indeed, a number of studies have indicated a role for posttranscriptional regulation in modulating *per* expression10,25–28. Here we demonstrate with multiple lines of evidence that TYF activates PER translation to sustain behavioural rhythms, revealing a novel and important role for translational control in the *Drosophila* circadian clock. We observe robust *tyf* effects on PER and lesser effects on TIM yet no detectable reduction of other core clock components we assayed. This observation suggests that impairing *tyf*-dependent translation is not critical for the expression of most clock components. Importantly, transgenic induction of PER, but not other clock components including TIM, can rescue *tyf* mutant phenotypes.

TYF function is especially important in pacemaker neurons. Both *tyf* and *per* expression in the PDF+ LN_v is sufficient to strongly rescue behavioural rhythms in *tyf* mutants. Moreover, *tyf* effects on PER are, by far, most evident in these pacemaker neurons. The brain pacemaker neurons are also among the few clock cells, which demonstrate robust free-running molecular rhythms in constant dark8,9. Thus, TYF-mediated translational control may be a specialization of networked pacemakers in the brain crucial for sustaining free-running rhythmicity.

Our data also strongly support the model that TYF acts at the level of translational control. TYF associates with *per* and *tim* RNAs, as well as translational regulatory components such as the eIF4E-containing 5′ cap binding complex, and PABP, the latter of which are insensitive to RNase treatment. In addition, TYF tethered to a reporter RNA via MS2 can activate reporter expression without altering RNA levels in transfected cells and importantly, in cell extracts providing exogenous RNA templates and purified TYF-MS2.

How might TYF control translation of its target RNAs? We observe specific effects on PER and TIM yet not on other clock components while we find that TYF interacts with translation components such as the eIF4E-containing cap binding complex and PABP. We hypothesize that RNA-binding translational repressors associate with newly transcribed *per* RNA, temporarily postpone translation and thus, delay feedback PER repression on its own transcription (Supplementary Fig. 15). Such a delay could contribute to the observed lag between protein and RNA particularly in pacemaker neurons, although posttranslational mechanisms may also contribute at least in the eyes29. TYF, which does not have a known

RNA recognition motif, could then be recruited to target transcripts by these translational repressors, releasing them to stimulate initiation of *per* translation. We have not been able to biochemically or genetically link TYF to RNA-binding proteins FMR, LARK, or the translation regulator *Thor*/4E-BP that have been shown to contribute to circadian clock function21,22,24. Nonetheless, TYF association with eIF4E and their similar polysome profiles implicates TYF as a novel translation initiation factor. In addition, TYF effects may be more evident on poorly adenylated transcripts based on our in vitro data (Fig. 5c). Of note, the fly homolog of the clock-regulated deadenylase *nocturnin*30 has been shown to be important in DNs for circadian light responses but neither an LN function nor an RNA target has been described24. Nevertheless, unique features of TYF-regulated transcripts may mediate the highly selective TYF effects on clock components in vivo.

Posttranscriptional regulation on *per* RNA has been considered to be modulatory to clock function. The identification of critical role for TYF highlights an important role for PER translation in the *Drosophila* neural clockwork. It will be of interest to determine if proteins functionally analogous to TYF serve similarly important and specific functions in the mammalian clock.

METHODS SUMMARY

Plasmids

Total RNA from adult fly heads was isolated using Trizol reagent and reverse-transcribed using Superscript III according to the manufacturer's instructions (Invitrogen). *tyf* cDNA was PCR-amplified by Platinum *Pfx* polymerase (Invitrogen) with the appropriate primer sets and inserted into pUAST vector for regular germ-line transformation and into its modified version with *attB* site and C-terminal V5-tag for site-specific germ-line transformation.

Fly stocks

All flies were reared with standard cornmeal-yeast-agar medium at 25°C under LD (12-h light/12-h dark) cycles. EP lines G10872 and G14151 were obtained from KAIST-GenExel *Drosophila* library. To generate a *tyf* deletion line (tyf^{Δ}), P-element excision lines were established from the G14151 line and molecularly characterized by genomic DNA-PCR with appropriate primer sets. *Df(1)HC244*, *Df(1)rb23*, and UAS-mCD8-GFP lines were obtained from Bloomington *Drosophila* stock center. UAS-tyf^{RNAi} line was obtained from National Institute of Genetics (Japan). Several independent germ-line transformants were established from w^{1118} embryos injected with UAS-*tyf* transgenic construct (BestGene Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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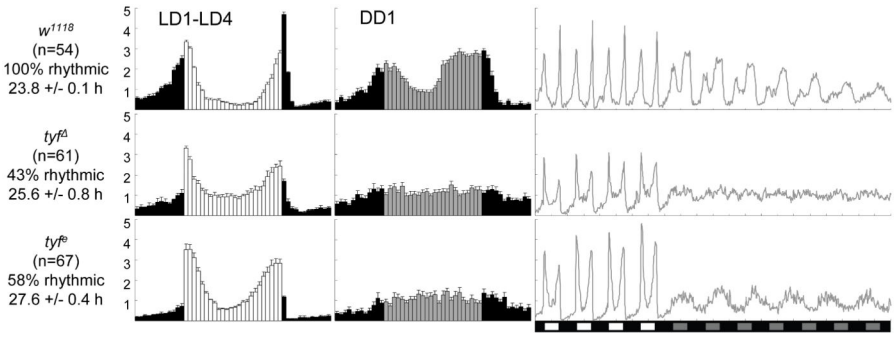

Figure 1. Robust behavioural rhythms require *tyf*

Averaged activity profiles during light-dark (LD)/first constant dark (DD) cycles (middle panel) and actograms throughout the behavioural analyses (right panel) (n=54–67). Percentage of rhythmic flies and period +/− SEM (n=26–54) under DD cycles are given at the left. Error bars indicate SEM. White/black bars, LD cycle; Grey/black bars, DD cycle.

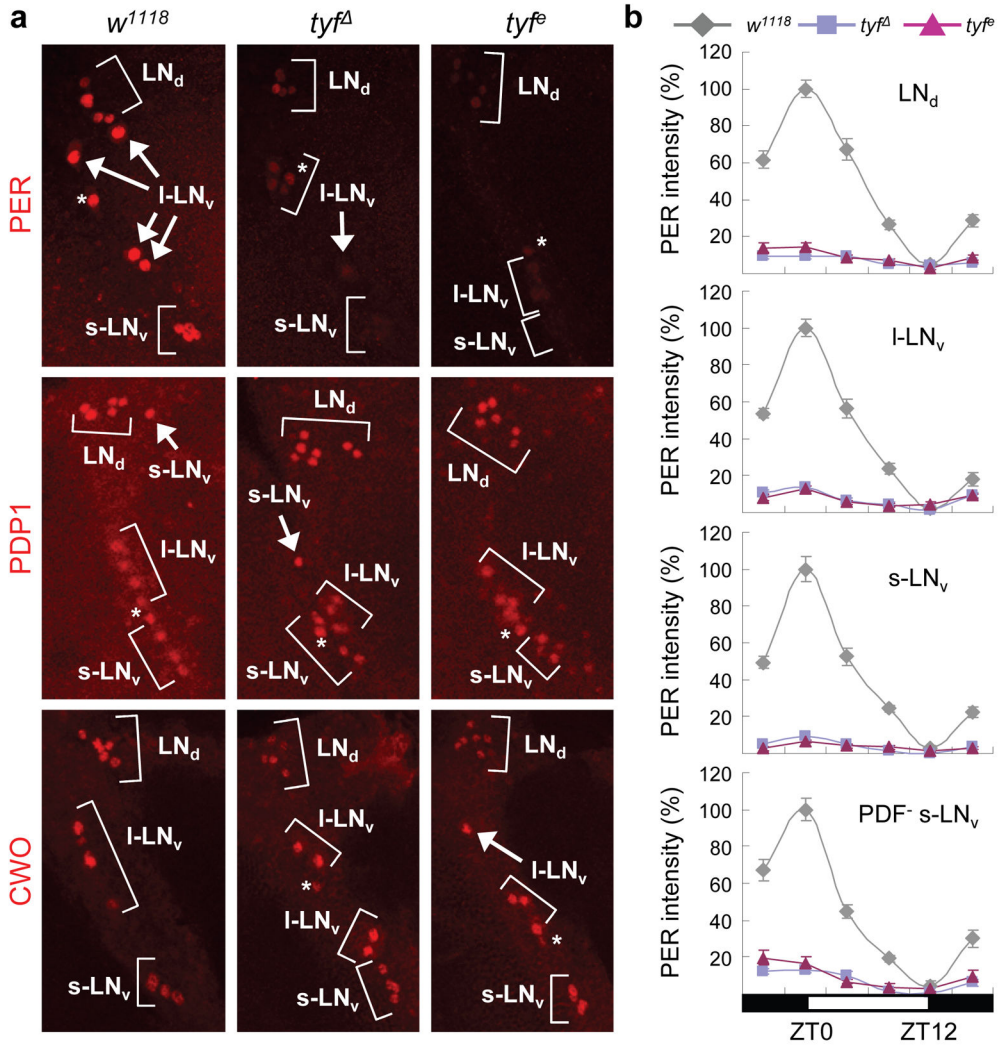

Figure 2. *tyf* is crucial for PER expression in pacemaker neurons

a, Adult brains were immunostained with anti-PER (upper, ZT0), anti-PDP1 (middle, ZT21), and anti-CWO (lower, ZT3) antibodies. Clock cell groups were identified by co-staining with anti-PDF antibody (data not shown). **b**, PER intensity in each clock cell group was quantified, averaged (n=7–10), and normalized to the value of wild-type fly at ZT0 which was set as 100%. Error bars indicate SEM. LN_d, dorsal lateral neuron; l-LN_v, large ventral LN; s-LN_v, small ventral LN; asterisk, PDF-negative s-LN_v.

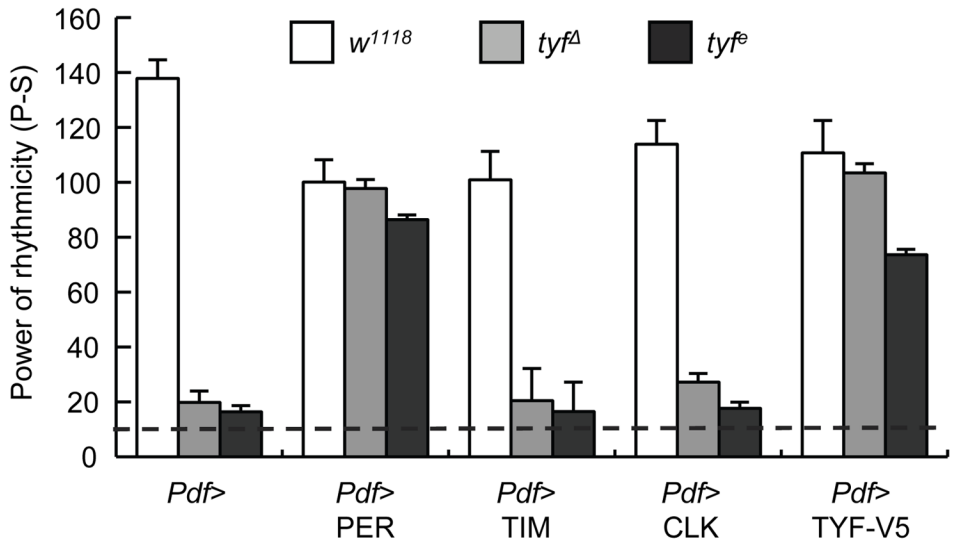

Figure 3. PER induction rescues *tyf* mutant rhythms

Each clock gene was overexpressed in PDF+ cells of wild-type or *tyf* mutants and their rhythmicity under DD cycles was measured by P-S values. Of note, each fly with P-S values greater than 10 (dotted line) is defined as rhythmic. Data represent average + SEM (n=18–48).

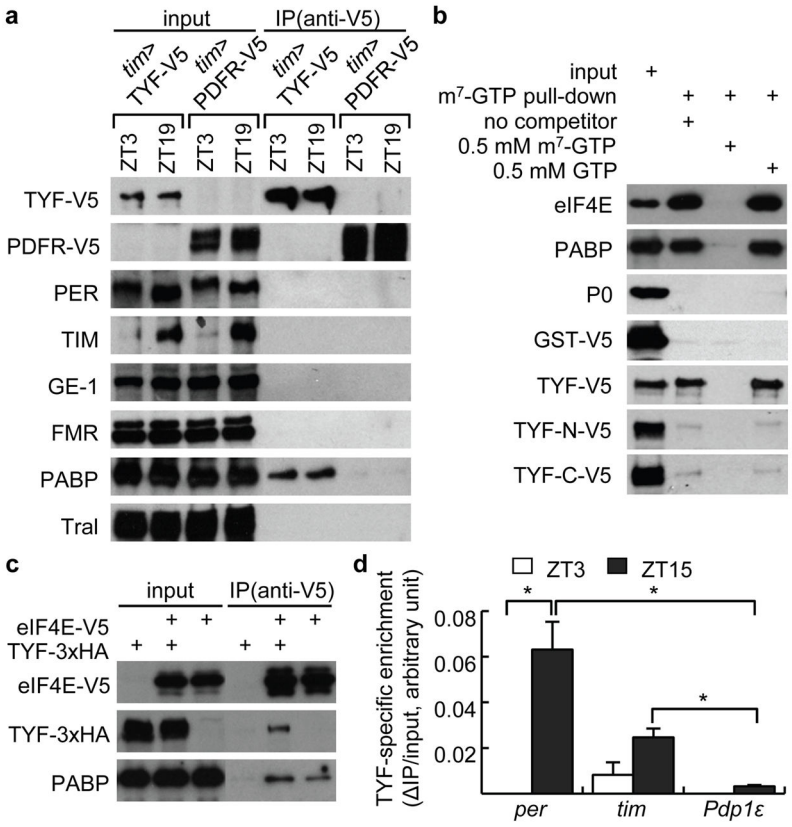

Figure 4. TYF specifically associates with the 5′ cap-binding complex, PABP, and target gene transcripts

a, Fly head extracts were immunoprecipitated with anti-V5 antibody. Bound proteins were probed with antibodies shown on the left. **b–c**, S2 cell extracts expressing epitope-tagged TYF were incubated with m^7-GTP sepharose beads (**b**) or immunoprecipitated with anti-V5 agarose beads (**c**). Where indicated, 0.5 mM of soluble competitor was added to the extracts. Bound proteins were detected similarly as in **a**. Input, 3% of extract included in each binding assay. **d,** After the immunoprecipitation of TYF-V5 from fly head extracts, bound RNAs were quantitatively analyzed by real-time RT-PCR (n=2, in triplicates). TYF-specific enrichment fold was calculated by normalizing RNA levels in TYF immunoprecipitation to input levels and then subtracting the normalized value of control immunoprecipitation (PDFR-V5). Error bars indicate SEM. *p<0.05 as determined by Student's t tests.

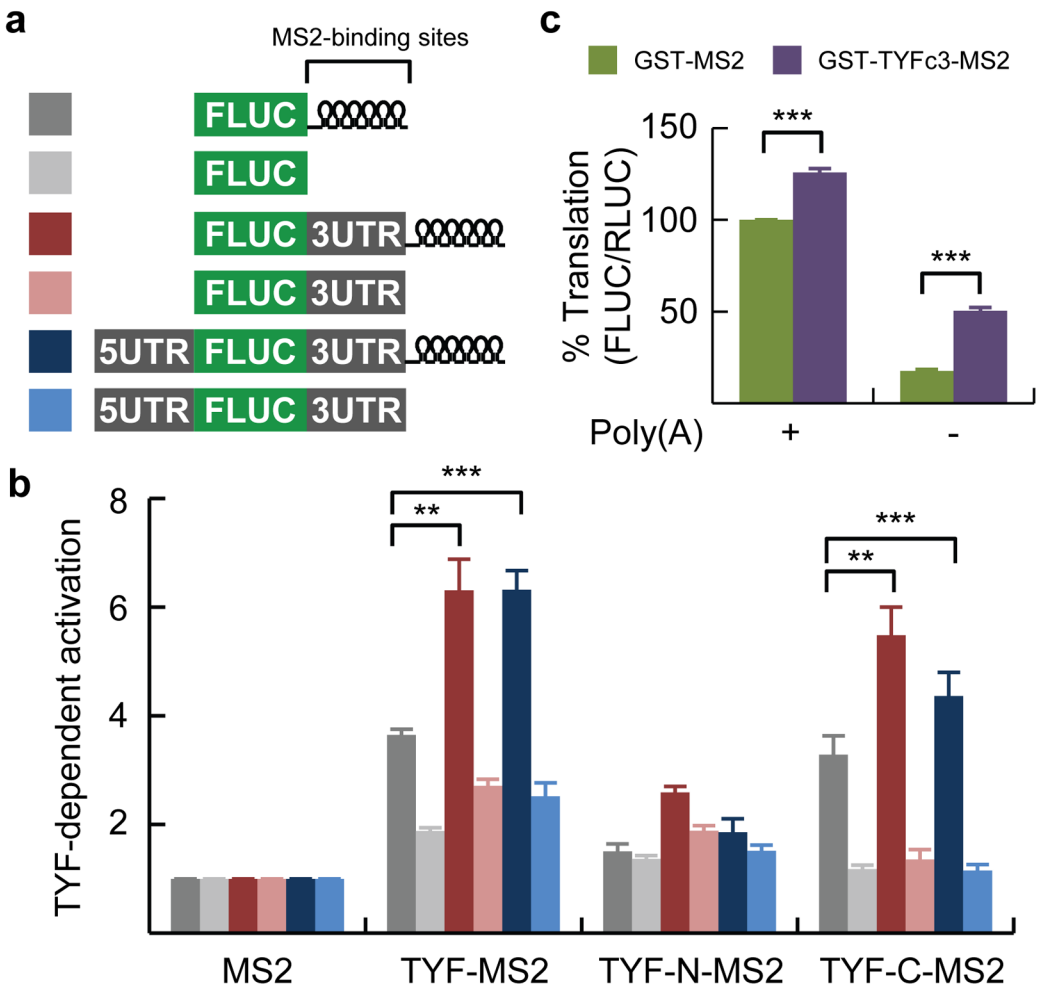

Figure 5. TYF activates reporter expression when tethered to its RNA

a–b, S2 cells were transfected with MS2 reporter plasmid (depicted in **a**) and expression vector for MS2 fusion protein. Firefly luciferase activity (FLUC) was first normalized to *Renilla* luciferase activity (RLUC). Activation fold was then calculated by normalizing to the value in the presence of MS2. Data represent average + SD (n=4–6). 5UTR, *per* 5′ UTR; 3UTR, *per* 3′ UTR; TYF-N, 1–1167 amino acids of TYF; TYF-C, 1161–1911 amino acids of TYF. **p<0.01 and ***p<0.001 as determined by Student's t tests. **c**, m^7-G-capped MS2 reporter RNAs were synthesized using in vitro transcription, polyadenylated by bacterial poly(A) polymerase, and incubated with translation-competent S2 cell extracts and purified GST-MS2 fusion protein in in vitro translation reactions. Luciferase assays were performed as in **b**. Data represent average + SEM (n=3–7). ***p<0.001 as determined by one-way ANOVA, Turkey post hoc.