METHODS

Plasmids. Pabp cDNA was amplified and inserted into pGEX-4T-1 (GE Healthcare) to purify GST-PABP protein from bacteria. To make a DNA construct for tyf-GAL4 transgenic fly, tyf gene promoter region ranging from -3.0 kb to +0.5 kb relative to the transcription start site was PCR-amplified from wild-type genomic DNA and inserted into pPTGAL4 vector. cDNAs encoding an oligomerization-defective MS2 coat protein mutant and 6 tandem repeats of MS2 coat protein binding site (6xBS) were PCR-amplified from pcNMS2-FLAG and pcB6bs, respectively, and inserted into pAc5.1/V5 (invitrogen). Firefly or Renilla luciferases cDNA with or without clock gene UTRs was inserted into pAc5.1/V5 or pAc/6xBS to generate MS2 reporter plasmids. cDNAs corresponding to wild type or deletion mutants of tyf were inserted into 1) pAc5/1/V5 or pAc/MS2-V5 to express C-terminal V5 or MS2-V5 fusion proteins in transfected S2 cells and 2) pcDNA3 (Invitrogen) for in vitro transcription and translation.

Fly stocks and behavioural assays. Pdf-GAL4, tim-GAL4, per-GAL4, Elav-Gene Switch-GAL4, Pdf-GAL80, cry-GAL80, UAS-per16, UAS-tim, UAS-Clk, perG, per(Δ)-HAHis, XLG-luc, and 7.2:9 transgenic lines were described previously. For phiC31 integrase-based transformation, UAS-tyf-V5 transgene was inserted into the attP40 landing site (Genetic Services Inc.). Locomotor activity of individual male or virgin female flies was measured using Drosophila Activity Monitors (Trikinetics) and analyzed using ClockLab analysis software (Actimetrics) as described previously. For adult-specific TYF overexpression, flies were fed with foods containing 0.5 mM RU486 (dissolved in 4% ethanol) or vesicle control (4% ethanol) from the first LD cycle of their
behavioural assays to activate Gene Switch-GAL4\textsuperscript{36} and induce TYF overexpression from UAS-\textit{tyf} transgenes.

**Quantitative RT-PCR.** Semi-quantitative and real-time RT-PCR using total RNA from adult fly heads and transfected S2 cells were performed as described previously\textsuperscript{39,40}. Primer sequences used in our transcript analyses are available upon request.

**Immunostaining.** Whole-mount immunostaining in adult fly brains was performed as described previously\textsuperscript{40}. For primary antibodies, we used rabbit anti-PER (a gift from Dr. Rosbash), rabbit anti-PDP1\textsuperscript{40} and guinea pig anti-TIM\textsuperscript{40}, guinea pig anti-CWO\textsuperscript{41} and anti-CLK\textsuperscript{42}, mouse anti-PDF (Developmental Studies Hybridoma Bank) and anti-V5 (Invitrogen) antibodies. Anti-mouse IgG Alexa 488, anti-mouse IgG Alexa 594, anti-rabbit IgG Alexa 594, anti-guinea pig IgG Alexa 594 and 633 antibodies (Invitrogen) were used for fluorescence-conjugated secondary antibodies. Images were obtained using confocal laser-scanning microscopes (Nikon C1 or Carl Zeiss Pascal). For the quantitative analysis, signal intensity from each group of clock cells was quantified using ImageJ software as described previously\textsuperscript{43}.

**Immunoprecipitation.** Immunoprecipitation from fly head and S2 extracts were performed as described previously\textsuperscript{40}. For RNA analysis, \~200 fly heads were homogenized in a lysis buffer (25 mM Tris-Cl pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) containing 20 U/ml DNase I (Promega), 100 U/ml RNasin (Promega) and 8 mM vanadyl ribonucleoside complex (Sigma). After the immunoprecipitation with anti-V5 antibody, bound RNA was purified using Trizol reagent (Invitrogen) and subsequently used in transcript analyses by semi-quantitative and real-time RT-PCR.
**Antibodies.** Mouse anti-V5 (Invitrogen), rat anti-HA (Roche), mouse anti-dFMR 6A15 (Abcam), human anti-P0 (Immunovision), rabbit anti-PER40, guinea pig anti-TIM40, rat anti-GE-144, rat anti-Tral44, rabbit anti-dPABP45, and rabbit anti-eIF4E46 antibodies were used in our protein analyses.

**GST pull-down assay.** In vitro binding assay was performed as described previously40 with minor modifications. TYF and deletion mutant proteins were in vitro translated in the presence of 35S-methionine using TNT T7 coupled reticulocyte lysate system (Promega). GST fusion proteins were purified from bacterial cultures using glutathione-Sepharose 4B beads (GE Healthcare) and incubated with 35S-labeled proteins in a binding buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). After the incubation for 1 h at 4°C, the beads were washed three times in the same buffer. Bound proteins were eluted, resolved by SDS-PAGE, and detected by autoradiography.

**Cap pull-down assay.** S2 cells in 100 mm dish were transfected with 1.2 µg of expression vector for TYF-V5 or deletion mutants. Cells were harvested at 48 h after transfection, washed in PBS, and resuspended in a lysis buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors). Extracts were clarified by centrifugation and incubated with pre-equilibrated 7-methyl-GTP Sepharose 4B (GE Healthcare) for 1.5 h at 4°C. Where indicated, 0.5 mM m7-GTP or GTP was added to the lysis buffer. The beads were washed four times with the same buffer. Bound proteins were eluted by boiling in 1x SDS sample buffer, resolved by SDS-PAGE and immunoblotted with antibodies as shown.
**Sucrose density gradient fractionation.** Sucrose gradient sedimentations were performed as described previously\(^{47,48}\) with minor modifications. S2 cell were cultured in 100 mm dishes and transfected with 2 µg of Ac/TYF-3xHA using Effectene reagent (Qiagen). At 48 h after transfection, 200 µg/ml of cycloheximide was added to the media and incubated for additional 10 min. Cells were harvested, washed twice with PBS and lysed in 800 µl of polysome gradient buffer (15 mM Tris-Cl pH 7.5, 300 mM NaCl, 10 mM MgCl\(_2\), 0.2 mg/ml cycloheximide, 50 U/ml RNasin, 0.1 mg/ml heparin, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor) containing 0.5% Nonidet P-40. For EDTA treatment, 10 mM MgCl\(_2\) in the lysis buffer was replaced by 25 mM EDTA and cycloheximide was omitted. The lysates were incubated for 15 min at 4°C and centrifuged twice. Clarified lysates were loaded on 10-50% linear sucrose gradient solution. Samples were spun in a SW 41Ti rotor (Beckman) at 36,000 rpm for 2 h. Using tube piercer (Brandel) and FRAC-100, each fraction was collected from the bottom while UV absorbance at 254 nm was monitored continuously with UA-6 detector (ISCO). Proteins in each fraction were precipitated with chloroform/methanol\(^{49}\) and used for Western blotting analyses.

**RNA tethering assay.** S2 cells on 24-well plates were cotransfected with 5 ng of Ac/FLUC-6xBS (or its derivatives), 25 ng of Ac/RLUC, and 250 ng of Ac/MS2-V5 (or its derivatives). For competition experiments, S2 cells were cotransfected with 5 ng of Ac/5UTR-FLUC-3UTR-6xBS, 5 ng of Ac/RLUC, 125 ng of Ac/MS2-V5 or Ac/TYF-MS2-V5, and the increasing amount (125 or 375 ng) of Ac/TYF-V5 (or TYF deletion constructs). Cells were harvested at 48 h after transfection and dual luciferase assays were performed according to the manufacturer’s instructions (Promega).
**Subcellular fractionation.** S2 cells were treated with 200 μg/ml of cycloheximide for 10 min before harvesting at 48 h after transfection. Cell pellets were resuspended in a hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol) on ice for 10 min. Nonidet P-40 were added to a final concentration of 0.5% and incubated for additional 5 min on ice. Lysates were centrifuged at 5,000 rpm for 5 min. RNA was isolated from supernatant (cytoplasmic fraction) and pellet (nuclear fraction), separately, using Trizol LS and Trizol reagents (Invitrogen), treated with DNase I and Dpn I, reverse-transcribed by M-MLV reverse transcriptase (Promega), and quantified by real-time RT-PCR. Fluc RNA levels were first normalized to cyc RNA levels in each fraction and the ratio of normalized Fluc RNA levels in cytoplasm fraction to nuclear fraction was compared between MS2 and TYF-MS2 transfected cells.

**Sucrose cushion centrifugation.** Transfected cells were resuspended in 400 μl of the polysome gradient buffer containing 0.5% Nonidet P-40, homogenized by 20 strokes with a pestle homogenizer and further incubated for 15 min at 4°C. Lysates were cleared by centrifugation at 12,000 rpm for 5 min at 4°C, and layered onto a 2 ml of 45% (wt/wt) sucrose cushion in the polysome gradient buffer. Samples were centrifuged in a SW55Ti (Beckman) for 2 h at 50,000 rpm at 4°C. After the centrifugation, RNA was isolated from sucrose cushion and pellet fractions, separately, using RNeasy mini kit (Qiagen) and quantified as similarly above. Based on the quantification of 18S rRNA levels, 32+/-5% of total ribosomes were pelleted by their higher density under these conditions. The ratio of normalized Fluc RNA levels in pellet fraction to sucrose cushion fraction was compared between MS2 and TYF-MS2 transfected cells.
In vitro translation assay. In vitro translation assays using S2 cell extracts were performed as described previously\textsuperscript{50,51} with some modifications. S2 cells were harvested and washed three times in PBS. Cell pellets were resuspended and homogenized in 1 volume of hypotonic lysis buffer (10 mM Hepes-KOH pH 7.4, 10 mM KOAc, 0.5 mM Mg(OAc)\textsubscript{2}, 5 mM dithiothreitol, and protease inhibitors). After 10-min incubation on ice, the cell extracts were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. Supernatants were immediately used in in vitro translation reaction or stored at -80°C. For the template of MS2 reporter RNAs, firefly luciferase cDNA along with \textit{per} 3’UTR and MS2-binding sites was subcloned into pcDNA3. m\textsuperscript{7}G-capped RNAs were in vitro transcribed using mMESSAGE mMACHINE kit (Ambion) and divided into two aliquots. Poly(A) tail (>150 nucleotides) was added to RNA transcripts in one aliquote using Poly(A) Tailing kit (Ambion) according to the manufacturer’s instruction. For poly(A)\textsuperscript{−} reporter RNA, poly(A) polymerase was omitted in a parallel reaction using the other aliquot. All RNAs were further purified using MEGAclear kit (Ambion), quantified, and stored in aliquots at -80°C until use. m\textsuperscript{7}G-capped and polyadenylated RNA for \textit{Renilla} luciferase was similarly prepared and included as an internal control in in vitro translation reactions. Each in vitro translation reaction included 5 ng of firefly luciferase RNA containing \textit{per} 3’UTR and MS2-binding sites, 10 ng of \textit{Renilla} luciferase RNA, 100 ng of bacterially purified GST-MS2 or GST-TYFc3-MS2, 24 mM Hepes-KOH pH7.4, 60 mM KOAc, 1.5 mM Mg(OAc)\textsubscript{2}, 0.1 mM spermidine, 1.2 mM dithiothreitol, 20 mM creatine phosphate, 60 \textmu M amino acids, 120 \textmu g/ml creatine kinase, 0.1 mg/ml yeast tRNA, 4 U RNAsin (Promega), and 40% S2 extracts in 12.5 \mu l reaction. The reaction was incubated for 90 min at 25°C, stopped by adding 50 \mu l of 1x Reporter lysis buffer (Promega), and kept on
ice. Ten microliter of the mixture was used for dual luciferase assay as above. Total RNA was purified from the rest of the in vitro translation reaction using Trizol and quantified by realtime RT-PCR.

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