PNAS PLUS

mir-276a strengthens *Drosophila* circadian rhythms by regulating *timeless* expression

Xiao Chen (陈霄)[a] and Michael Rosbash[a,b,1]

[a]Brandeis University National Center for Behavioral Genomics, Department of Biology, Brandeis University, Waltham, MA 02454; and [b]Howard Hughes Medical Institute, Brandeis University, Waltham, MA 02454

Contributed by Michael Rosbash, April 12, 2016 (sent for review January 21, 2016; reviewed by Isaac Edery and Patrick Emery)

Circadian rhythms in metazoan eukaryotes are controlled by an endogenous molecular clock. It functions in many locations, including subsets of brain neurons (clock neurons) within the central nervous system. Although the molecular clock relies on transcription/translation feedback loops, posttranscriptional regulation also plays an important role. Here, we show that the abundant *Drosophila melanogaster* microRNA mir-276a regulates molecular and behavioral rhythms by inhibiting expression of the important clock gene *timeless* (*tim*). Misregulation of mir-276a in clock neurons alters *tim* expression and increases arrhythmicity under standard constant darkness (DD) conditions. mir-276a expression itself appears to be light-regulated because its levels oscillate under 24-h light–dark (LD) cycles but not in DD. mir-276a is regulated by the transcription activator Chorion factor 2 in flies and in tissue-culture cells. Evidence from flies mutated using the clustered, regularly interspaced, short palindromic repeats (CRISPR) tool shows that mir-276a inhibits *tim* expression: Deleting the mir-276a–binding site in the *tim* 3′ UTR causes elevated levels of TIM and ∼50% arrhythmicity. We suggest that this pathway contributes to the more robust rhythms observed under light/dark LD conditions than under DD conditions.

microRNA | circadian rhythms | light regulation | CRISPR

In diverse organisms from bacteria to human many biological pathways are regulated by an endogenous circadian clock, which is entrained by environmental cues such as light, food, and temperature (1, 2). The fruit fly *Drosophila melanogaster* has been a traditional model for studying circadian rhythms, and its locomotor rhythms can be monitored easily with the TriKinetics Activity Monitoring system. In the laboratory, flies are usually entrained with 12-h light–dark (LD) cycles, with lights on at Zeitgeber time (ZT) 0 and lights off at ZT12. Wild-type flies manifest rhythmic behavior in LD, which persists under constant darkness (DD) conditions.

Rhythmic behavior is controlled by the ∼150 clock neurons, ∼75 on each side of the fly brain, eight pairs of which express the neuropeptide pigment-dispersing factor (PDF). They are known to be key pacemakers of the clock system (3). For example, flies are arrhythmic when these cells are ablated, and artificial firing of these few neurons is sufficient to phase shift the fly rhythms (4, 5).

In these pacemaker neurons and elsewhere, rhythms are controlled by a conserved molecular clock including a transcription/translation feedback loop (TTFL). It includes four core clock proteins: TIMELESS (TIM), CLOCK (CLK), CYCLE (CYC), and PERIOD (PER). The CLK/CYC heterodimer activates the transcription of hundreds of genes, including *tim* and *per* (6, 7). TIM and PER then enter the nucleus to suppress the activity of the CLK/CYC dimer, resulting in a decrease in their own levels (8). When TIM/PER levels are sufficiently low, CLK/CYC activates transcription again, and a new cycle begins. Each molecular cycle takes ∼24 h and generates the behavioral and physiological cycles that are commonly assayed.

Posttranscriptional regulation and even posttranslational regulation also contribute to the TTFL (9–12). In addition, there are substantial differences between the expression levels and the cycling patterns of nascent and mature transcripts (9, 13). For example, 193 of the 263 fly head mRNAs with cycling expression had lower amplitudes or even flat expression at the nascent RNA levels (9). MicroRNA (miRNA) regulation is an important type of posttranscriptional regulation (14) that might influence the oscillations of mRNAs and/or proteins. miRNAs generally base pair with the 3′ UTR of mRNAs; this interaction triggers mRNA degradation and/or translational inhibition, leading to decreased levels of gene expression (15). To date, only a few examples of miRNAs regulating circadian gene expression in *Drosophila* have been reported (16–20).

Here we demonstrate that the abundant miRNA mir-276a affects *Drosophila* rhythmicity by suppressing the expression of the core clock protein TIM. mir-276a levels oscillate throughout the day and are controlled by the transcription factor Chorion factor 2 (CF2). Manipulation of CF2 expression phenocopies the circadian effects of manipulating mir-276a expression. Importantly, flies missing only the mir-276a–binding site in the 3′ UTR of *tim* are ∼50% arrhythmic and show weakened rhythmic strength. Taken together, our data indicate a previously unidentified posttranscriptional pathway that regulates *tim* expression. We suggest that proper TIM levels are critical for a proper balance of output gene transcription, which in turn is necessary for robust rhythmic behavior.

Results

Manipulation of mir-276a Levels Affects Behavioral Rhythmicity in *Drosophila*. To search for circadian-relevant miRNAs, we screened for circadian rhythm abnormalities by using the GAL4 system to drive overexpression of individual miRNA in PDF-containing neurons (*UAS*-miRNA, *Pdf-GAL4*). We focused on miRNAs with some evidence for cycling levels, as described below. Fifty percent of the flies are arrhythmic when mir-276a is overexpressed (OE) in

Significance

The circadian clock regulates biochemical, physiological, endocrine, and behavioral features of higher organisms. Although the timekeeping mechanism and many of the functions it governs rely on transcriptional regulation, posttranscriptional regulation also plays an important role. We show here that levels of the abundant *Drosophila* microRNA mir-276a oscillate under 24-h light–dark cycles and are regulated by the transcription factor Chorion factor 2. mir-276a is important for robust rhythmicity and binds to a single site in the 3′ UTR of the important clock gene *timeless* (*tim*). We used clustered, regularly interspaced, short palindromic repeats to generate fly strains missing this mir-276a–binding site. Because these strains have elevated TIM levels and enhanced arrhythmicity, the data indicate that mir-276a helps maintain proper TIM levels, which are important for robust rhythmicity.

Author contributions: X.C. and M.R. designed research; X.C. performed research; X.C. and M.R. analyzed data; and X.C. and M.R. wrote the paper.

Reviewers: I.E., Center for Advanced Biotechnology and Medicine; and P.E., University of Massachusetts Medical School.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. Email: rosbash@brandeis.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605837113/-/DCSupplemental.

PDF-containing neurons (Fig. 1*A*). The phenotype is even more dramatic (~80% arrhythmicity) if mir-276a is overexpressed in all ~150 clock neurons with *Tim-GAL4* (Fig. 1*A*). To address the possibility that this phenotype is caused by a developmental effect, we used the inducible *Pdf* driver (*Pdf-geneswitch*) to turn on mir-276a overexpression only in adults; the use of *Pdf-geneswitch* resulted in ~67% arrhythmicity, indicating that the phenotype is caused predominantly by adult expression (Fig. 1*A*).

mir-276a is one of the most abundant miRNAs in fly heads. Although not conserved in mammals, mir-276a is conserved in insects and belongs to a family that also includes mir-276b. The two miRNAs share identical seed sequences and therefore may act redundantly. We focus here on mir-276a because it is substantially (approximately sevenfold more, according to ref. 17) more abundant than mir-276b in fly heads.

To learn more about the circadian role of mir-276a, we took advantage of the mir-276a KO mutant generated by the Cohen laboratory (21). mir-276a is essential because homozygous KO flies are inviable. We therefore assayed the heterozygous KO strain, which had an ~70% decrease in mir-276a levels (Fig. S1). The mir-276a heterozygous KO flies are more arrhythmic than wild-type flies (18% vs. 0%) and also have a significantly lower rhythmicity index than wild-type flies (Fig. 1*B*). Similar results were obtained by decreasing mir-276a levels only in adults with a mir-276a sponge expressed under *Pdf-geneswitch* control, further indicating that flies are also less rhythmic when mir-276a levels decrease in adult PDF-containing neurons (Fig. 1*C*) (22). In conclusion, manipulating mir-276a levels up or down in circadian neurons reduces rhythmicity. Notably, there is no change in the free-running period in any of these mir-276a–altered flies (Fig. S2).

The Molecular Clock Within PDF-Containing Neurons Is Disrupted by mir-276a Overexpression. Two hypotheses can explain the enhanced arrhythmicity caused by altered mir-276a expression: (*i*) The core molecular clock is affected, or (*ii*) one or more key circadian output pathways is misregulated. To address these possibilities, we assayed PER and TIM levels in *Pdf-GAL4*–driven mir-276a OE flies with immunostaining. Flies were entrained in LD for 3 d and collected at ZT10 (2 h before lights-off) and ZT22 (2 h before lights-on). PER and TIM levels cycle throughout the day, with maximal expression around ZT22 and low expression at ZT10 (23, 24). Because manipulating miR-276a expression has no effect on circadian period, the peak expression time should not change.

As expected, signals for both PER and TIM are almost invisible at ZT10 (Fig. 2*A*), and there was no discernable difference between control and OE animals at this time because of this very low PER/TIM staining intensity (Fig. 2*A*). However, signals were much stronger at ZT22, and both PER and TIM staining was decreased in mir-276a OE flies (Fig. 2 *A* and *B*). To validate and extend these effects, mir-276a was overexpressed in fly heads with *Tim-GAL4,* and PER and TIM levels quantified with Western blots. Consistent with the immunostaining, both proteins are down-regulated in mir-276a OE flies (Fig. 2*C*). In sum, both the immunostaining and Western blot results support the first hypothesis, that the arrhythmicity seen with mir-276a OE is caused by disruption of the core molecular clock.

mir-276a Expression Is Regulated by Light. To learn more about mir-276a regulation, we examined mir-276a levels throughout the day. To this end, we entrained flies in LD and collected RNA samples every 4 h for quantitative RT-PCR (qRT-PCR) as illustrated in Fig. 3*A*. mir-276a levels cycle with peak expression at ZT10 (Fig. 3*B*). Although the cycling amplitude is modest (1.8-fold), the pattern is reproducible and disappears completely on the first day of constant darkness (DD1) (Fig. 3*C*), suggesting that light may play a role in regulating mir-276a levels.

To address this possibility, we assayed mir-276a in arrhythmic *period*-null (per^0) flies under LD conditions. mir-276a cycling amplitude and phase were rather similar to those in wild-type flies (Fig. 3*D*), suggesting that mir-276a levels are regulated predominantly by light. However, the mir-276a expression patterns in wild-type and per^0 flies are not identical, indicating that the core clock also may impact mir-276a expression.

CF2 Regulates mir-276a Expression both in Vitro and in Vivo. To gain insight into the regulation of mir-276a expression, we used the online prediction program MATCH (www.gene-regulation.com/pub/programs.html#match) to identify potential transcriptional activators. The region upstream of premir-276a is enriched in the binding sites of several transcriptional activators including CF2 (Fig. S3).

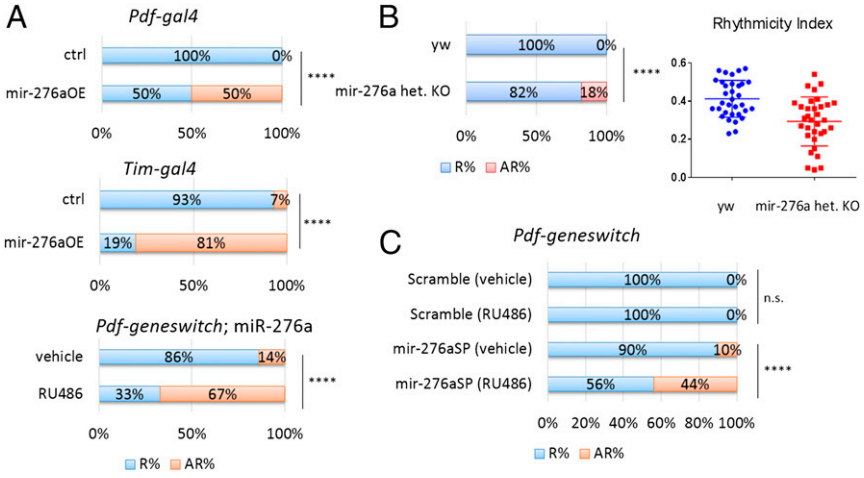

Fig. 1. Manipulation of miR-276a expression levels causes increased arrhythmicity in flies. Quantification of numbers of rhythmic vs. arrhythmic flies. R% represents the percentage of rhythmic flies. AR% represents the percentage of arrhythmic flies. $n = 30–35$. (*A*) Comparison of rhythmicity in mir-276a OE and control flies. mir-276a OE flies are generated by crossing *UAS*-mir-276a to either *Pdf-GAL4* (*Top*), *Tim-GAL4* (*Middle*), or *Pdf-geneswitch* (*Bottom*). Control flies are GAL4 lines crossed with background wild-type flies (w1118). For *Pdf-geneswitch,* control flies were fed on vehicle-containing food, and an experimental group was fed on RU486-containing food. (****$P < 0.0001$; χ^2 test.) (*B*, *Left*) Comparison of rhythmicity in mir-276a heterozygous KO and control flies (yw). (*Right*) The rhythmicity index measures rhythm strength. Each dot represents one fly. Error bars represent mean ± SD. (****$P < 0.0001$; χ^2 test.) (*C*) Comparison of rhythmicity in mir-276a adult-specific knockdown and control flies. mir-276aSP represents the *UAS*-mir-276a sponge for knockdown. Scramble contains the scramble sequence serving as a negative control for mir-276aSP. (n.s., nonsignificant; ****$P < 0.0001$; χ^2 test.)

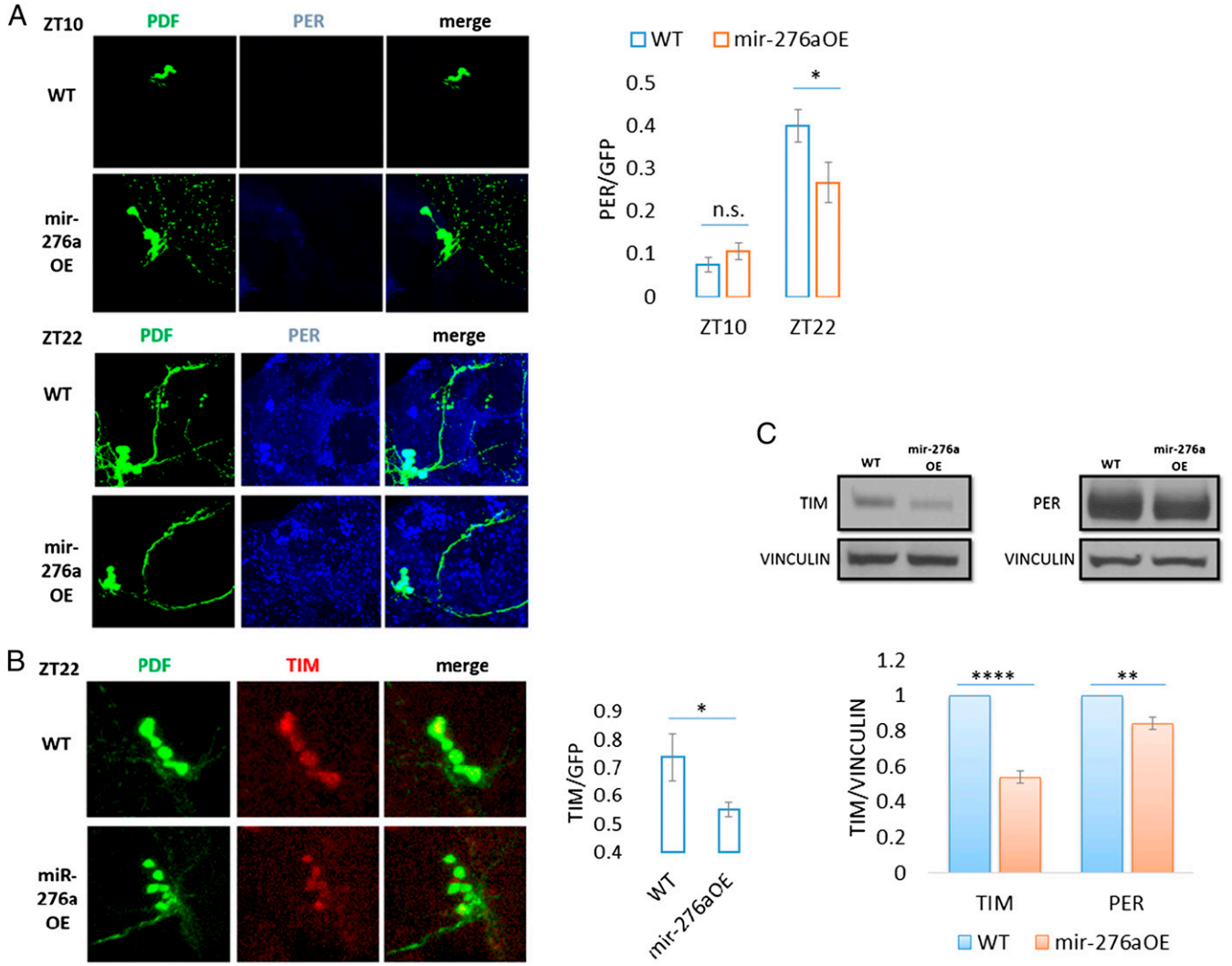

Fig. 2. The molecular clock in core circadian neurons is disrupted by overexpression of mir-276a. (*A*, *Left*) Immunostaining of control (wild-type, *Pdf-GAL4; UAS-mCD8GFP*) and mir-276a OE (*Pdf-GAL4; UAS-mCD8GFP*; *UAS*-mir-276a) flies against GFP labeling of PDF-containing neurons (green) and PER (blue) at ZT10 (*Upper*) or ZT22 (*Lower*). (*Right*) The PER signal is normalized to the GFP signal. (n = 12–14 hemispheres; error bars indicate SEM; n.s., nonsignificant; *$P < 0.05$; two-tailed t test.) (*B*, *Left*) Immunostaining of the flies in *A* against GFP labeling PDF-containing neurons (green) and TIM (red) at ZT22. (*Right*) The TIM signal is normalized to the GFP signal. (n = 12–14 hemispheres; error bars indicate SEM; *$P < 0.05$; two-tailed t test.) (*C*, *Upper*) Western blots against TIM or PER in control or *Tim-GAL4-UAS*-mir-276a OE flies at ZT18. VINCULIN is used as a loading control. (*Lower*) Protein levels are normalized to VINCULIN levels using ImageJ and are quantified in bar graphs. (n = 4; error bars indicate SEM; **$P < 0.01$; ****$P < 0.0001$; two-tailed t test.)

Like mir-276a, *cf2* mRNA levels cycle in fly heads under LD conditions with peak expression at ZT10 (Fig. 4*A*).

We first used the S2 cell reporter system to address the regulation of mir-276a expression by CF2 and fused the mir-276a upstream region to a luciferase reporter (Fig. 4*B*). As a positive control, the luciferase reporter was fused to a minimal *Alcohol dehydrogenase* (*adh*) promoter downstream of five consecutive CF2-binding sites (Fig. 4*B*). The luciferase reporter controlled by the mir-276a upstream region increase by approximately fivefold with increasing levels of the *cf2*-expressing plasmid (Fig. 4*C*). This result is comparable to the ∼5.8-fold induction of luciferase in the positive control, indicating that CF2 activates transcription of mir-276a in vitro (Fig. 4*C*).

To confirm that CF2 also regulates mir-276a expression in vivo, *cf2* was either overexpressed or knocked down in fly heads with *Tim-GAL4*. Overexpression caused an ∼1.7-fold increase in *cf2* mRNA levels and an ∼2.4-fold increase in mir-276a RNA levels in the same head RNA preparations (Fig. 4*D*). Conversely, *cf2* knockdown via RNAi [*Tim-GAL4; UAS-cf2* RNAi (VDRC)] caused an ∼30% decrease in *cf2* mRNA and an ∼40% decrease in mir-276a levels (Fig. 4*D*).

A further prediction of the regulation of mir-276a expression by *cf2* is that *cf2* overexpression or depletion should phenocopy the behavioral effects of mir-276a overexpression or depletion. Indeed, arrhythmicity was increased by altering CF2 levels. Overexpression of *cf2* caused ∼86% arrhythmicity, whereas *cf2* knockdown caused ∼27% arrhythmicity (Fig. 4*E*). Together with the in vitro data, these results indicate that CF2 probably regulates mir-276a expression both in vitro and in vivo.

mir-276a Regulates Behavioral Rhythmicity via Suppressing TIM. miRNAs usually regulate gene expression by binding to the 3′ UTRs of mRNAs and causing mRNA degradation and/or translational inhibition. To identify candidate mRNA target(s) of mir-276a, we performed high-throughput sequencing of mRNA from mir-276a mutant (heterozygous KO and OE) fly heads and compared genes with decreased expression levels in mir-276a OE flies to genes with increased expression levels in mir-276a heterozygous

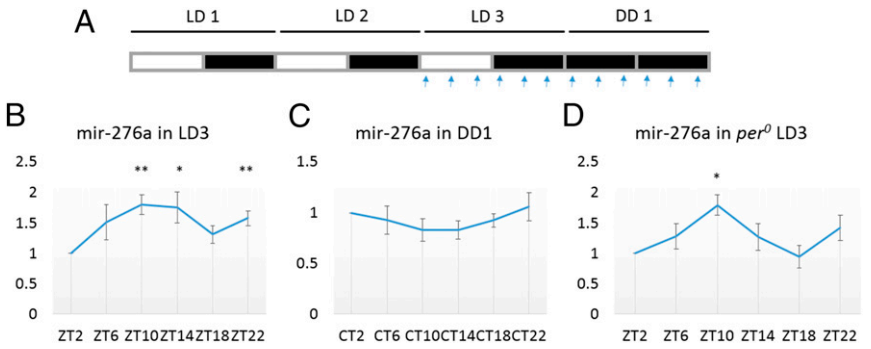

Fig. 3. mir-276a expression in fly heads is regulated by light. (*A*) Entrainment schedule for time-point collection. DD 1, constant darkness day 1; LD, 12-h LD cycles. Samples were collected every 4 h as indicated by arrows. (*B*) mir-276a levels in the heads of wild-type (Canton-S) flies entrained in 12-h LD cycles for 3 d quantified with RT-qPCR. ($n = 4$; error bars indicate SEM; each point was compared with ZT2; $^*P < 0.05$; $^{**}P < 0.01$; two-tailed *t* test.) (*C*) mir-276a levels in the heads of wild-type flies at DD day 1 quantified with RT-qPCR. ($n = 3$; error bars indicate SEM). (*D*) mir-276a levels in the heads of per^0 flies at LD day 3 quantified with RT-qPCR. ($n = 3$; error bars indicate SEM; each point was compared with ZT2; $^*P < 0.05$; two-tailed *t* test.)

KO flies. *tim* is among the fewer than 20 genes met both criteria (Fig. 5*A*). There also is a conserved mir-276a–binding site in the *tim* 3′ UTR with an exact match from positions 2–8 seed of the miRNA as predicted by the two online target prediction tools TargetScan (www.targetscan.org/) and microRNA.org (www.microRNA.org/) (Fig. S4*A*).

To test whether mir-276a targets *tim* in vitro, the *tim* 3′ UTR was fused downstream of a luciferase reporter and transfected into S2 cells for an activity assay (Fig. S4*B*). The reporter activity is reduced by approximately two- to threefold with mir-276a overexpression compared with the negative controls, i.e., overexpression of either an irrelevant gene, *dsRed*, or mir-184, which does not have a binding site in the *tim* 3′ UTR (Fig. 5*B*). Additionally, reporter activity is increased when the mir-276a–binding site within the *tim* 3′ UTR is mutated (Fig. 5*C*). No overexpression was used for this experiment because mir-276a is endogenously expressed in S2 cells according to miRBase (www.mirbase.org/).

Changes in TIM levels also are evident by Western blotting after mir-276a levels are manipulated in fly heads. As expected, very little signal is observed during the day (ZT0–12), but TIM signals during the night (ZT12–24) are consistently higher in mir-276a heterozygous KO flies and are lower in mir-276a OE flies driven by *Tim-GAL4* than in control flies (Fig. 5*D*). Consistent with these effects and as predicted by the positive regulation of mir-276a expression by CF2, TIM levels decreased with *cf2* overexpression and increased with a *cf2* knockdown (Fig. S5).

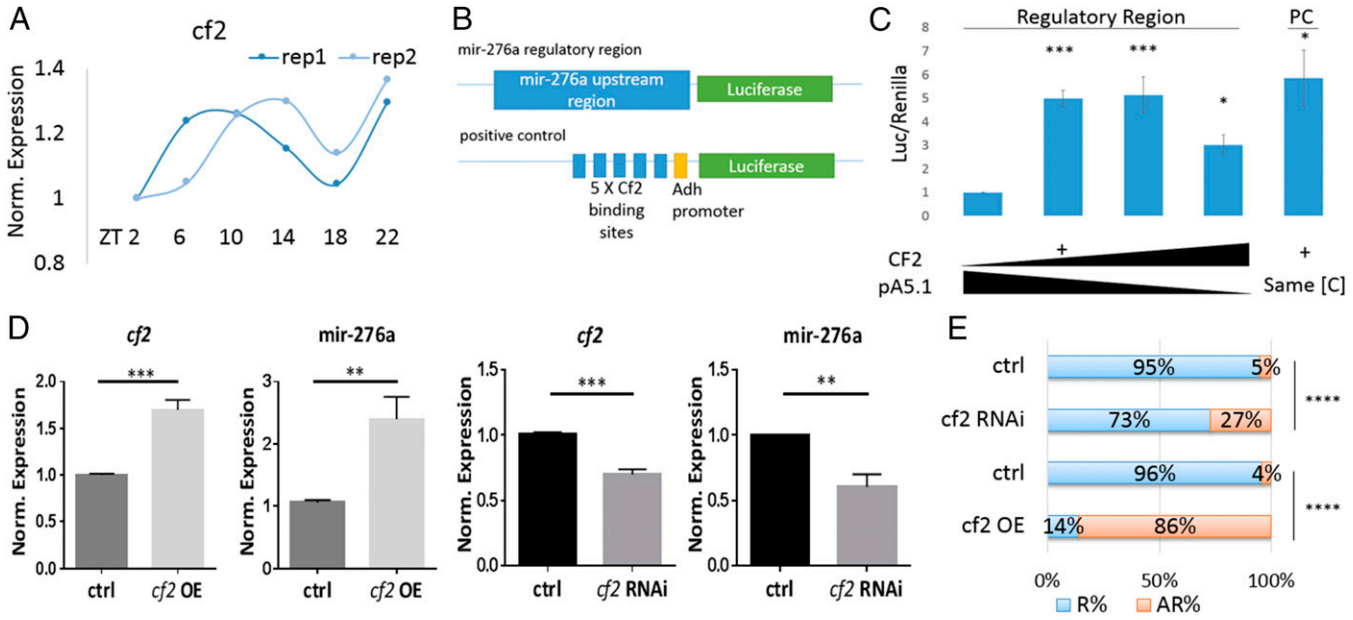

Fig. 4. CF2 regulates mir-276a expression both in vitro and in vivo. (*A*) *cf2* mRNA levels cycle in fly heads throughout the day (data are from ref. 9). Expression levels are normalized to 1 for the first time point of both biological replicates (rep). (*B*) Scheme of vector construction, the mir-276a regulatory region, and the positive control for the S2 cell assay. (*C*) CF2 is expressed by cotransfection of the pAC5.1-CF2 vector. Empty pAc5.1 vector is used to keep the total amount of DNA transfected to S2 cells the same in all conditions. PC, positive control. The same amount of pAC5.1-CF2 was used for the two conditions marked with a plus sign. pRL-Renilla is cotransfected as a transfection control. Luciferase levels in all samples are normalized to Renilla levels. ($n = 3$; error bars indicate SEM; $^*P < 0.05$; $^{***}P < 0.001$; one-way ANOVA was used among samples of regulatory regions, and a two-tailed *t* test was used for PC.) (*D*) qRT-PCR quantification of *cf2* mRNA and mir-276a in *Tim-GAL4*–driven CF2 OE or RNAi fly heads. ($n = 4$; error bars indicate SEM; $^{**}P < 0.01$; $^{***}P < 0.001$; two-tailed *t* test.) (*E*) Quantification of rhythmic vs. arrhythmic flies in CF2 mutants used in *D*. ($n = 28$–32; $^{****}P < 0.0001$; χ^2 test.)

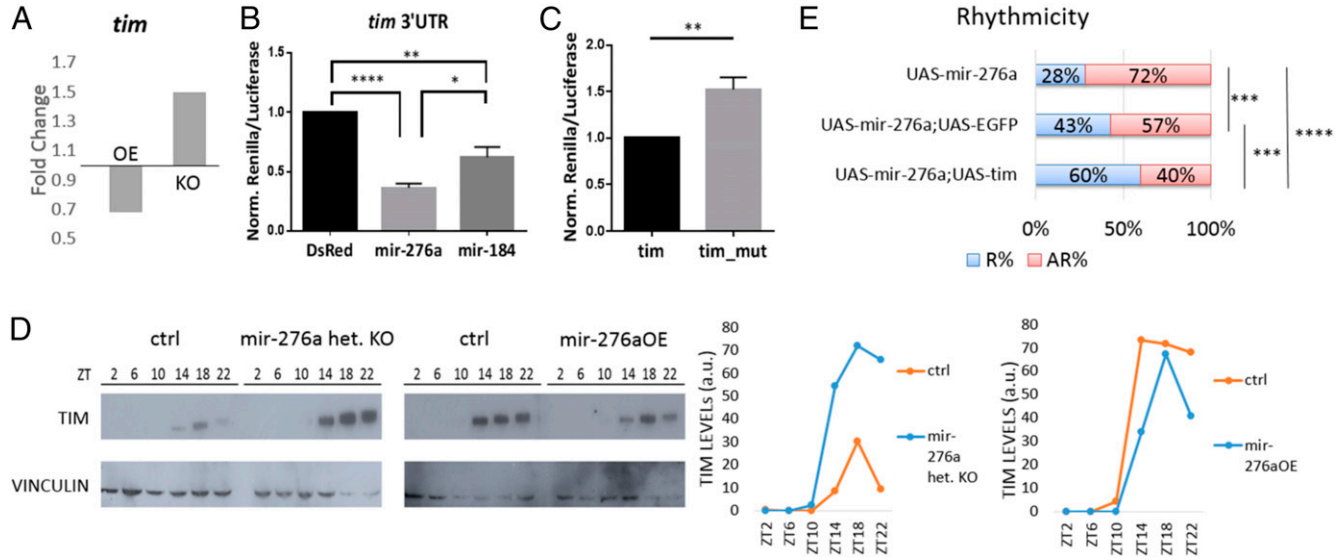

Fig. 5. mir-276a regulates the circadian clock by suppressing TIM. (*A*) Fold changes in *tim* mRNA levels in fly heads in mir-276a OE and mir-276a heterozygous KO flies compared with control flies. (*B*) *tim* 3′ UTR activity reporter Renilla signals normalized to an internal luciferase control when dsRed, mir-276a, or mir-184 is overexpressed in S2 cells. ($n = 4$; error bars indicate SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; one-way ANOVA.) (*C*) Renilla signals in the *tim* wild-type 3′ UTR and in the 3′ UTR in which the mir-276a–binding site was mutated, normalized to the internal luciferase control. Because mir-276a is endogenously expressed in S2 cells, no mir-276a–expressing plasmid is added in the assay. ($n = 4$; error bars indicate SEM; $^{**}P < 0.01$; two-tailed *t* test.) (*D*, *Right*) Western blots against TIM at different time points during a day in control, mir-276a heterozygous KO, and mir-276a OE fly heads. VINCULIN was used as loading control. (*Left*) Quantification. This example is representative of three biological replicates of six time points. (*E*) Quantification of the percentage of rhythmic (R%) and arrhythmic(AR%) flies in *UAS*-mir-276a OE (control), *UAS*-mir-276a OE;*UAS-EGFP* (control), or *UAS*-mir-276a OE;*UAS-tim* (rescue) flies driven by *Pdf-geneswitch*. (n = 16–32; $^{***}P < 0.001$; $^{****}P < 0.0001$; χ^2 test.)

To connect the behavioral phenotypes caused by altering mir-276a levels to *tim*, we overexpressed *tim* along with mir-276a within PDF-containing neurons (*Pdf-geneswitch*). Overexpression of mir-276a alone caused 72% arrhythmicity in this experiment, similar to previous observations shown in Fig. 1*A*. The addition of a control UAS transgene (*UAS-EGFP*) reduced the arrhythmicity to 57%, presumably because competition between the two UAS transgenes for Gal4 reduces mir-276a overexpression. The addition of *UAS-tim* further reduced the arrhythmicity to 40%, indicating that *tim* overexpression partially rescues the effects of mir-276a overexpression. This rescue probably underestimates the role of *tim* as a mir-276a target, because expression of *UAS-tim* alone results in high levels or arrhythmicity (25). These data further indicate that *tim* is a behaviorally relevant target of mir-276a (Fig. 5*E*).

Knockout of a mir-276a–Binding Site in the *tim* 3′ UTR Leads to Increased TIM and Behavioral Arrhythmicity. To support the connection between *tim* and mir-276a further, we used the clustered, regularly interspaced, short palindromic repeats (CRISPR) tool and a single guide RNA to make genomic DNA deletions missing the single mir-276a–binding site within the *tim* 3′ UTR. We chose four fly lines with deletions of 1 nt to 10 nt in the binding site for further

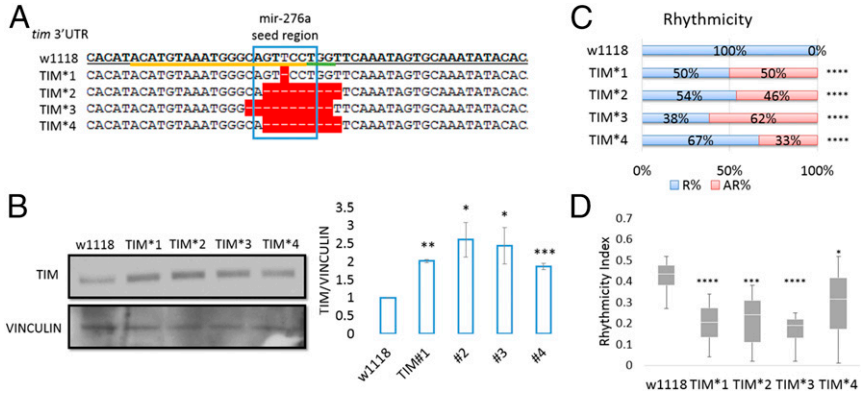

Fig. 6. CRISPR KO of the mir-276a–binding site in the *tim* 3′ UTR leads to increased amounts of TIM and behavioral arrhythmicity. (*A*) Genomic deletion of the mir-276a–binding site in the *tim* 3′ UTR using CRISPR. The underlined sequence is the wild-type sequence obtained from w1118 wild-type flies, which is identical to the annotated sequence. Yellow underlining indicates the guide RNA used for CRISPR genomic editing. The protospacer adjacent motif (PAM) sequence is indicated by green underlining. The rectangle indicates the mir-276a–binding site. TIM*1, TIM*2, TIM*3, and TIM*4 are four mutated fly lines with deletions in the binding site indicated by the white dashed lines against a red background. (*B*) Western blots against TIM from fly heads of w1118 and TIM-mutated lines (*Left*) and quantification (*Right*) at ZT18. ($n = 3$; error bars indicate SEM; each mutant was compared with w1118; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; two-tailed *t* test.) (*C*) Quantification of rhythmicity in wild-type and mutant flies. (n = 16; $^{****}P < 0.0001$; χ^2 test.) (*D*) Rhythmicity indices are scatter-plotted for wild-type and mutant flies. (n =16; each mutant was compared with w1118; $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; two-tailed *t* test.)

analysis (Fig. 6*A*). As predicted, TIM levels are increased in all four of these strains, including the one with a 1-nt deletion, as compared with wild-type flies (Fig. 6*B*). These strains also are ∼50% arrhythmic with significantly reduced rhythmicity indices (Fig. 6 *C* and *D*; note that all four strains were extensively backcrossed to a wild-type strain). In conclusion, flies with disrupted *tim* 3′ UTR mir-276a–binding sites show changes molecularly and behaviorally similar to those seen in mir-276a heterozygous KO flies. This result indicates that mir-276a binding to the *tim* 3′ UTR is important for maintaining proper TIM levels, which in turn are important for robust behavioral rhythmicity.

Discussion

Posttranscriptional regulation is important for circadian rhythms, and miRNAs are important mediators of posttranscriptional regulation. However, only a few have been reported to affect fly circadian rhythms (16–20, 26). Although most of these control clock output pathways, two abundant miRNAs, *bantam* and *let*-7, directly target core clock proteins, CLK and CLOCKWORK ORANGE (CWO), respectively (16, 20). Here we report that a third abundant miRNA, mir-276a, regulates fly circadian behavior by targeting a different core clock protein, TIM. Manipulation of mir-276a levels, either up or down, disrupts or weakens free-running rhythms in DD (Fig. 1). This disruption or weakening is not caused by developmental effects, because the arrhythmic phenotypes also were observed when mir-276a levels were altered only during adulthood (Fig. 1 *A* and *C*).

The arrhythmic phenotypes observed in mir-276a OE flies is caused predominantly by effects on the core molecular clock, because PER and TIM levels are decreased significantly in mir-276a OE flies (Fig. 2). TIM and PER form a stable heterodimer in vivo, and decreases in TIM levels affect levels of the heterodimer as well as of PER (10, 27, 28). Because our experiments indicate that mir-276a targets *tim* expression directly, decreased PER levels are likely a secondary consequence of the reduced TIM levels (Fig. 5).

It is worth noting that neither the daily oscillation of TIM and PER nor the circadian period is detectably affected in mir-276a OE or heterozygous KO flies, despite the strong effects on behavioral rhythmicity (Figs. 1 and 5*D*). The partial behavioral rescue of mir-276a overexpression with TIM overexpression indicates that at least some of the enhanced arrhythmicity by mir-276a overexpression is caused by its effect on TIM levels (Fig. 5*E*). Although TIM overexpression alone increases the fraction of arrhythmic flies (25), the failure to observe complete rescue may indicate that mir-276a also targets other rhythm-relevant mRNAs. The lack of an effect on circadian period is consistent with previous results indicating that an alteration in *tim* gene dose does not affect the circadian period (25). Taken together, the results suggest that the behavioral phenotypes are caused not by the misregulation of circadian timekeeping but rather by the impact of altered TIM levels on the expression of numerous circadian output genes. This interpretation reflects the interaction of all four core clock transcription factors (CLK, CYC, PER, and TIM) with numerous output genes as well as with the core clock genes themselves (29, 30).

A relatively short half-life is a prerequisite for circadian cycling of an RNA, because miRNAs generally are quite stable, and this requirement explains why only a few of them have been reported as being rhythmically expressed (16–18). [Mammalian neurons may be exceptional in having many miRNAs with short half-lives (31).] Cycling fly miRNAs now include mir-276a, which here we show oscillates throughout the day under LD conditions. This finding is consistent with the miRNA deep-sequencing results from ref. 17 (Fig. 3*B*). However, the amplitude of mir-276a cycling is low, presumably because of modest circadian oscillations in transcription rate and/or an insufficiently short half-life. Another possibility is some modest circadian oscillation in the mir-276 half-life, e.g., a rather long half-life during the day but a shorter half-life at night. In addition, assays from whole heads may show reduced amplitudes because a miRNA cycles only in subsets of neurons or cycles with different phases in different neurons.

In any case, the biological significance of the mir-276a oscillation is not clear. Although it shows an expression pattern antiphasic to TIM, mir-276a oscillation probably does not contribute to the daily oscillations in TIM levels. For example, the mir-276a heterozygous KO strain has low mir-276a levels (Fig. S1) but has an even greater TIM cycling amplitude than wild-type strains (Fig. 5*D*). The same is true for *let-7*: Despite oscillations in expression, it suppresses CWO with equal efficiency at all time points tested (16). It is possible that these miRNAs are so abundant that the circadian differences in their levels across time points have no effect on their circadian targets, perhaps suggesting that their oscillations are more important for the regulation of other target(s). Therefore we prefer the interpretation that mir-276a does not function to strengthen TIM oscillations but instead acts as a buffer to keep TIM levels within a proper range.

The transcription factor CF2 activates mir-276a expression, as shown using a luciferase reporter in S2 cells and genetic manipulations of fly strains (Fig. 4). Changing *cf2* expression alters TIM levels and behavioral rhythmicity in a manner similar to the direct manipulation of mir-276a expression (Fig. 4*E* and Fig. S5). *cf2* mRNA also is expressed similarly to mir-276a throughout the day (Fig. 4*A*). An attractive possibility is that the higher mir-276a levels during the daytime are caused by light-mediated up-regulation of CF2 activity. This pathway might complement the more traditional pathways that also lower TIM levels in response to light (24, 28, 32–34).

Although CF2 could identify another light-regulated pathway that regulates TIM, it could be difficult to test: (*i*) CF2 activity may not reflect *cf2* mRNA levels, meaning light-mediated posttranscriptional regulation of CF2 activity may be more important, or (*ii*) CF2 coactivators may be light-regulated. Because these coactivators are unidentified, it may be even more difficult to address the mechanism whereby light impacts mir-276a expression. However, the S2 cell assays (Fig. 4 *B* and *C*) could be adapted to flies to assay for in vivo light regulation of CF2 activity.

We also took advantage of the newly developed genomic-editing technique CRISPR to delete precisely the mir-276a–binding site from the *tim* 3′ UTR (Fig. 6*A*). Head extracts from these flies have increased TIM levels, and the flies are ∼50% arrhythmic, even with the deletion of a single nucleotide from the binding site (Fig. 6 *B* and *C*). The phenotypes are consistent with those from the mir-276a heterozygous KO flies but are even stronger, presumably because the deletion efficiently eliminates the activity of mir-276b as well as mir-276a on *tim* expression. This binding-site region of the *tim* 3′ UTR is conserved among all *Drosophila* species, unlike the rest of the 3′ UTR (University of California, Santa Cruz genome browser), suggesting that mir-276a regulation of *tim* extends well beyond *D. melanogaster*.

As originally suggested by Ebert and Sharp (35) for miRNA regulation of gene expression, we surmise that mir-276a prevents abrupt changes in *tim* mRNA levels from affecting TIM expression and thereby keeps TIM levels within an acceptable biological range. A similar interpretation for circadian biology has been proposed previously for the regulation of *Drosophila* CLK levels by the miRNA *bantam* (19). It is intriguing that the mir-276a misregulation of TIM has an effect on rhythmicity with no discernable effect on timekeeping. One possibility is that this effect reflects some unknown buffering mechanism that impacts timekeeping, perhaps related to the enigmatic mechanism(s) that underlie temperature compensation (36). Because *tim* missense alleles originally were identified in a screen for altered circadian period, there may be a fundamental distinction between the relationship of TIM to period determination and its contribution to output gene expression and rhythmic strength (37). If so, it further suggests that rhythmic strength is a better readout of clock gene levels than circadian period.

Materials and Methods

***Drosophila* Stocks.** *D. melanogaster* were reared on standard cornmeal/agar medium supplemented with yeast under 12-h LD cycles at 25 °C. *Pdf-GAL4* was described in ref. 38. *Tim-GAL4* (yw; *Tim-GAL4*/CyO) was described in ref. 17. *Pdf-geneswitch* was described in ref. 39. *UAS*-mir-276a and *UAS-cf2* were from the Bloomington Stock Center. *UAS*-mir-276a sponge was a kind gift from the Van Vactor laboratory, Harvard Medical School, Boston. mir-276a heterozygous KO was a kind gift from the Cohen laboratory, University of Copenhagen, Copenhagen, and is described in ref. 21. *UAS-mCD8GFP;Pdf-GAL4* was described in ref. 40. *UAS-cf2* RNAi lines (nos. 48895 and 103664) were from the Vienna Drosophila RNAi Center. CRISPR lines were generated by injecting pCFD3-dU6:3gRNA (from Addgene) containing a *tim*-targeting insert guide RNA (gRNA) into BLS #25710 flies using Rainbow Transgenic services. The mutated lines were backcrossed to wild-type flies (w1118) three times before testing.

Plasmids. For the CF2 regulation in vitro S2 cell assay, ∼10 kb of the mir-276a upstream region was amplified using AAAAGAGCTCGTGCCGCCAAGACAATTAGGCATTTGTTAT and AAAACCCGGGGAGAGACAGACGTGGCCCATTAATATCACAT and then was digested with SacI and XmaI and cloned into pGL3-Basic. The positive control plasmid consists of five CF2-binding sites and an *adh* promoter. It was cloned by annealing CATTAGTATATATAGGTCATTAGTATATATAGGTCATTAGTATATATAGGTCATTAGTATATATAGGTCATTAGTATATATAGGTCAGATAAC and CCGGGTTATCTGACCTATATATACTAATGACCTATATATACTAATGACCTATATATACTAATGACCTATATATACTAATGACCTATATATACTAATGACCTATATATACTAATGGTAC and ligation into KpnI- and XmaI-digested pGL3-Basic. pAc5.1-CF2 was generated by amplifying CF2 coding sequences from KHBD00180 (Addgene) with AAAAGATATCATGATAAAGTCCACCACGAA and AAAAGCGGCCGCGAGCGGATGCAGCTTGGTCGTGT and ligation into EcoRV- and NotI-digested pAc5.1. Renilla loading control plasmid was a kind gift from Michael Marr, Department of Biology and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA. For the mir-276a regulation on *tim* in vitro assay, the *tim* 3′ UTR was amplified with AAAACTCGAGGGATCCAATTCCAATCGATCCTAACCG and AAAAGCGGCCGCGGCTACAGGGAAAGCTTTATTACTGCTAAGCAT, digested with XhoI and NotI, and cloned into digested psiCHECK2. psiCHECK2-*tim*-mut was mutagenized from psiCHECK2-*tim* with the Agilent QuikChange II Site-Directed Mutagenesis Kit using the PAGE-purified oligos CATGTAAATGGGCACAAGGTGGTTCAAATAGTGC
and GCACTATTTGAACCACCTTGTGCCCATTTACATG.

Expression of dsRed, mir-276a, and mir-184 in S2 cells was achieved by cotransfecting *Ub-GAL4* and *UAS-dsRed* or *UAS*-miR. These vectors were kind gifts from Eric Lai, Department of Developmental Biology, Sloan-Kettering Institute, New York. The *tim*-targeting guide RNA insert for CRISPR was cloned by annealing gtcgACATGTAAATGGGCAGTTCC and aaacGGAACTGCCCATTTACATGT and ligation into the BbsI-digested pCFD3-dU6:3gRNA.

Locomotor Activity Assay. TriKinetics activity monitors were used to measure the locomotor activity of individual male flies (∼7 d old), and data were analyzed using MATLAB (MathWorks) as described (5). Flies with a rhythmicity index lower than 0.2 were considered arrhythmic.

Fly Brain Immunocytochemistry. Fly brains were immunostained as described (41). Briefly, fly heads were fixed in PBS with 4% (vol/vol) paraformaldehyde supplemented with 0.008% Triton X-100 for 1 h at 4 °C, dissected in PBS, and stained with rabbit anti-PER (1:1,000), a rat anti-TIM (1:200), or a mouse anti-GFP antibody (1:1,000; Invitrogen). Alexa Fluor 488 and Alexa Fluor 633 were used as secondary antibodies. Antibodies were also described in ref. 5. Brains were imaged at 20× on a Leica SP5 confocal microscope. ImageJ was used for signal quantification.

Western Blots. Twenty fly heads were homogenized in lysis buffer on ice and heat-denatured in SDS buffer to run on a 3–8% Tris-acetate gel (Invitrogen). iBlot Dry Blotting (Invitrogen) was used for transfer. Anti-TIM (1:4,000) was described in ref. 41. Anti-VINCULIN (Santa Cruz) was used as a loading control. ImageJ was used for signal quantification.

RNA Extraction and qRT-PCR. Total RNA from fly heads was extracted using TRIzol reagent (Invitrogen) following the supplier's protocol. Total RNA was reverse transcribed using SuperScript II and random hexamers and quantified with MasterCycler RealPlex from Eppendorf. mir-276a was basically quantified as described in ref. 17, except that MasterCycler RealPlex was used for quantification.

S2 Cell Luciferase Assay. S2 cells plated in 96-well plates (3610; Costar) were cotransfected with 8–25 ng of each plasmid mixed with 2 μL of Cellfectin II Reagent (Thermo Fisher). The total amounts of transfected plasmids were identical among samples. The Promega Dual-Luciferase Reporter Assay System was used to measure luciferase levels 3 d posttransfection.

ACKNOWLEDGMENTS. We thank Dr. Stephen Cohen (University of Copenhagen) and Dr. Davie Van Vactor (Harvard Medical School) for kindly sharing the mir-276a heterozygous KO and mir-276a sponge strains, respectively. We also thank members of the M.R. laboratory, especially Kate Abruzzi, Weifei Luo, Hua Jin, Fang Guo, and Aoife McMahon, for thoughtful discussions.

1. Allada R, Chung BY (2010) Circadian organization of behavior and physiology in Drosophila. *Annu Rev Physiol* 72:605–624.
2. Johnson CH, Elliott JA, Foster R (2003) Entrainment of circadian programs. *Chronobiol Int* 20(5):741–774.
3. Nitabach MN, Taghert PH (2008) Organization of the Drosophila circadian control circuit. *Curr Biol* 18(2):R84–R93.
4. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 99(7):791–802.
5. Guo F, Cerullo I, Chen X, Rosbash M (2014) PDF neuron firing phase-shifts key circadian activity neurons in Drosophila. *eLife* 3:3.
6. Allada R, White NE, So WV, Hall JC, Rosbash M (1998) A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* 93(5):791–804.
7. Rutila JE, et al. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* 93(5):805–814.
8. Meyer P, Saez L, Young MW (2006) PER-TIM interactions in living Drosophila cells: An interval timer for the circadian clock. *Science* 311(5758):226–229.
9. Rodriguez J, et al. (2013) Nascent-Seq analysis of Drosophila cycling gene expression. *Proc Natl Acad Sci USA* 110(4):E275–E284.
10. Price JL, et al. (1998) double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. *Cell* 94(1):83–95.
11. Li Y, Guo F, Shen J, Rosbash M (2014) PDF and cAMP enhance PER stability in Drosophila clock neurons. *Proc Natl Acad Sci USA* 111(13):E1284–E1290.
12. Luo W, et al. (2012) CLOCK deubiquitylation by USP8 inhibits CLK/CYC transcription in Drosophila. *Genes Dev* 26(22):2536–2549.
13. Menet JS, Rodriguez J, Abruzzi KC, Rosbash M (2012) Nascent-Seq reveals novel features of mouse circadian transcriptional regulation. *eLife* 1:e00011.
14. Mehta N, Cheng HY (2013) Micro-managing the circadian clock: The role of microRNAs in biological timekeeping. *J Mol Biol* 425(19):3609–3624.
15. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120(1):15–20.
16. Chen W, et al. (2014) Regulation of Drosophila circadian rhythms by miRNA let-7 is mediated by a regulatory cycle. *Nat Commun* 5:5549.
17. Vodala S, et al. (2012) The oscillating miRNA 959-964 cluster impacts Drosophila feeding time and other circadian outputs. *Cell Metab* 16(5):601–612.
18. Yang M, Lee JE, Padgett RW, Edery I (2008) Circadian regulation of a limited set of conserved microRNAs in Drosophila. *BMC Genomics* 9:83.
19. Lerner I, et al. (2015) Clk post-transcriptional control denoises circadian transcription both temporally and spatially. *Nat Commun* 6:7056.
20. Kadener S, et al. (2009) A role for microRNAs in the Drosophila circadian clock. *Genes Dev* 23(18):2179–2191.
21. Chen YW, et al. (2014) Systematic study of Drosophila microRNA functions using a collection of targeted knockout mutations. *Dev Cell* 31(6):784–800.
22. Fulga TA, et al. (2015) A transgenic resource for conditional competitive inhibition of conserved Drosophila microRNAs. *Nat Commun* 6:7279.
23. Edery I, Zwiebel LJ, Dembinska ME, Rosbash M (1994) Temporal phosphorylation of the *Drosophila period* protein. *Proc Natl Acad Sci USA* 91(6):2260–2264.
24. Hunter-Ensor M, Ousley A, Sehgal A (1996) Regulation of the *Drosophila* protein *timeless* suggests a mechanism for resetting the circadian clock by light. *Cell* 84(5):677–685.
25. Yang Z, Sehgal A (2001) Role of molecular oscillations in generating behavioral rhythms in Drosophila. *Neuron* 29(2):453–467.
26. Luo W, Sehgal A (2012) Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. *Cell* 148(4):765–779.
27. Gekakis N, et al. (1995) Isolation of timeless by PER protein interaction: Defective interaction between timeless protein and long-period mutant PERL. *Science* 270(5237):811–815.
28. Zeng H, Qian Z, Myers MP, Rosbash M (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature* 380(6570):129–135.
29. Abruzzi KC, et al. (2011) Drosophila CLOCK target gene characterization: Implications for circadian tissue-specific gene expression. *Genes Dev* 25(22):2374–2386.
30. Menet JS, Abruzzi KC, Desrochers J, Rodriguez J, Rosbash M (2010) Dynamic PER repression mechanisms in the Drosophila circadian clock: From on-DNA to off-DNA. *Genes Dev* 24(4):358–367.
31. Krol J, et al. (2010) Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell* 141(4):618–631.
32. Ceriani MF, et al. (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* 285(5427):553–556.
33. Koh K, Zheng X, Sehgal A (2006) JETLAG resets the Drosophila circadian clock by promoting light-induced degradation of TIMELESS. *Science* 312(5781):1809–1812.

34. Myers MP, Wager-Smith K, Rothenfluh-Hilfiker A, Young MW (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* 271(5256):1736–1740.
35. Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. *Cell* 149(3):515–524.
36. Kidd PB, Young MW, Siggia ED (2015) Temperature compensation and temperature sensation in the circadian clock. *Proc Natl Acad Sci USA* 112(46): E6284–E6292.
37. Rothenfluh A, Abodeely M, Price JL, Young MW (2000) Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in Drosophila. *Genetics* 156(2):665–675.
38. Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature* 431(7010): 862–868.
39. Depetris-Chauvin A, et al. (2011) Adult-specific electrical silencing of pacemaker neurons uncouples molecular clock from circadian outputs. *Curr Biol* 21(21): 1783–1793.
40. Nagoshi E, et al. (2010) Dissecting differential gene expression within the circadian neuronal circuit of Drosophila. *Nat Neurosci* 13(1):60–68.
41. Tang CH, Hinteregger E, Shang Y, Rosbash M (2010) Light-mediated TIM degradation within Drosophila pacemaker neurons (s-LNvs) is neither necessary nor sufficient for delay zone phase shifts. *Neuron* 66(3):378–385.