miR-263b controls circadian locomotor activity and the structural plasticity of small ventral lateral neurons by inhibition of Beadex

Xiaoge Nian¹,², Wenfeng Chen²,³, Weiwei Bai¹, Zhangwu Zhao¹, and Yong Zhang²*

¹Department of Entomology and MOA Key Lab of Pest Monitoring and Green Management, College of Plant Protection, China Agricultural University, Beijing, 100193, China.
²Department of Biology, University of Nevada Reno, Reno, NV 89557, USA
³Institute of Life Sciences, Fuzhou University, Fuzhou, Fujian, 350108, China

*Corresponding authors:
zhaozw@cau.edu.cn, yongzhang@unr.edu
Abstract

Circadian clocks drive rhythmic physiology and behavior to allow adaption to daily environmental changes. In Drosophila, the small ventral lateral neurons (sLNvs) are the master pacemakers that control circadian rhythms. Circadian changes are observed in the dorsal axonal projections of the sLNvs, but their physiological importance and the underlying mechanism are unclear. Here we identified miR-263b as an important regulator of circadian rhythms in Drosophila. Flies depleted of miR-263b (miR-263bKO) exhibited dramatically impaired rhythms under constant darkness. Indeed, miR-263b is rhythmically expressed and controls circadian output by affecting the structural plasticity of sLNvs through inhibition of expression of the LIM-only protein Beadex (Bx). The misexpression of Bx in flies phenocopied miR-263bKO in behavior and molecular characteristics. In addition, the circadian phenotypes of miR-263bKO were recapitulated by mutating the miR-263b binding sites in the Bx 3'UTR. Together, these results establish miR-263b as an important regulator of circadian locomotor behavior.

Keywords

Drosophila, circadian rhythms, pacemaker neurons, structural plasticity, miRNA

Introduction

Circadian clocks are intracellular pacemakers that generate approximately 24-hour rhythms of behavior and physiology in most organisms. In animals, the core circadian oscillator consists of a conserved autoregulatory transcriptional-translational negative feedback loop (Crane & Young, 2014; Hardin & Panda, 2013; Tataroglu & Emery, 2015). In Drosophila, transcription factors CLOCK (CLK) and CYCLE (CYC) activate rhythmic transcription of clock-controlled genes. Among these genes, PERIOD (PER) is a key repressor in the core circadian negative feedback loop that inhibits CLK/CYC activity and represses per transcription. Post-translational modifications such as phosphorylation,
glycosylation, and ubiquitination also play important roles in setting the pace of circadian
clock (J. C. Chiu, Ko, & Edery, 2011; Grima, Dognon, Lamouroux, Chelot, & Rouyer,
2012; Hardin & Panda, 2013; Kim et al., 2012; Ko, Jiang, & Edery, 2002; W. F. Luo et
al., 2012).

Circadian locomotor rhythms of flies are generated by a neuronal network consisting of
about 150 circadian neurons in the brain (Beckwith & Ceriani, 2015; Dubowy & Sehgal,
2017; Johard et al., 2009; Nitabach & Taghert, 2008). Based on location and function,
these circadian neurons are divided into clusters: three groups of ventral lateral neurons
(LNvs), three groups of dorsal neurons (DN1s, DN2s, and DN3s), dorsal lateral neurons
(LNds), and lateral posterior neurons (LPNs). The large LNvs (lLNvs) and four of the
small LNvs (sLNvs) express the neuropeptide PDF, whereas the fifth sLNv is PDF
negative (Nitabach & Taghert, 2008). Flies exhibit bimodal locomotor activity rhythms,
peaking around dawn and dusk. PDF-positive sLNvs are responsible for promoting the
morning peak before daylight, and the fifth sLNv and the LNds are mainly responsible for
generating the evening activity (Grima, Chelot, Xia, & Rouyer, 2004; Stoleru, Peng,
Agosto, & Rosbash, 2004). DN1s can integrate light or temperature inputs and are able
to generate either morning or evening activity; DN1s also promote sleep (Guo et al.,
2016; Zhang, Liu, Bilodeau-Wentworth, Hardin, & Emery, 2010). A recent study
indicated that the DN1 activity is acutely modulated by temperature, thus DN1s sense
temperature to regulate sleep (Yadlapalli et al., 2018). On the other hand, DN2s play
important roles in the temperature preference of circadian rhythm (Kaneko et al., 2012).

As master pacemaker neurons, the PDF-positive sLNvs synchronize other circadian
neurons, thus enabling flies to maintain robust circadian locomotor rhythms in constant
conditions (Renn, Park, Rosbash, Hall, & Taghert, 1999). Elimination of sLNvs or
mutation of pdf or its receptor causes arrhythmic behavior (Mertens et al., 2005; Renn et
al., 1999). The PDF positive sLNvs also send axonal projections toward the dorsal protocerebrum region of fly brain, where DN1s and DN2s are located (Helfrichforster, 1995; Helfrichforster & Homberg, 1993). PDF immunoreactivity in the dorsal axonal terminal of sLNvs has a clearly circadian pattern with high intensity during the daytime and low intensity at nighttime (Park et al., 2000). The sLNvs dorsal projections also show arborization rhythms with higher complexity of axon terminals found in the early day and lower complexity at the night (Fernandez, Berni, & Ceriani, 2008). This structural plasticity of the sLNvs dorsal projections is controlled by both circadian and activity dependent mechanisms (Depetris-Chauvin et al., 2011; Muraro, Pirez, & Ceriani, 2013). The molecular mechanisms underlying this plasticity of sLNv are poorly understood, however. The transcription factor Mef-2 was found to control circadian plasticity of sLNvs through regulation of the cell adhesion molecule Fas2 (Sivachenko, Li, Abruzzi, & Rosbash, 2013). In addition, two matrix metalloproteinases (MMP1, and MMP2) are also shown to be required for the structural remodeling control of sLNv projections (Depetris-Chauvin et al., 2014). Recently the circadian protein Vrille was found to control sLNv arborization rhythms through unknown mechanisms (Gunawardhana & Hardin, 2017).

MicroRNAs (miRNAs) are small non-coding RNAs of about 22 nucleotides that regulate gene expression post-transcriptionally (Bartel, 2004). miRNAs repress expression of their target genes through mRNA degradation and/or translation inhibition. Recent studies have uncovered critical roles of miRNAs in the regulation of different aspects of animal circadian clocks (Mendoza-Viveros et al., 2017; Xue & Zhang, 2018). In mouse, miR-132/212 modulates circadian entrainment to day length, thus influencing seasonal adaptation, while miR-219 and miR-24 affect circadian period length (Cheng et al., 2007; Yoo et al., 2017). In Drosophila, overexpression of the miRNA bantam lengthens circadian locomotor period by repressing clk, and timeless and clockwork orange are
targets of miR-276a and let-7, respectively (W. F. Chen et al., 2014; X. Chen & Rosbash, 2016; Kadener et al., 2009). miRNAs also play important roles in circadian output pathways. We showed previously that the disruption of general miRNA functions resulting from depletion of GW182 affects circadian rhythms by interfering with PDF-receptor signaling (Zhang & Emery, 2013). In addition, the miR-279 and miR959-964 cluster miRNAs modulate circadian locomotor behavior output and circadian timing of feeding and immune responses (W. Y. Luo & Sehgal, 2012; Vodala et al., 2012). Others and we have demonstrated that miR-124 controls the phase of circadian locomotor activity (Garaulet et al., 2016; Zhang, Lamba, Guo, & Emery, 2016).

To further understand the post-transcriptional regulation of circadian clock functions by miRNAs, we screened for circadian clock phenotypes in miRNA mutants. Here we show that a conserved miRNA, miR-263b, is an important regulator of circadian rhythms in Drosophila. miR-263b regulates circadian locomotor behavior by affecting the structural plasticity of sLNvs through inhibition of Beadex (Bx) expression.

**Results**

**miR-263b regulates circadian behavior**

To identify miRNAs that regulate circadian rhythms, we first used miRNA quantitative real-time PCR to identify rhythmically expressed miRNAs in fly heads. We found that the expression of miR-263b is rhythmic in fly heads, consistent with previous microarray data, (Figure 1A, (Yang, Lee, Padgett, & Edery, 2008)). Importantly, the oscillation of miR-263b was abolished in the Clk\(^{\text{ARK}}\) mutant confirming that the expression of miR-263b is under circadian control (Figure 1A).

To examine the circadian role of miR-263b, we analyzed the previously generated miR-263b knockout (miR-263b\(^{\text{KO}}\)) flies (Hilgers, Bushati, & Cohen, 2010). In constant darkness (DD) condition, flies lacking miR-263b displayed a severely disrupted circadian
locomotor rhythm (Figure 1B, Table 1). In contrast to the wild-type flies, more than 60% of the miR-263b KO flies were arrhythmic (Figure 1B). Moreover, the amplitude of behavioral rhythms was clearly reduced even in rhythmic miR-263b KO flies (Figure 1C). Because of the high percentage of arrhythmia of the miR-263b KO flies in DD, we examined the locomotor activity under light dark cycles (LD). Under LD, wild-type flies gradually increase their activity before light-on, an anticipatory behavior controlled by the clock. In miR-263b KO flies, however, the morning anticipation was abolished (Figure 1D and 1E).

Next, we utilized a GAL4 knock-in replacement (Δ263b-GAL4, (Hilgers et al., 2010)) to drive expression of miR-263b, resulting in a 9-fold increase of miR-263b abundance (Figure S1). The overexpression of miR-263b disrupted circadian locomotor activity rhythms in DD (Figure 1B and 1C). In addition, a significant reduction of morning anticipation was also observed in these flies (Figure 1D and 1E). Together, these results indicate that miR-263b KO and overexpression of miR-263b both decrease the robustness of circadian locomotor activity rhythms and suppress the morning anticipation in LD. Notably, the free-running period was not significantly altered in the flies in which miR-263b levels were abnormal (Figure 1F, Table 1).
Figure 1. *miR-263b* is rhythmically expressed and regulates circadian behavior.

Quantitative-PCR analysis of *miR-263b* from adult brains of wild-type and *Clk<sup>pik</sup>* mutant flies at the indicated time points. The relative expression levels were normalized to 2S rRNA and were further normalized to *w<sup>1118</sup>* at ZT0. Each time point was compared to ZT0. Data represent mean ± SEM. ***P< 0.001 determined by Student’s t test. (B-D) Comparison of B) rhythmicity, C) power, and D) morning anticipation index in indicated fly strains. Data represent mean ± SEM (n=45-79). *P<0.05, **P<0.01, ***P< 0.001 determined by Student’s t test. (E) Locomotor activity of indicated strains measured during 3 days of LD. The white and black bars indicate day and night, respectively. (F) Actograms showing the average activities on the last day in LD and 5 days in DD. Light represents the day and gray darkness. See also Figure S1.
Figure S1. miR-263b expression level in relevant flies.

Quantitative realtime PCR analysis of total RNA prepared from adult brains at ZT13. The relative expression levels were normalized to 2S RNA levels and were further normalized to w^1118 control. Data represent mean ± SEM. n.s. no significant, *P<0.05, **P<0.01, ***P< 0.001 determined by Student’s t test.

Table 1: Locomotor activity of flies with altered miR-263b and Bx levels in DD

| Genotype                  | N  | % Rhythmic | Period (hr) ± S.E.M. | Power* ± S.E.M. |
|---------------------------|----|------------|----------------------|-----------------|
| w^1118                    | 79 | 98.5±1.5   | 24.4±0.1             | 86.0±2.7        |
| miR-263b^KU               | 60 | 41.5±10.4  | 24.3±0.1             | 58.7±4.8        |
| Bx mutant                 | 105| 40.1±4.8   | 25.7±0.2             | 44.7±2.8        |
| Δ263b-GAL4/UAS-263b       | 45 | 68.8±2.6   | 24.2±0.2             | 61.4±4.5        |
| UAS-Bx/+; Δ263b-GAL4/+     | 48 | 25.1±10.9  | 24.4±0.4             | 48.6±6.3        |
| Δ263b-GAL4/+              | 76 | 96.6±3.3   | 24.5±0.0             | 94.1±3.7        |
| UAS-263b/+                | 48 | 95.8±4.2   | 23.8±0.1             | 99.2±3.7        |
| UAS-Bx/+                  | 45 | 100.0±0.0  | 24.0±0.1             | 105.7±3.6       |
| Bx*1                      | 71 | 43.6±11.7  | 24.1±0.1             | 69.5±4.3        |
| Bx*2                      | 69 | 31.1±6.7   | 24.3±0.1             | 54.3±5.4        |
| yw, Act5C-Cas9            | 48 | 100.0±0.0  | 24.4±0.2             | 89.1±4.0        |

*Power is a measure of rhythm amplitude and corresponds to the height of the periodogram peak above the significance line.
**miR-263b is required for the circadian structural plasticity of sLNv axonal projections**

To examine whether the impaired circadian locomotor rhythmicity in miR-263b\(^{KO}\) flies is due to a defect in the molecular pacemaker or in the circadian output pathway, we examined the oscillation of the key pacemaker protein PER in DD in three important groups of circadian neurons: sLNvs, LNds, and DN1s. We found no obvious changes in PER oscillation in these neurons known to control locomotor behavior (Figure S2), suggesting that miR-263b may regulate circadian behavior by modulating the output pathway.

![Image of confocal images and graphs](Figure S2. The molecular pacemaker is not affected in miR-263b\(^{KO}\) flies. (A) Representative confocal images of sLNvs from \(^{w1118}\) and miR-263b\(^{KO}\) flies dissected at six time points (circadian time, CT) during the second day of DD and stained with anti-PDF (green) and anti-PER (red) antibodies. Scale bar, 10 µm. (B-D) Quantification of PER staining in sLNvs, LNds, and DN1s at different circadian time points. Data represent mean ± SEM (16-19).)
PDF-positive sLNvs send axonal projections toward the dorsal protocerebrum region (Helfrichforster & Homberg, 1993). In addition, circadian structural remodeling of sLNv dorsal projections has been observed (Fernandez et al., 2008). To determine whether miR-263b affects the circadian plasticity of PDF-positive projections, we examined the termini of sLNv dorsal projections in miR-263bKO flies with a PDF-specific antibody at early day (Zeitgeber time 2 (ZT2), ZT0 is light on and ZT12 is light off) and early night (ZT14). Similar to previous reports, we found that the wild-type flies had more branches of axon terminals at ZT2 than at ZT14 (Figure 2A and 2B). The axonal crosses were quantified with Sholl’s analysis to assay the interactions between axon branches and concentric circles (see Methods, (Sivachenko et al., 2013)). Remarkably, this difference was abolished in the miR-263bKO flies due to a significant decrease of axonal crosses at ZT2 compared to control w1118 flies (Figure 2B and 2C). No significant changes of PDF levels were detected in the sLNv soma, indicating that the decrease of axonal branches was not due to the decrease of PDF staining (Figure 2A, Figure S3).

Figure 2. miR-263b modulates the structural plasticity of sLNv axonal projections.
(A) Representative confocal images of whole brains from the indicated genotypes stained for PDF at ZT2. Scale bar, 75 µm. (B) Representative images of sLNv dorsal projections from the indicated genotypes stained with anti-PDF at ZT2 and ZT14. Flies were entrained for at least 3 days under LD conditions prior to the assay. Note “open”, defasciculated axonal conformation at ZT2 and fasciculated axons at ZT14 in control w^{1118} flies. Scale bar, 10 µm. (C) Quantification of axonal morphology (fasciculation) of sLNv dorsal termini in LD conditions by Sholl’s analysis (see also STAR* Methods). Data represent means ± SEM (n=15-22). n.s. no significant, ***P< 0.001 determined by Student’s t test. (D) Representative images of sLNv dorsal projections from the indicated genotypes stained with anti-PDF at CT2 or CT14 during the second day of DD. Scale bar, 10 µm. (E) Quantification analysis of axonal morphology of sLNv dorsal termini in DD conditions. Data represent means ± SEM (n=15-21). n.s. no significant, ***P< 0.001 determined by Student’s t test.

Figure S3. PDF levels in sLNvs soma are not significantly changed under LD conditions.

(A-B) Quantification of PDF staining in sLNvs at ZT2 and ZT14. Data represent mean ± SEM (n=18-21). n.s. no significant, *P<0.05, **P<0.01, ***P< 0.001 determined by Student’s t test.
To test whether sLNv dorsal projections were present in the absence of miR-263b, a membrane-tethered GFP (mCD8-GFP) was used to mark the PDF projections under the control of a pdf-specific promoter. As observed by PDF staining, this marker revealed that the dorsal axonal branches of sLNVs were dramatically reduced (Figure S4). Interestingly, overexpression of miR-263b caused similar defects in PDF axonal projections (Figure 2B and 2C). These data indicate that the proper level of miR-263b is required for the circadian structural plasticity of sLNv dorsal projections.

Figure S4 shows the axonal projection of an sLNv labeled by a membrane-tethered GFP.

(A) Representative confocal images of fly brains stained for GFP (green) and PDF (red) dissected at ZT2 and ZT14. Scale bar, 10 µm. (B) Quantification analysis of axonal morphology of sLNv dorsal termini. Data represent mean ± SEM (n=15-21). n.s. no significant, ***P< 0.001 determined by Student's t test.

The structural plasticity of sLNv dorsal projections is under circadian regulation and is activity dependent (Fernandez et al., 2008; Gorostiza, Depetris-Chauvin, Frenkel, Pirez, & Ceriani, 2014; Petsakou, Sapsis, & Blau, 2015; Sivachenko et al., 2013). To further confirm miR-263b affects the circadian structural plasticity of sLNVs, we dissected fly brains in DD (Figure 2D). In wild-type flies, we observed more axonal branches at
circadian time 2 (CT2) in the subjective morning and fewer branches in the subjective
night (CT14) (Figure 2D and 2E). Similar to the results in LD, the rhythmic change in
structural plasticity was abolished in the \(miR-263b^{\text{KO}}\) flies and in flies that overexpressed
\(miR-263b\) in DD (Figure 2D and 2E).

sLNv axonal complexity is modified in response to electrical activity, and adult-specific
activation of sLNvs results in increased complexity (Depetris-Chauvin et al., 2011). Thus
we examined whether this activity-dependent structural plasticity requires \(miR-263b\). We
took advantage of the temperature-gated TrpA1 channel to increase the neuronal activity
of sLNvs (Hamada et al., 2008). Increasing the environment temperature to 30°C for 2
hours beginning at ZT12 specifically activated sLNvs and caused defasciculation of
sLNv dorsal termini at ZT14 in flies expressing TrpA1 (Figure 3A and 3B). Activation of
sLNvs by temperature treatment also increased the dorsal projection complexity (Figure
3B and 3C). Effects were dependent on TrpA1 expression and were similar in all
genotypes, including \(miR-263b^{\text{KO}}\). Together, these results indicate that \(miR-263b\) is
required for the circadian structural plasticity but dispensable for the activity-dependent
rhythms in sLNv dorsal projections.
Figure 3. Activity-dependent remodeling of sLNvs axonal fasciculation is intact in miR-263bKO flies.

(A) Diagram of activity induction by heat. Flies were raised at 18°C and entrained in LD cycles at 18°C for at least 3 days before shifting to 30°C at ZT12. Flies were dissected 2hr later (ZT14) for anti-PDF staining. (B) Representative confocal images of sLNvs projections at ZT14 with or without heat shock (HS). Induction of TrpA1 in PDF cells by 2-h temperature elevation to 30°C leads to open conformation of s-LNvs dorsal projections at ZT14 in all strains. Scale bar, 10 µm. (C) Quantification of TrpA1-induced changes in axonal fasciculation. “+” or “-” represents that the relative genes are present or absent in the flies. Data represent means ± SEM (n=26-34). n.s. No significant. ***P< 0.001 determined by Student’s t test.

**Bx regulates circadian rhythms, and its expression is suppressed by miR-263b**

miRNAs usually regulate gene expression by binding to the 3’ untranslated regions (UTRs) of target mRNAs and causing mRNA degradation and/or translational repression (Bartel, 2004). We used an in silico prediction algorithm to identify putative miR-263b targets by using the annotated Drosophila genome (http://www.targetscan.org/fly_12/).
One of the potential mRNA targets was Beadex (Bx), which encodes a LIM-only protein. A disrupted circadian rhythm was previously observed in flies with mutations in Bx (Tsai, Bainton, Blau, & Heberlein, 2004). Target prediction indicated that the Bx 3'UTR has two putative miR-263b binding sites, which are highly conserved across Drosophila species (Figure S5).

Figure S5. Predicted miR-263b binding site conservation in the Bx 3'UTR among Drosophila species. Blue letters indicate conserved sequences. Green letters indicate miR-263b seed region; red letters indicate positions of mutations in Bx 3'UTR made for S2 cell reporter gene assay.

To test whether miR-263b directly binds to the Bx 3'UTR and inhibits its expression, the Bx 3'UTR was fused downstream of a luciferase reporter and transfected into S2 cells. Luciferase activity was significantly suppressed when miR-263b was co-transfected with the reporter. In contrast, the luciferase activity was observed in the presence of miR-263b when the putative miR-263b binding sites within the Bx 3'UTR were mutated (Figure 4A). Encouraged by these S2 cell results, we further tested whether Bx abundance is suppressed by miR-263b in fly brain. Since the BX antibody we generated in our lab did not work for staining, we decided to use an enhanced GFP (EGFP) reporter strategy. We tested whether miR-263b regulates BX levels by expressing an
EGFP reporter fused to the wild-type Bx 3'UTR or 3'UTR with miR-263b binding sites mutated (same construct as Figure 4A). We expressed these two reporters using a Bx-GAL4 line, which is expressed in the PDF-positive sLNvs and ILNvs, as well as other region of fly brain (Figure S6). Strikingly, we found that the expression of EGFP under the control of the wild-type Bx 3'UTR is significantly lower than the mutant 3'UTR in both sLNvs and ILNvs (Figure 4B and 4C). Thus, our S2 cell and imaging results suggest that Bx is a direct target of miR-263b and is negatively regulated by miR-263b.

miRNAs are normally negative regulators of gene expression, so we reasoned that overexpression of Bx should mimic miR-263bKO. As expected, overexpression of Bx using Δ263b-GAL4 caused approximately 75% arrhythmicity in DD, which phenocopied the miR-263bKO (Figure 4D-F, and Table 1). Furthermore, similar to miR-263b overexpression, even in the rhythmic Bx-mutant flies amplitude of behavior rhythms, and morning anticipation were significantly reduced (Figure 4G and 4H). Taken together, these results demonstrate that Bx is required for robust circadian locomotor rhythms and is a potential target of miR-263b.
Figure 4. Bx regulates circadian rhythms, and its expression is suppressed by miR-263b.

(A) pAC or pAC-miR-263b were co-transfected with pAc-fluc-Bx 3'UTR or pAc-fluc-Bx 3'UTR (mut) and with pCopia-Renilla luciferase into S2 cells. After two days luciferase activity was quantified. For each condition, a normalized firefly/Renilla luciferase value is plotted with SEM.

(B) Representative confocal images of Bx 3'UTR (Bx-GAL4/+;UAS-EGFP-Bx 3'UTR/+) and Bx 3'UTR(mut) (Bx-GAL4/+;UAS-EGFP-Bx 3'UTR(mut)/+). The brains were stained with anti-GFP.
antibody (green) and anti-PDF antibody (red) at ZT13. Scale bar, 10 µm. (C) Quantification of GFP signals in sLNvs and ILNvs from Bx 3'UTR and Bx 3'UTR(mut) flies. Data represent means ± SEM (n=16-17). *P<0.05, **P<0.01 determined by Student’s t test. (D) Locomotor activity of adult male flies of the indicated genotypes measured during 3 days of LD cycle. The white and black bars indicate day and night, respectively. (E) Actograms showing the average activities on the last day in LD and the 5 days in DD. Light represents the day and gray darkness. (F-H) Comparison of E) rhythmicity, F) power, and G) morning anticipation in indicated lines. The numbers of tested flies are shown in each column. Each experiment was conducted three times. Data represent means ± SEM (n=45-105). **P<0.01, ***P<0.001 determined by Student’s t test.

Figure S6: Bx is expressed in the PDF positive sLNvs and ILNvs.

(A-C) Representative confocal images of fly brains, ILNvs and sLNvs stained for GFP (green) and PDF (red) dissected at ZT13. Scale bar, 100 µm.

Bx regulates the arborization rhythms of sLNv dorsal projections
If miR-263b regulates circadian rhythms by downregulating Bx expression, the Bx mutant should phenocopy the circadian structural plasticity defects in miR-263b overexpression. We therefore examined the dorsal axonal projections of sLNvs in Bx mutant flies. As expected, PDF projections were maintained in the fasciculated state during the day as well as the night in Bx mutants. At ZT2, the Bx mutants showed reduced maximal axonal crosses relative to those in control flies that were not significantly different from those observed at ZT14 (Figure 5A, 5B, and 5C). Interestingly, when Bx was overexpressed in miR-263b expressing cells results were similar to those in the miR-263b\textsuperscript{KO} flies (Figure 5A, 5B, and 5C). The rhythmic change in structural plasticity was abolished in the Bx mutants or Bx overexpression in DD (Figure 5D and 5E). These results support our hypothesis that miR-263b regulates the fasciculation-defasciculation state of the PDF termini via effects on Bx expression. In addition, activation of PDF cells with TrpA1 in the Bx mutant background resulted in defasciculated sLNv dorsal termini at ZT14 (Figure 5F and 5G).
Figure 5. Bx regulates the arborization rhythms of sLNv dorsal projections.

(A) Representative confocal images of brains of the indicated genotypes stained for PDF at ZT2. Scale bar, 75 µm. (B) Dorsal projections of sLNvs at ZT2 and ZT14. Flies were entrained for at least 3 days under LD conditions prior to the assay. Scale bar, 10 µm. (C) Quantification analysis of axonal morphology (fasciculation) of sLNv dorsal termini in LD conditions by Sholl’s analysis. Data represent means ± SEM (n=15-23). n.s. no significant. ***P< 0.001 determined by Student’s t test. (D) Representative confocal images of the projections at CT2 or CT14 during the second day of DD. Scale bar, 10 µm. (E) Quantification of axonal morphology of sLNv dorsal termini in DD conditions. Data represent means ± SEM (n=15-21). n.s. no significant. B***P< 0.001 determined by Student’s t test. (F) Confocal images of sLNv projections from flies stained with anti-PDF at ZT14. Conformation changes in response to 2-hour temperature elevation (HS) is shown on the right. The left show a control not subjected to temperature elevation. (G)
Quantification of TrpA1-induced changes in axonal fasciculation. Data represent means ± SEM (n=19-34). n.s. no significant, ***P< 0.001 determined by Student's t test.

**miR-263b binding sites in the Bx 3'UTR are essential for circadian function of Bx**

To further confirm that Bx is a direct miR-263b target, we used the CRISPR/Cas9 system to mutate the potential miR-263b binding sites within the Bx 3'UTR in flies. The seed region of miRNAs (positions 2-7) are critical for activity (Brennecke, Stark, Russell, & Cohen, 2005). It has been shown that substitution of one nucleotide in the seed sequence is sufficient to reduce miRNA binding to the target mRNA (Brennecke et al., 2005). We recovered two fly lines (Bx*1 and Bx*2) with mutations in the miR-263b binding sites (Figure 6A). While Bx*1 had a T to G point mutation in the seed sequences of one of the two miR-263b binding sites in the Bx 3'UTR, Bx*2 had mutations in both sites. There were also several base changes near the seed sequence that differed in Bx*1 and Bx*2 (Figure 6A). We predicted that if miR-263b suppresses Bx abundance through the binding sites in the 3'UTR, Bx*1 and Bx*2 mutants should phenocopy miR-263bKO. As expected, both Bx*1 and Bx*2 mutants showed significantly impaired locomotor activity rhythms and reduced power of rhythms (Figure 6B-6E). The morning anticipation was also dramatically reduced or abolished, especially in the Bx*2 mutants (Figure 6B and 6F). Furthermore, similar to the miR-263bKO flies, Bx*2 mutants also had fasciculated sLNv projections and significantly decreased numbers of axonal crosses at ZT2 (Figure 6G and 6H). As flies with disrupted miR-263b binding sites within Bx 3'UTR show anatomical and behavioral phenotypes similar to those seen in miR-263bKO flies, we conclude that miR-263b regulates circadian rhythm by directly regulating Bx expression.
Figure 6. Mutation of the miR-263b binding site in the Bx 3'UTR leads to behavioral arrhythmicity.

(A) Sequence in the miR-263b binding site region in the Bx 3'UTR and mutations induced using the CRISPR/Cas9 system. (B) Locomotor activity of adult male flies of indicated strains measured during 4 days of LD cycles and 5 days of DD. The white and black bars indicate day and night, respectively. (C) Actograms showing the average activities on the last day in LD and the 5 days in DD. Light represents the day and gray darkness. (D-F) Comparison of rhythmicity, power, and F) morning anticipation in indicated genotypes. The numbers of tested flies are shown in each column. Data represent means ± SEM (n=48-69). (G) Representative confocal images of control and Bx*2 fly brains stained for PDF at ZT2. Scale bar, 75 µm for the whole-mount figure and 10 µm for the magnified images. (H) Quantification of axonal morphology from control and Bx*2 flies. Data represent means ± SEM (n=18-21). *P<0.05, **P<0.01, ***P< 0.001 determined by Student's t test.

Discussion

Here we demonstrate that miR-263b is critical for circadian behavior rhythms and axonal structural plasticity of pacemaker neurons. miR-263b modulates circadian rhythms via repression of Bx. Alterations in levels of miR-263b or Bx led to behavior arrhythmia and defects of arborization rhythm in sLNvs.
The mechanisms underlying the structural plasticity of sLNv dorsal projections are poorly understood. This plasticity is controlled by both circadian regulation and activity-dependent mechanisms. The maximal numbers of axon terminal branches are found in the early day and the minimal numbers in the night. This arborization rhythm exists even in constant darkness indicating this process is under circadian control. Manipulating the activity of sLNvs can change the axon plasticity, however. For example, electrical silencing the sLNvs during adulthood can reduce axon complexity (Depetris-Chauvin et al., 2011). Whether the circadian and activity regulatory mechanisms are independent of each other or interconnected is unknown. Recently, the transcription factor Mef-2 has been shown to control both circadian- and activity-dependent axonal fasciculation of sLNvs through the neural cell adhesion molecule Fas2 (Sivachenko et al., 2013). This indicates that the two mechanisms might be interconnected through Mef-2. Here we showed that miR-263b specifically regulates the circadian structural plasticity by suppressing Bx expression. Interestingly, the activity-dependent axonal changes are intact in miR-263b and Bx mutants. Thus, it is possible that different mechanisms influence circadian regulation and activity-dependent regulation of structural plasticity. It will be interesting to test whether there is a mechanism that only affects the activity-dependent structural changes in sLNvs.

Consistent with a report by Yang et al. (Yang et al., 2008), our data indicate that there is a cyclic expression of miR-263b in fly heads and that this expression is under clock control. Whereas the effect of miR-263b on structural plasticity occurred in the early morning, the maximal expression of miR-263b was late in the day and in early evening (Figure 1A). This apparent discrepancy between the expression peak and function peak may be due to the mechanism of miRNA action. Most miRNAs function through translational inhibition or degradation of target mRNAs so it is possible that the peak in
protein abundance of miRNA targets is opposite the expression peak of the miRNA. In the future, it will be interesting to see whether Bx has a peak of abundance at early morning that matches its function.

Evidence suggests that \textit{miR-263b} regulates Bx expression in the sLNv cell bodies.

A previous study and data shown here clearly show that Bx is enriched in sLNvs (Fig S5, (Tsai et al., 2004)). That Bx is expressed in sLNvs suggests an autonomous mechanism since the \textit{miR-263b} binding sites are critical. \textit{miR-263b} is enriched in central nervous neurons (Yang et al., 2008); however, we cannot exclude a glial contribution of \textit{miR-263b}. A recent study showed that glial expression of \textit{miR-263b} is also required for circadian locomotor behavior (You, Fulga, Van Vactor, & Jackson, 2018). Tissue-specific knock out of \textit{miR-263b} will help answering this question.

Here we demonstrated that \textit{miR-263b} regulates \textit{Drosophila} circadian locomotor rhythms and axonal structural plasticity of pacemaker neurons. A similar mechanism may exist in mammals. The suprachiasmatic nucleus (SCN) is the mammalian master circadian pacemaker (Mohawk, Green, & Takahashi, 2012; Reppert & Weaver, 2002). In SCN, a group of neurons expressing vasoactive intestinal peptide (VIP) receive retinal light input to synchronize the clock (Morin & Allen, 2006). VIP is the functional homologue of \textit{Drosophila} PDF. Interestingly, circadian structural plasticity was found in the glutamatergic synapse on the VIP neurons, which maybe important for light entrainment (Girardet et al., 2010). As a highly conserved miRNA, \textit{miR-263b} has a homologue in vertebrates: \textit{miR-183}. Expression of \textit{miR-183} is enriched in both retina and the pineal gland (Clokie, Lau, Kim, Coon, & Klein, 2012; Xu, Witmer, Lumayag, Kovacs, & Valle, 2007), Furthermore, in the rat pineal gland, \textit{miR-183} is rhythmically expressed with a peak at around ZT12 (Clokie et al., 2012). This oscillation of \textit{miR-183} suggests a potential circadian function. Bx is a \textit{Drosophila} LIM-only protein. \textit{In silico}
miRNA target prediction with targetscan identified a highly conserved binding site of miR-183 in the 3'UTR of Lmo3, a LIM-only protein conserved from rodents to primates (Dambal, Shah, Mihelich, & Nonn, 2015). Our results establish miR-263b as an important regulator of circadian locomotor behavior and suggest that a highly conserved miRNA-LIM-only protein pathway regulates circadian rhythms in organisms as diverse as flies and mammals.

**Materials and Methods**

**Fly stocks**

The following strains were used in this study: w^{1118}, Clk^{Jrk}, y w; Pdf-GAL4/CyO, UAS-TrpA1, Δ263b-GAL4/TM6B (Hilgers et al., 2010); UAS-Bx and Bx mutant (Bejarano, Smibert, & Lai, 2010); Bx-GAL4, UAS-miR-263b, miR-263b^{KO} (obtained from the Bloomington Stock Center). All the flies were raised on standard cornmeal/agar medium at 25 °C under 12 hour:12 hour LD cycle.

**Behavioral experiments and analysis**

Adult male flies (2-5 days old) were used to test locomotor activity rhythms. Flies were entrained for 4 days LD cycle at 25 °C, using about 500 lux light intensities, and then released into constant darkness (DD) at 25 °C for at least 5 days. Locomotor activity was recorded with a TriKinetics Activity Monitor in an I36-LL Percival Incubator. FAAS-X software was used to analyze behavioral data (Grima et al., 2002). Actograms were generated with a signal-processing toolbox for MATLAB. The morning anticipation amplitude was determined by assaying for the locomotor activity as described (Zhang & Emery, 2013).

**Whole-mount immunohistochemistry and quantification**
Whole-mount immunohistochemistry of fly brains were done as previously described (Zhang et al., 2010). For PDF staining, adult flies were entrained to LD for 4 days and dissected at ZT2 or 14. For PER staining, flies were entrained to LD for 4 days and then release into DD. Brains were dissected on the second day of DD at six time points. Mouse anti-PDF (1:400), rabbit anti-GFP (1:600), rabbit anti-GFP (1:1500) antibodies were used. All samples were imaged using a Leica TCS SP8 confocal microscope with a constant laser setting for each time point. Eight to ten brains for each genotype were dissected for imaging. ImageJ software (Schneider, Rasband, & Eliceiri, 2012) was used for GFP, PER and PDF quantification from at least five brains. For quantification, the average signals of three neighboring background areas were subtracted from signal intensity in each circadian neuron. Each experiment was conducted three times.

Quantitative real-time PCR

30-40 flies were collected at the indicated time points, heads were isolated on dry ice and stored at -80 °C. Total RNA, including miRNA, was purified with miRcute miRNA isolation kits (TIANGEN). Reverse transcription and real-time PCR of miR-263b and 2s rRNA were performed with first-stand cDNA synthesis kits and miRcute miRNA qPCR detection kits (SYBR Green) (TIANGEN). For miR-263b the following primer was used: 5’GCGTTTCTCCTTGGCACTGGG. 2s was used for normalization and the following primer was used: 5’-TGCTTGGACTACATATGGTTGAGG. Each experiment was conducted three times.

S2 cell luciferase assay

The full-length 3’UTR of Bx and about 400 base pairs of coding region of miR-263b were amplified by PCR using primeSTAR HS DNA Polymerase (TaKaRa). The Bx 3’UTR was cloned into a pAc5.1-firefly luciferase-V5-His vector and the miR-263b coding region was cloned into a pAc5.1-V5-His vector (Invitrogen). The miR-263b seed-
targeted sequence in the 3'UTR of Bx was mutated from GTGCCAA to CTACTCG using site-directed, ligase-independent mutagenesis (J. Chiu, March, Lee, & Tillett, 2004). We co-transfected 100 ng pAc-firefly luc-Bx 3'UTR (pAc-firefly luc-Bx 3’UTR mutant), 1 µg pAc-miR-263b, and 100 ng pAc-Renilla luc (transfection control) into S2 cells. Luciferase activity (Dual Luciferase System, Promega) was measured two days after transfection. Each experiment was conducted three times.

**Analysis of axonal morphology by modified Sholl’s method**

The following fly genotypes were used: pdf-GAL4/UAS-TrpA1 (control), Bx mutant; UAS-TrpA1, Bx mutant; pdf-GAL4/UAS-TrpA1, UAS-TrpA1/+; miR-263bKO, pdf-GAL4/UAS-TrpA1; miR-263bKO. For the analysis of activity-dependent changes in axonal morphology, pdf-GAL4/UAS-TrpA1, Bx mutant; pdf-GAL4/UAS-TrpA1, pdf-GAL4/UAS-TrpA1; miR-263bKO, UAS-TrpA1; miR-263bKO, Bx mutant; UAS-TrpA1 flies were raised at 18 °C and entrained in LD cycles at 18 °C for at least 3 days, then shifted to 30 °C at ZT12. Flies were dissected 2 hours later (ZT14) and stained with anti-PDF. Structural plasticity was analyzed as reported (Sivachenko et al., 2013). Each experiment was conducted three times.

**Statistics analysis**

Statistical analysis was performed with SPSS statistics 17.0. P values was obtained with t-test and considered n.s. no significant, significant at *P < 0.05 and extremely significant at ***P < 0.001.

**Acknowledgements**

We thank Dr. Yi Liu and Dr. Patrick Emery for carefully reading the manuscript and suggesting improvements. We thank Dr. Steve Cohen for the miR-263bKO and Δ263b-GAL4 fly strains and Dr. Eric Lai for the Bx mutant and UAS-Bx fly strains. We also
thank Dr. Ralf Stanewsky for the anti-PER antibodies. We thank the Bloomington stock Center for various fly stocks. We also thank the Developmental Studies Hybridoma Bank for PDF antibodies. This work is supported by grants from the National Nature Science Foundation of China (Grant numbers 31572317 and 31730076) to Zhangwu Zhao and the China Scholarship Fund. Wenfeng Chen’s work is supported by the National Natural Science Foundation of China (grant number 31601894), and the Fujian Natural Science Foundation (grant number 2017J0106). Yong Zhang’s lab is supported by the National Institutes of Health COBRE Grant P20 GM103650.

Author Contributions

Z.Z. and Y.Z. supervised the project and designed the experiments. X.N., W.C., and W.B. performed the experiments and analysis. Y.Z., Z.Z., and X.N. wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

References

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell, 116(2), 281-297.
Beckwith, E. J., & Ceriani, M. F. (2015). Experimental Assessment of the Network Properties of the Drosophila Circadian Clock. Journal of Comparative Neurology, 523(6), 982-996.
Bejarano, F., Smibert, P., & Lai, E. C. (2010). miR-9a prevents apoptosis during wing development by repressing Drosophila LIM-only. Developmental Biology, 338(1), 63-73.
Brennecke, J., Stark, A., Russell, R. B., & Cohen, S. M. (2005). Principles of MicroRNA-target recognition. Plos Biology, 3(3), 404-418.
Chen, W. F., Liu, Z. X., Li, T. J., Zhang, R. F., Xue, Y. B., Zhong, Y., . . . Zhao, Z. W. (2014). Regulation of Drosophila circadian rhythms by miRNA let-7 is mediated by a regulatory cycle. Nature Communications, 5.
Chen, X., & Rosbash, M. (2016). mir-276a strengthens Drosophila circadian rhythms by regulating timeless expression. Proceedings of the National Academy of Sciences of the United States of America, 113(21), E2965-E2972.
Cheng, H. Y. M., Papp, J. W., Varlamova, O., Dziema, H., Russell, B., Curfman, J. P., . . . Obrietan, K. (2007). microRNA modulation of circadian-clock period and entrainment. Neuron, 54(5), 813-829.

Chiu, J., March, P. E., Lee, R., & Tillett, D. (2004). Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. Nucleic Acids Research, 32(21).

Chiu, J. C., Ko, H. W., & Edery, I. (2011). NEMO/NLK Phosphorylates PERIOD to Initiate a Time-Delay Phosphorylation Circuit that Sets Circadian Clock Speed. Cell, 145(3), 357-370.

Clokie, S. J. H., Lau, P., Kim, H. H., Coon, S. L., & Klein, D. C. (2012). MicroRNAs in the Pineal Gland miR-483 REGULATES MELATONIN SYNTHESIS BY TARGETING ARYLALKYLAMINE N-ACETYLTRANSFERASE. Journal of Biological Chemistry, 287(30), 25312-25324.

Crane, B. R., & Young, M. W. (2014). Interactive Features of Proteins Composing Eukaryotic Circadian Clocks. Annual Review of Biochemistry, Vol 83, 83, 191-219.

Dambal, S., Shah, M., Mihelich, B., & Nonn, L. (2015). The microRNA-183 cluster: the family that plays together stays together. Nucleic Acids Research, 43(15), 7173-7188.

Depetris-Chauvin, A., Berni, J., Aranovich, E. J., Muraro, N. I., Beckwith, E. J., & Ceriani, M. F. (2011). Adult-Specific Electrical Silencing of Pacemaker Neurons Uncouples Molecular Clock from Circadian Outputs. Current Biology, 21(21), 1783-1793.

Depetris-Chauvin, A., Fernandez-Gamba, A., Gorostiza, E. A., Herrero, A., Castano, E. M., & Ceriani, M. F. (2014). Mmp1 Processing of the PDF Neuropeptide Regulates Circadian Structural Plasticity of Pacemaker Neurons. Plos Genetics, 10(10).

Dubowy, C., & Sehgal, A. (2017). Circadian Rhythms and Sleep in Drosophila melanogaster. Genetics, 205(4), 1373-1397.

Fernandez, M. P., Berni, J., & Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. Plos Biology, 6(3), 518-524.

Garaulet, D. L., Sun, K. L., Li, W. H., Wen, J. Y., Panzarino, A. M., O’Neil, J. L., . . . Lai, E. C. (2016). miR-124 Regulates Diverse Aspects of Rhythmic Behavior in Drosophila. Journal of Neuroscience, 36(12), 3414-3421.

Girardet, C., Blanchard, M. P., Ferracci, G., Leveque, C., Moreno, M., Francois-Bellan, A. M., . . . Bosler, O. (2010). Daily changes in synaptic innervation of VIP neurons in the rat suprachiasmatic nucleus: contribution of glutamatergic afferents. European Journal of Neuroscience, 31(2), 359-370.

Gorostiza, E. A., Depetris-Chauvin, A., Frenkel, L., Pirez, N., & Ceriani, M. F. (2014). Circadian Pacemaker Neurons Change Synaptic Contacts across the Day. Current Biology, 24(18), 2161-2167.

Grima, B., Chelot, E., Xia, R., & Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. Nature, 431(7010), 869-873. doi:10.1038/nature02935
Grima, B., Dognon, A., Lamouroux, A., Chelot, E., & Rouyer, F. (2012). CULLIN-3 Controls TIMELESS Oscillations in the Drosophila Circadian Clock. Plos Biology, 10(8).

Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limboureg-Bouchon, B., & Rouyer, F. (2002). The F-box protein Slimb controls the levels of clock proteins Period and Timeless. Nature, 420(6912), 178-182.

Gunawardhana, K. L., & Hardin, P. E. (2017). VRILLE Controls PDF Neuropeptide Accumulation and Arborization Rhythms in Small Ventrolateral Neurons to Drive Rhythmic Behavior in Drosophila. Current Biology, 27(22), 3442-+.

Guo, F., Yu, J. W., Jung, H. J., Abruzzi, K. C., Luo, W. F., Riffith, L. C. G., & Rosbash, M. (2016). Circadian neuron feedback controls the Drosophila sleep-activity profile. Nature, 536(7616), 292-+.

Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J., & Garrity, P. A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature, 454(7201), 217-U255.

Hardin, P. E., & Panda, S. (2013). Circadian timekeeping and output mechanisms in animals. Current Opinion in Neurobiology, 23(5), 724-731.

Helfrichforster, C. (1995). The Period Clock Gene Is Expressed in Central-Nervous-System Neurons Which Also Produce a Neuropeptide That Reveals the Projections of Circadian Pacemaker Cells within the Brain of Drosophila-Melanogaster. Proceedings of the National Academy of Sciences of the United States of America, 92(2), 612-616.

Helfrichforster, C., & Homberg, U. (1993). Pigment-Dispersing Hormone-Immunoreactive Neurons in the Nervous-System of Wild-Type Drosophila-Melanogaster and of Several Mutants with Altered Circadian Rhythmicity. Journal of Comparative Neurology, 337(2), 177-190.

Hilgers, V., Bushati, N., & Cohen, S. M. (2010). Drosophila microRNAs 263a/b Confer Robustness during Development by Protecting Nascent Sense Organs from Apoptosis. Plos Biology, 8(6).

Johard, H. A. D., Yoishii, T., Dircksen, H., Cusumano, P., Rouyer, F., Helfrich-Forster, C., & Nassel, D. R. (2009). Peptidergic Clock Neurons in Drosophila: Ion Transport Peptide and Short Neuropeptide F in Subsets of Dorsal and Ventral Lateral Neurons. Journal of Comparative Neurology, 516(1), 59-73.

Kadener, S., Menet, J. S., Sugino, K., Horwich, M. D., Weissbein, U., Nawathean, P., . . . Rosbash, M. (2009). A role for microRNAs in the Drosophila circadian clock. Genes & Development, 23(18), 2179-2191.

Kaneko, H., Head, L. M., Ling, J., Tang, X., Liu, Y., Hardin, P. E., . . . Hamada, F. N. (2012). Circadian rhythm of temperature preference and its neural control in Drosophila. Current Biology, 22(19), 1851-1857. doi:10.1016/j.cub.2012.08.006

Kim, E. Y., Jeong, E. H., Park, S., Jeong, H. J., Edery, I., & Cho, J. W. (2012). A role for O-GlcNAcylation in setting circadian clock speed. Genes & Development, 26(5), 490-502.

Ko, H. W., Jiang, J., & Edery, I. (2002). Role for Slimb in the degradation of Drosophila Period protein phosphorylated by Doubletime. Nature, 420(6916), 673-678.
Luo, W. F., Li, Y., Tang, C. H. A., Abruzzi, K. C., Rodriguez, J., Pescatore, S., & Rosbash, M. (2012). CLOCK deubiquitylation by USP8 inhibits CLK/CYC transcription in Drosophila. *Genes & Development, 26*(22), 2536-2549.

Luo, W. Y., & Sehgal, A. (2012). Regulation of Circadian Behavioral Output via a MicroRNA-JAK/STAT Circuit. *Cell, 148*(4), 765-779.

Mendoza-Viveros, L., Chiang, C. K., Ong, J. L. K., Hegazi, S., Cheng, A. H., Bouchard-Cannon, P., . . . Cheng, H. Y. M. (2017). miR-132/212 Modulates Seasonal Adaptation and Dendritic Morphology of the Central Circadian Clock. *Cell Reports, 19*(3), 505-520.

Mertens, I., Vandingenen, A., Johnson, E. C., Shafer, O. T., Li, W., Trigg, J. S., . . . Taghert, P. H. (2005). PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. *Neuron, 48*(2), 213-219.

Mohawk, J. A., Green, C. B., & Takahashi, J. S. (2012). Central and Peripheral Circadian Clocks in Mammals. *Annual Review of Neuroscience, Vol 35*, 445-462.

Muraro, N. I., Pirez, N., & Ceriani, M. F. (2013). The Circadian System: Plasticity at Many Levels. *Neuroscience, 247*, 280-293.

Nitabach, M. N., & Taghert, P. H. (2008). Organization of the Drosophila circadian control circuit. *Current Biology, 18*(2), R84-R93.

Park, J. H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M., & Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America, 97*(7), 3608-3613.

Petsakou, A., Sapis, T. P., & Blau, J. (2015). Circadian Rhythms in Rho1 Activity Regulate Neuronal Plasticity and Network Hierarchy. *Cell, 162*(4), 823-835.

Renn, S. C. P., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (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.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature, 418*(6901), 935-941.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675.

Sivachenko, A., Li, Y., Abruzzi, K. C., & Rosbash, M. (2013). The Transcription Factor Mef2 Links the Drosophila Core Clock to Fas2, Neuronal Morphology, and Circadian Behavior. *Neuron, 79*(2), 281-292.

Stoleru, D., Peng, Y., Agosto, J., & Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature, 431*(7010), 862-868. doi:10.1038/nature02926.

Tataroglu, O., & Emery, P. (2015). The molecular ticks of the Drosophila circadian clock. *Current Opinion in Insect Science, 7*, 51-57.

Tsai, L. T., Bainton, R. J., Blau, J., & Heberlein, U. (2004). Lmo mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. *Plos Biology, 2*(12), e408. doi:10.1371/journal.pbio.0020408.

Vodala, S., Pescatore, S., Rodriguez, J., Buescher, M., Chen, Y. W., Weng, R. F., . . . Rosbash, M. (2012). The Oscillating miRNA 959-964 Cluster Impacts
Drosophila Feeding Time and Other Circadian Outputs. *Cell Metabolism*, 16(5), 601-612.

Xu, S. B., Witmer, P. D., Lumayag, S., Kovacs, B., & Valle, D. (2007). MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *Journal of Biological Chemistry*, 282(34), 25053-25066.

Xue, Y. B., & Zhang, Y. (2018). Emerging roles for microRNA in the regulation of Drosophila circadian clock. *Bmc Neuroscience*, 19.

Yadlapalli, S., Jiang, C., Bahle, A., Reddy, P., Eyhofer, E. M., & Shafer, O. T. (2018). Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature*, 555(7694), 98-.

Yang, M., Lee, J. E., Padgett, R. W., & Edery, I. (2008). Circadian regulation of a limited set of conserved microRNAs in Drosophila. *Bmc Genomics*, 9.

Yoo, S. H., Kojima, S., Shimomura, K., Koike, N., Buhr, E. D., Furukawa, T., . . . Takahashi, J. S. (2017). Period2 3'-UTR and microRNA-24 regulate circadian rhythms by repressing PERIOD2 protein accumulation. *Proceedings of the National Academy of Sciences of the United States of America*, 114(42), E8855-E8864.

You, S., Fulga, T. A., Van Vactor, D., & Jackson, F. R. (2018). Regulation of Circadian Behavior by Astroglial MicroRNAs in Drosophila. *Genetics*, 208(3), 1195-1207.

Zhang, Y., & Emery, P. (2013). GW182 Controls Drosophila Circadian Behavior and PDF-Receptor Signaling. *Neuron*, 78(1), 152-165.

Zhang, Y., Lamba, P., Guo, P. Y., & Emery, P. (2016). miR-124 Regulates the Phase of Drosophila Circadian Locomotor Behavior. *Journal of Neuroscience*, 36(6), 2007-2013.

Zhang, Y., Liu, Y. X., Bilodeau-Wentworth, D., Hardin, P. E., & Emery, P. (2010). Light and Temperature Control the Contribution of Specific DN1 Neurons to Drosophila Circadian Behavior. *Current Biology*, 20(7), 600-605.