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Splice variants of DOMINO control *Drosophila* circadian behavior and pacemaker neuron maintenance

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Abstract

Circadian clocks control daily rhythms in behavior and physiology. In *Drosophila*, the small ventral lateral neurons (sLNv,s) expressing PIGMENT DISPERSING FACTOR (PDF) are the master pacemaker neurons generating locomotor rhythms. Despite the importance of sLNv,s and PDF in circadian behavior, little is known about factors that control sLNv,s maintenance and PDF accumulation. Here, we identify the *Drosophila* SWI2/SNF2 protein DOMINO (DOM) as a key regulator of circadian behavior. Depletion of DOM in circadian neurons eliminates morning anticipatory activity under light dark cycle and impairs behavioral rhythmicity in constant darkness. Interestingly, the two major splice variants of DOM, DOM-A and DOM-B have distinct circadian functions. DOM-A depletion mainly leads to arrhythmic behavior, while DOM-B knockdown lengthens circadian period without affecting the circadian rhythmicity. Both DOM-A and DOM-B bind to the promoter regions of key pacemaker genes *period* and *timeless*, and regulate their protein expression. However, we identify that only DOM-A is required for the maintenance of sLNv,s and transcription of *pdf*. Lastly, constitutive activation of PDF-receptor signaling rescued the arrhythmia and period lengthening of DOM downregulation. Taken together, our findings reveal that two splice variants of DOM play distinct roles in circadian rhythms through regulating abundance of pacemaker proteins and sLNv,s maintenance.

Author summary

Circadian rhythms are critical for timing of animal bodily functions. In flies, sLNv,s are the master pacemaker neurons regulating locomotor rhythms, which release the neuropeptide PDF. Little is known about factors that control sLNv,s maintenance and PDF accumulation. Here, we identified the *Drosophila* chromatin remodeler DOMINO (DOM) as a new regulator of circadian behavior. Depletion of DOM in circadian neurons impaired behavioral rhythmicity in constant darkness. Interestingly, two splice variants of DOM have...
distinct functions. DOM-A depletion mainly led to arrhythmia, while DOM-B knockdown lengthened circadian period. Furthermore, we found DOM-A is critical for the maintenance of sLN_{vs} and transcription of pdf. Our findings reveal that DOM splice variants play distinct roles in rhythms through different mechanisms.

**Introduction**

Circadian clocks allow animals to anticipate daily oscillations in behavior, physiology and metabolism [1]. The core of the molecular clock is a negative transcriptional-translational feedback loop, which is evolutionarily conserved across species [2]. The fruit fly *Drosophila melanogaster* has been a powerful model in dissecting the molecular and neuronal mechanisms of circadian rhythms. In *Drosophila*, a heterodimeric complex of CLOCK (CLK) and CYCLE (CYC) activates rhythmic transcription of clock-controlled genes, including the transcriptional repressor *period* (*per*) and *timeless* (*tim*). PER and TIM dimerize and repress their own transcription by blocking CLK-CYC transactivation [3]. A number of kinases (CK1, SGG, NEMO, etc) and phosphatases (PP1, PP2A) also control the circadian clock at the post-translational level [3].

Circadian locomotor rhythms are controlled by a small set of clock neurons expressing core pacemaker proteins in the brain. In each hemisphere of the fly brain, there are ~75 clock neurons, which are divided into clusters based on their anatomical locations and functions in circadian behavior [4]. There are three groups of dorsal neurons (DN1s, DN2s, and DN3s), the lateral dorsal neurons (LNd{s}), the lateral posterior neurons (LPN{s}), the large ventral lateral neurons (lLN_{vs}), and the small ventral lateral neurons (sLN_{vs}). The lLN_{vs} and four sLN_{vs} express the neuropeptide PIGMENT DISPERSING FACTOR (PDF), while the fifth sLN_{v} is PDF negative. The PDF positive sLN_{vs} are the key pacemaker neurons: they control the circadian rhythmicity under constant darkness (DD) [4]. They are also critical for generating morning anticipatory activity before lights on (i.e. dawn) under light-dark cycle (LD) [5, 6]. The sLN_{vs} send axonal projections towards the DN1s and DN2s, and the structural plasticity of dorsal projections is under circadian control [7–9]. PDF positive sLN_{vs} and their dorsal projections are formed in 4 hrs old first instar larvae after hatching [10]. Despite the importance of sLN_{vs} in the circadian behavior, little is known about the mechanisms that control the maintenance of sLN_{vs}.

PDF is the most prominent neuropeptide in the regulation of circadian behavioral rhythms [11]. PDF plays a critical role in the synchronization of different groups of clock neurons by binding to PDF receptor (PDFR), and activating cAMP-activated protein kinase A (PKA) signaling [12–15]. Loss of PDF or PDFR abolishes morning anticipation and significantly impairs behavioral rhythmicity in DD. Not only as a critical circadian output signal, PDF/PDFR signaling has also been shown to control the molecular clock by regulating the stability of pacemaker proteins PER and TIM recently [16–18]. PDF abundance in the sLN_{vs} is regulated by CLK and CYC [19–21]. Another pacemaker protein VRILLE also promotes PDF levels [22]. Despite these studies, mechanisms regulating PDF abundance remain poorly understood.

DOM is a chromatin-remodeling protein, which belongs to the SW1/SNF2 DNA-dependent ATPase family [23]. DOM is in the TIP60 complex and plays a critical role for incorporation of the phosphorylated Histone H2A variant (H2Av) and exchange with H2Av in *Drosophila* [24]. It has been found that DOM is involved in oogenesis, wing development, cell viability and proliferation, neuroblast maintenance and polarity, as well as in dendrite development [23, 25–27]. In the fly genome, two alternative splicing variants of *dom* encode DOM-A
and DOM-B. Interestingly, a recent study identified that DOM-A and DOM-B play distinct roles in cell-type specific development during Drosophila oogenesis [25].

A few chromatin remodelers have been shown to regulate circadian photoresponses and gene expression [28–30]. However, the role played by chromatin remodeling in the control of Drosophila circadian clocks is still largely unknown. Here, we report the functions of DOM in the regulation of circadian rhythms. Using isoform-specific RNAi and rescue, we demonstrate distinct functions of DOM-A and DOM-B in the regulation of circadian locomotor rhythms. Depletion of DOM-A in tim-expressing circadian neurons leads to arrhythmic behavior and long period, while DOM-B downregulation specifically lengthens the circadian period in flies. Both DOM-A and DOM-B bind to the promoters of per and tim, and regulate the abundance of PER and TIM levels, which is critical for the control of circadian period. However, DOM-A is specifically necessary for maintenance of sLN,s as well as accumulation of PDF in these neurons. Indeed, activation of PDFα signaling restores the locomotor rhythms of DOM downregulation. Together, our results suggest that the two alternative spliced variants of DOM play distinct roles in circadian rhythms.

**Results**

**DOM regulates circadian locomotor rhythms**

Mass spectrometry (MS) label-free quantitative proteomics approach was previously performed to identify the BRAHMA (BRM) chromatin-remodeling protein complex as interactor of circadian clock proteins [31]. In the same data set, we observed that several core subunits of the ATP-dependent DOM chromatin-remodeling complex, including DOM, NIPPED-A, PONT, REPT, and Mrg15, interact with CLK in the nucleus of Drosophila S2 cells (Table 1). Since DOM is the ATPase subunit of the protein complex and shows significant binding to CLK (especially C-terminal FLAG-tagged CLK) according to SAINT (Significant Analysis of

| Symbol | FlyBaseID | peptide^a Count(3 reps) | Avg^b | Max^c | False^d Discovery Rate (FDR) | peptide^a Count(3 reps) | Avg^b | Max^c | False^d Discovery Rate (FDR) | Control AP-MS |
|--------|-----------|--------------------------|-------|-------|-------------------------------|--------------------------|-------|-------|-------------------------------|---------------|
| Domino | FBgn0020306 | 37|0|46^e | 0.652 0.9825 0.0663 | 30|17|16 | 1 | 1 | 0 | 0|0|0 | 0|0|0 |
| Nipped- A | FBgn0053554 | 53|2|114 | 1 | 1 | 0 | 75|45|55 | 1 | 1 | 0 | 0|0|0 | 0|0|0 |
| His2AV | FBgn001197 | 7|1|8 | 0.8631 0.9896 0.0172 | 1|0|3 | 0.5466 0.8827 0.1643 | 0|0|0 | 0|0|0 | 0|0|0 |
| Pont | FBgn0040078 | 27|15|34 | 1 | 1 | 0 | 25|22|26 | 1 | 1 | 0 | 0|0|0 | 0|0|0 | 0|0|0 |
| Rept | FBgn0040075 | 28|16|30 | 1 | 1 | 0 | 30|9|14 | 0.9994 1 | 0.0001 | 2|0|1|0 | 0|0|0 | 0|0|0 | 0|0|0 |
| E(Pc) | FBgn0000581 | 17|0|20 | 0.6398 0.9638 0.1001 | 34|17|17 | 1 | 1 | 0 | 0|0|0 | 0|0|0 | 0|0|0 | 0|0|0 | 0|0|0 |
| Mrg15 | FBgn0027378 | 4|3|2 | 0.9874 0.9937 0.0010 | 6|0|3 | 0.6367 0.9718 0.0875 | 0|0|0 | 0|0|0 | 0|0|0 | 0|0|0 | 0|0|0 | 0|0|0 |

^a Number of peptides mapped to the specified prey protein in affinity purification (AP) followed by mass spectrometry (MS).

^b Average probability that protein interactions between the bait and prey protein are *bona fide*. Values were from all biological replicates as calculated by SAINT (Significant Analysis of Interactome).

^c Highest probability of protein interaction between the bait and prey protein across all replicate as calculated by SAINT.

^d False discovery rate as calculated by SAINT using all biological replicates.

^e Number of peptides mapped to prey protein in control AP-MS.

^f Peptide counts for 3 replicate proteins are shown.

^g peptide counts for 4 biological replicates are shown.

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INTeractome) scoring [32], we decided to further investigate its role in circadian regulation. Interestingly, prior studies indicated that dom mRNA levels in the sLNvs are clearly enriched about 4.4-fold compared to other neurons [33].

Homozygous dom mutants are lethal, therefore we used RNAi to deplete DOM in circadian neurons. Dicer 2 was co-expressed to enhance RNAi efficiency and has been shown to have no effects on circadian rhythms [34, 35]. When we expressed dom dsRNAs using tim-GAL4, a circadian tissue-specific driver [36], most of the flies became arrhythmic, and the amplitude of rhythm was significantly reduced (Fig 1A and S1 Table). Only 31% of flies with dom downregulation (41% for another independent line dom RNAi#2) were rhythmic, and interestingly the circadian period of these lines was about 1.5 hours longer than the control. The reduced rhythmicity and amplitude were also observed when we targeted all PDF negative circadian cells, using a combination of tim-GAL4 with the repressor transgene pdf-GAL80 (Fig 1A and S1 Table). Only a slight period-lengthening of activity rhythm (~0.4hr) was detected. These results indicated that DOM primarily functions in the LNvs to control circadian behavior, but could function in PDF negative circadian neurons to fine-tune circadian period length.

Fig 1. Depletion of DOM disrupted circadian rhythms. A. Free-running period (top panel) and power (bottom panel) of DOM depleted flies. The percentages of rhythmic flies are shown above each column. The number of tested flies is shown in each column. dom mRNA targeted by the two non-overlapping RNAi lines from the TRIP stock center. domRNAi#1 (JF01502) is a long double-stranded RNAi line, while domRNAi#2 (HMS01855) is a short hairpin RNAi line. TD2 = tim-GAL4, UAS-dcr2, PD2 = pdf-GAL4, UAS-dcr2, TD2; PG80 = TD2, pdf-GAL80. n = 50–90; error bars represent ± SD; n.s., non-significant, "p < 0.01, ""p < 0.001, """"p < 0.0001; one-way ANOVA. B. Average locomotor activity of flies under 3 days of 12:12 hr LD conditions. Dark activity bars represent the night, and white bars represent the day. The significant differences of the values (shown above bars) indicate morning anticipation is severely disrupted in dom RNAi lines. C. Actograms showing the average activities on the last day of LD followed by 5 days in DD. Light represents the day and gray darkness. From left to right: (Left panel) Gal4 control; (middle panel) domRNAi#1 flies; and (right panel) domRNAi#2 flies (knockdown of dom in all circadian neurons). Depletion of DOM caused arrhythmia in DD.

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Because of the high arrhythmia in DD, we also examined the behavioral phenotype of dom downregulation in tim-expressing circadian neurons under LD. We found that DOM knockdown abolished the morning peak of anticipatory activity in flies (Fig 1B). Importantly, two independent dom RNAi lines targeting non-overlapped regions of dom exhibited similar phenotypes in both LD and DD (Fig 1C and S1 Fig). Thus, it is unlikely that off-target effect of dom RNAi cause these circadian phenotypes. Furthermore, quantitative RT-PCR results showed that dom expression were significantly reduced in fly heads of DOM knockdown as compared to controls (S1 Fig). Together, these results suggest that DOM is important for regulation of circadian locomotor behavior.

DOM regulates the abundance of PER and TIM

Depletion of DOM severely decreased the rhythmicity and lengthened circadian period. To understand how DOM affects circadian rhythms, we first measured the oscillations of mRNA abundance of three core pacemaker genes in fly heads with expression of dom dsRNAs using tim-GAL4. For clk, the abundance and oscillation of mRNA under LD and DD were not affected in dom RNAi flies (Fig 2A). However, we found that the abundance of per and tim mRNA was reduced, especially at the time point of peak expression under LD (Fig 2A). Under...
DD, depletion of DOM also decreased the level of per and tim mRNA, which indicates that the effects are not due to masking effects of light (Fig 2A). We hypothesized that DOM may facilitate CLK-driven rhythmic transcription by regulating the balance between H2Av and phospho-H2Av via binding to CLK. So we examined the H2Av occupancy at per and tim promoter by performing chromatin immunoprecipitation (ChIP) in fly heads using the commercialize Drosophila H2Av antibodies. We entrained flies under LD and collected head samples at ZT4 and ZT16, close to the trough and peak time of CLK binding [37]. Consistent with our hypothesis, we observed a significant increase in H2Av binding at both promoters when dom is knocked down in tim-expressing circadian cells after normalized to an intergenic region control (Fig 2B). This data indicate that DOM interacts with CLK to regulate transcription of circadian genes by replacing H2Av at the promoters. However, we did not observe significant difference in binding between ZT4 and ZT16 (Fig 2B), which indicates that the effect of DOM might not be time-dependent.

Next we examined the oscillation of PER protein in major groups of pacemaker neurons with tim-GAL4 expressing dom dsRNA across 24 hours under DD. Consistent with the changes at mRNA level, the level of PER was also significantly reduced in the sLNv with DOM depletion (Fig 2C and 2D). In other groups of circadian neurons, such as the LNds and DN1s, decreased abundance of PER was also observed (Figs 2D and S2). Taken together, our results suggest that DOM regulates the H2Av occupancy at promoters of circadian genes and controls the abundance of PER.

**DOM-A but not DOM-B is regulated by CLK**

There are two major splice variants of dom: dom-A and dom-B, which has distinct functions during Drosophila oogenesis [25]. We wondered whether these two isoforms would have different functions in circadian rhythms. We first examined the expression pattern of dom-A and dom-B in the fly head across different times of the day. Interestingly, dom-A exhibited a strong oscillation pattern with a trough around zeitgeber time 9 (ZT9, as ZT0 is light on and ZT12 is light off) and a peak expression near ZT21 (S3A Fig). Oscillation of dom-A was also observed under DD. In addition, the oscillation of dom-A expression was abolished in the Clock null mutant Clkout (S3A Fig) [38]. Together, these data indicate that dom-A is controlled by circadian clock. However, we did not observe a clear oscillation for dom-B expression (S3B Fig). In order to detect changes at protein level, we took advantage of isoform-specific antibodies to DOM-A or DOM-B [25]. We confirmed the specificity of these antibodies by altering the level of DOM-A or DOM-B using RNAi and overexpression (S3C and S3D Fig). Similar to mRNA level, DOM-A protein abundance was dramatically reduced in Clkout, however, no oscillation of DOM-A was observed in wild type flies (S3E and S3F Fig). This loss of DOM-A oscillation at protein level indicates that DOM-A protein might be quite stable. The levels of DOM-B were not affected in Clkout (S3G and S3H Fig).

**DOM-A and DOM-B have distinct roles in circadian regulation**

Differential regulations of DOM-A and DOM-B by CLK suggest that they might have different roles in circadian rhythm. We therefore performed isoform-specific downregulation in circadian tissues using small hairpin RNA (shRNA) targeting dom-A or dom-B. These transgenic fly lines have been previously shown to specifically knockdown these alternative isoforms (S1 Fig) [25]. We first quantified the efficiency of dom-A and dom-B downregulation by expressing shRNAs in circadian cells of fly heads using quantitative real-time PCR. Consistent with previous report [25], we observed that each shRNA line specifically downregulated dom-A or dom-B (S1B Fig). Interestingly, when we depleted DOM-A or DOM-B in all circadian clock neurons
by tim-GAL4, we observed two distinct circadian behavioral phenotypes. Most of the flies expressing dom-A shRNA lost locomotor rhythms, except for ~20% that were rhythmic and showed a longer period as compared to the control (Fig 3A and 3B; S1 Table). As with depletion of all dom isoforms, DOM-A specific knockdown also blunted the morning activity peak under LD (S4 Fig). However, for DOM-B knockdown, flies exhibited a lengthened period of ~1.5 hrs in DD (Fig 3A and 3B), but no effect on amplitude of rhythms or morning anticipation was observed under LD (Fig 3B and S4 Fig). When we expressed dom-A or dom-B shRNA only in LN\textsubscript{s} using pdf-GAL4, or in PDF negative circadian neurons, we found similar effects on rhythmicity or period-lengthening (Fig 3B). These results suggested that DOM-A and DOM-B are required in both PDF positive and negative circadian neurons for behavior. It is unlikely that the different circadian phenotype is due to greater efficiency of dom-A over dom-B shRNA (S1B Fig). Actually a previous study has also validated the knockdown efficiency and found that dom-B shRNA is in fact slightly stronger than dom-A in the fly nervous system [25]. However, we cannot fully exclude the possibility that DOM-A protein is more stable than DOM-B and cause this difference of circadian phenotypes.

Our isoform-specific knockdown indicated that DOM-A and DOM-B have distinct functions in circadian rhythms. Although the shRNA lines have been shown here and in a previous study, DOM-A and DOM-B have distinct functions in regulating circadian locomotor rhythms. A. Actograms showing the average activities on the last day of LD followed by 5 days in DD. Light represents the day and gray darkness. From top to bottom: domA\textsuperscript{RNAi} (Top left panel); domA\textsuperscript{RNAi}; UAS-domA rescue (Top right panel); and domB\textsuperscript{RNAi} (Bottom left panel); domB\textsuperscript{RNAi}; UAS-domB rescue (Bottom right panel). Depletion of domA caused arrhythmia in DD, while knocking down domB lengthened circadian period. domA\textsuperscript{RNAi} and domB\textsuperscript{RNAi} phenotypes can be rescued by restoring domA and domB expressing in all circadian neurons respectively. B. Free-running period and percentage of rhythmicity of domA and domB depleted flies. The percentages of rhythmic flies are shown above the error bar. The numbers of tested flies are shown in each column. C. Free-running period and percentage of rhythmicity of flies with restoring DOM-A or DOM-B in domA or domB depletion. D. ChIP assays detecting DOM-A and DOM-B binding more at the per CRS and tim E-box in flies expressing domA (BL64261) and domB (BL64263) in tim-expressing cells as compared to control TG4 flies. Quantification was done same as Fig 2B. Error bars represent ± SEM; n.s., non significant; P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; one-way ANOVA. 

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study [25] to specifically knockdown the dom-A or dom-B isoforms, off-target effects could still contribute to the observed phenotypes. Thus, we performed rescue experiments with UAS transgenic flies expressing either dom-A or dom-B cDNA. Circadian behavior defects were rescued when we overexpressed corresponding UAS lines in the DOM-A or DOM-B knockdown (Fig 3A and 3C), which indicated that the isoform specific phenotype we observed was not due to off-target effects. Remarkably, with overexpression of dom-A, we were not able to rescue the long period phenotype of DOM-B knockdown (Fig 3C, and S1 Table). Similarly, overexpression of dom-B could not rescue the arrhythmic phenotype of DOM-A knockdown, although we did notice that the period-lengthening effect was partially rescued in the remaining 13% of rhythmic flies (Fig 3C).

Since we observed DOM regulates the occupancy of H2Av at per and tim promoters, we therefore examined whether DOM-A and DOM-B might be bound to the promoters of per and tim. We used the DOM-A- and DOM-B-specific antibodies to perform ChIP in fly heads with overexpression of dom-A or dom-B at two time points: ZT4 and ZT16. We observed significant enrichments of DOM-A and DOM-B binding on both per and tim promoters, compared to an intergenic region control (Fig 3D). Similar with H2Av ChIP, we did not observe significant difference in binding between ZT4 and ZT16 (Fig 3D), which indicates that the binding of DOM-A and DOM-B might not be time-dependent.

**DOM-A, but not DOM-B, is required for the maintenance of sLNv,s and PDF abundance**

The blunted morning activity peak and arrhythmia in DOM and DOM-A knockdown flies suggest that there might be defects in the sLNv,s or in PDF signaling. There are three major projections of PDF positive LNv,s. The ILNv,s send projections to the optic lobes and the contralateral brain hemisphere, while the sLNv,s send projections to the DN1s and DN2s region [39]. Based on whole mount immunohistochemistry in fly brains, we did not observe obvious defects in the brain structure or projections of the ILNv,s, however, PDF levels in the dorsal projections of the sLNv,s were barely detectable in DOM and DOM-A knockdown flies (Fig 4A). Absence of the dorsal projection or low PDF expression in the sLNv,s could lead to decrease of PDF in the dorsal projection [20, 40]. Our data are consistent with both possibilities. Using pdf-GAL4 and a membrane tethered GFP (CD8-GFP) to label axons of sLNv,s, we observed that the dorsal projections of sLNv,s were clearly shortened in DOM and DOM-A (Fig 4B–4D). Interestingly, the sLNv,s dorsal projections were normal in DOM-B downregulation, which is consistent with the behavior results. Furthermore, close observation of the PDF positive sLNv,s revealed that both the number of sLNv,s and PDF levels were reduced (Fig 5A and 5B). For each hemisphere, the average number of PDF positive sLNv,s in DOM and DOM-A knockdown flies was approximately 2, compared to 4 in the control flies or DOM-B knockdown (Fig 5A and 5B). However, with DOM-B knockdown, we did not observe any obvious loss of sLNv,s or reduction in PDF levels, which indicates that this process is specifically controlled by DOM-A (Fig 5A and 5B). Consistent with the ChIP results, the levels of PER and TIM were found to be significantly decreased in the sLNv,s with knockdown of DOM-A or DOM-B (Fig 5A and 5B). This may also explain why depletion of DOM-A or DOM-B had an effect on period-lengthening.

Next we examined which step of regulation causes the reduction of PDF levels in DOM and DOM-A knockdown. DOM regulates gene expression at the transcription level [24]. We therefore examined the expression of pdf using a transcriptional reporter line, pdfTomato [21]. Consistent with PDF staining, we observed significant reduction of pdf transcription in sLNv,s of both DOM and DOM-A knockdown (Fig 5C and 5D). Interestingly, the decrease in neuron
number and reduction of PDF level were unique to sLN\textsubscript{v}s, which were not detected in the lLN\textsubscript{v}s (Fig 5C and 5D).

In summary, consistent with differences in circadian behavior, DOM-A and DOM-B knockdown also exhibit differences at the molecular level. Knockdown of DOM-A causes loss of sLN\textsubscript{v}s and decrease in sLN\textsubscript{v} PDF levels, which is not observed in DOM-B downregulation.

Fig 4. Eliminations of dorsal projections in DOM and DOM-A downregulation were due to reduced number of sLN\textsubscript{v}s and decrease of PDF. A. Representative confocal images showing PDF expression in whole brain and dorsal axonal projection of sLN\textsubscript{v}s. Brains were dissected at ZT1 for anti-PDF (green) and anti-PER antibodies (red). Comparing to the control (Top left panel), dorsal projection of sLN\textsubscript{v}s was disrupted in dom (Top right panel) and dom\textsubscript{A} downregulation (Bottom left panel) no effects were observed in the presence of dom\textsubscript{B} RNAi (Bottom right panel) (Scale bar: left, 500 um; right, 100 um). B. Associated genotypes adults brains collected at ZT1 were immunostained with GFP (green) and PDF (red) antibodies and imaged (see Materials and Methods) (Scale bar: 500 um). C. Representative confocal images of dorsal axonal projection of sLN\textsubscript{v}s (Scale bar: 100 um). D. Representative images of sLN\textsubscript{v}s of associated lines. Merged GFP+PDF images were shown as yellow (Scale bar: 50 um).
Fig 5. Depletion of DOM-A, but not DOM-B affects s-LNv maintenance and PDF accumulation. A. Representative images of sLNv's of associated lines. Brains were dissected at ZT1 for anti-PDF (green) and anti-PER antibodies (red) or dissected at ZT23 for anti-PDF (green) and anti-TIM.
antibodies (red). Cell number of sLNv (marked as square brackets), PDF, PER and TIM levels in sLNv were decreased in dom and domA downregulation flies brains (Top right and bottom left panels), while only affected PER and TIM levels in domB downregulation flies (Bottom right panel). (Scale bar: 50 um). B. Quantification of sLNv numbers in each brain hemisphere, as well as PDF, PER and TIM levels in sLNv. n = 32 hemispheres was used for quantification. C. Pdf transcriptional level in lLNv and sLNv using TOMATO fluorescence signal. Pdf transcriptional levels in sLNv (marked as circle) were decreased in dom and domA downregulation fly brains (middle two panels), while sLNv numbers and pdf transcriptional levels in domB downregulation flies were normal (right panel) (Scale bar: 100 um.). D. Quantification of sLNv numbers and TOMATO fluorescence signal in sLNv and lLNv. n = 24 hemispheres were used. Error bars correspond to SEM. "p < 0.01, "p < 0.001, "p < 0.0001 as determined by t test.

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**DOM is required during development and adulthood for circadian rhythms**

Decrease of sLNv numbers and dorsal projections in adult flies might be due to abnormal development or maintenance defects. Therefore, we performed brain dissections at larval stage and pupal stage. The number of sLNv precursor in the DOM knockdown condition was unchanged compared to the control at 3rd instar larvae (Fig 6A and 6B), which suggests that DOM does not affect sLNv development. However, same as adulthood, even at early pupal stage (3 days after pupation), there were only 1–2 sLNv detected in dom downregulation (Fig 6C and 6D). These data indicate that DOM is required for the maintenance but not for the development of sLNv. DOM probably affects sLNv during larval-pupal metamorphosis. Consistent with adult flies, both PDF and PER levels of dom downregulation were reduced in sLNv compared to the control in larvae or pupae (Fig 6B and 6D).

The decrease of axonal projections and number of sLNv we observed in DOM knockdown flies (Figs 4 and 5) suggested that DOM might play a role during development to properly regulate circadian rhythms. Thus, we used tim-GAL4 and GAL80ts TARGET system to temporally express dom dsRNA during development or after eclosion [41]. GAL80 is active at 18°C thus blocks GAL4 function and prevents dsRNA production, while 30°C inactivates GAL80 and allows expression of dsRNA to downregulate DOM. When we grew flies at 18°C and tested circadian behavior at 30°C, we observed strong rhythmicity but around 1–2 hr period-lengthening with adult-specific DOM or DOM-A knockdown (Fig 6E and S1 Table). However, knockdown DOM-A only during development led to high arrhythmic activity (Fig 6E and S1 Table). Thus, developmental expression of dom or dom-A dsRNA appears to affect the rhythmicity, while adult specific depletion appears to be sufficient for arrhythmic activity (Fig 6E and S1 Table). Therefore, knockdown of DOM-B only in adulthood caused a ~1 hr increase in period length, suggesting that unlike DOM-A, the DOM-B splice form is mainly necessary for the regulation of circadian clocks in adult stage (Fig 6E and S1 Table). We observed a slight but significant lengthening of period (~0.6 hr, S1 Table), when we specifically depleted DOM-B in circadian neurons during development, which indicates that DOM-B might also play a role during development.

To exclude anatomical defects in the circadian circuitry, we grew flies at 18°C and entrain them under LD for 5 days at 30°C, then performed brain dissections at ZT1. As expected, the dorsal projections and number of sLNv in the flies with adult-specific DOM downregulation were normal, and PDF abundance was not affected (Fig 6F and 6G). These data further suggest that the arrhythmia we observed in DOM knockdown was mainly due to the function of DOM during development. Indeed, the abundance of PER in sLNv was still reduced, which could explain the lengthened period phenotype (Fig 6G). Since we observed DOM binding to the per and tim promoters, and since DOM is required at adulthood for the period-lengthening, we checked whether adulthood specific downregulation of DOM affects per and tim transcription by using quantitative RT-PCR. The pattern of per and tim oscillation was still present in adult-specific DOM knockdown, however the abundance of transcripts is dramatically reduced, especially for the early night time points of per (S5 Fig), which is consistent with the PER protein level (Fig 6F and 6G).
Taken together, these data indicate that DOM/DOM-A is required during development for the rhythmic activity, while both DOM-A and DOM-B are necessary for period determination post development.

**Activation of PDFR signaling in circadian neurons rescues the behavioral phenotype of DOM depletion**

Since we observed low PDF levels in the sLN_s with DOM knockdown, we wondered whether the decrease of PDF signaling was the cause of arrhythmia. To test this hypothesis, we determined whether hyperactivation of PDFR could rescue the phenotypes that accompany reduction in DOM expression. To increase PDFR signaling, we expressed a membrane-tethered...
PDF (t-PDF) in circadian cells with *tim-GAL4*, which has been shown to mimic high PDF levels [42]. A scrambled peptide sequence (t-PDF SCR) with similar length as PDF was used as a negative control. Strikingly, co-expression of t-PDF using *tim-GAL4* restored the rhythmicity to 89% for *dom* RNAi (71% for another independent *dom* RNAi line) (Fig 7A and 7B), whereas the t-PDF SCR control was unable to restore rhythmicity (Fig 7A and 7B). Not only the rhythmicity, but also the period of DOM knockdown was rescued with co-expression of t-PDF (Fig 7B). To exclude the possibility that the behavior rescue in t-PDF was due to ceased expression of *dom* dsRNA, we measured the *dom* transcripts in fly heads. Co-expression of t-PDF with *tim-GAL4* downregulated *dom* to the similar level as *dom* RNAi control (S6A Fig). Since the circadian period was also restored in dom knockdown with expression of t-PDF, we then stained fly brains with PDF and pacemaker proteins PER and TIM (Fig 7C and S6B Fig). With expression of t-PDF in DOM knockdown flies, we made two interesting observations. First, PDF level as well as the dorsal projection of sLN_v was restored, while the number of sLN_v was still lower (Fig 7C and 7D). Second, the abundance of PER and TIM in sLN_v was also restored (Fig 7C and 7D and S6B and S6C Fig). Together, our data showed that activation of PDFR signaling in circadian neurons rescues the arrhythmic and period-lengthening phenotype of *dom* knockdown.

**Discussion**

Here, we identify that two alternatively spliced variants of DOM play distinct roles in circadian rhythms. Both DOM-A and DOM-B determine the circadian period of locomotor rhythms by regulating core pacemaker protein PER/TIM abundance. However, DOM-A plays specific roles for maintenance of sLN_v and PDF abundance during development thus controls morning anticipatory activity and circadian rhythmicity.

The abolished morning anticipatory activity in light dark cycle and low rhythmicity under constant darkness in DOM/DOM-A knockdown flies is reminiscent of the phenotypes seen in *pdf* mutant (Fig 1 and Fig 3) [43]. Based on these observations, we hypothesized that PDF signaling is disrupted with *dom* or *dom-A* RNAi. Indeed, we found that PDF abundance was decreased both in the soma and dorsal axonal projections of sLN_v (Fig 4). Furthermore, by restricting RNAi to adulthood, we restored the PDF expression and projection, which also restored the rhythms (Fig 6). These results suggest that the arrhythmic phenotype is due to the decrease of PDF levels in *dom* or *dom-A* downregulation.

Despite the importance of sLN_v in circadian rhythms, the mechanism underlying sLN_v maintenance is still unclear. Here we find that DOM-A plays important roles in the maintenance of sLN_v. PDF positive sLN_v and dorsal projections are formed in first instar larvae [10]. During development, both DOM-A and DOM-B start to be expressed in embryos [23]. Our data suggest that DOM-A does not affect sLN_v development in larvae, but rather regulates sLN_v maintenance at later stages (Fig 6). Knockdown of DOM-A may trigger apoptosis or necrosis pathways and cause programmed cell death in sLN_v. In fact, chromatin-modifying pathway has been shown to regulate neuronal necrosis in flies [44]. In the future, it will be interesting to examine whether DOM-A is involved these programmed cell death pathways and blocking necrosis or apoptosis pathway can restore the sLN_v in DOM downregulation.

Here we found that depletion of DOM-A seems to specifically affect the maintenance of PDF positive sLN_v and PDF accumulation in sLN_v, while the PDF positive ILN_v is not affected (Fig 5). This suggests that as master pacemaker neurons, sLN_v may have some unique regulatory program for maintenance and gene expression, which requires DOM-A. Interestingly, a phosphatase LAR has recently been found to specifically regulate PDF expression in the dorsal projection of sLN_v during development [40]. The pacemaker protein VRI also
played important roles in controlling PDF abundance and dorsal arborization rhythms of sLNVs [22]. Future efforts to identify specific regulatory mechanisms in sLNVs will help us understand the maintenance and function of circadian neuronal network.

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Why does DOM-A, but not DOM-B, regulate sLN_v maintenance and pdf transcription? One possibility might be that DOM-A and DOM-B are associated with different protein complexes. Compared to DOM-B, DOM-A has two unique domains in the C-terminus: a SANT domain and a poly-Q domain. Both of these two domains are known to mediate protein interactions. Interestingly, only DOM-A is identified to bind the Tip60 complex when Tip60 was purified by a tagged protein subunit from Drosophila S2 cells [24]. A previous study finds that depletion of Tip60 or overexpression of a histone acetyltransferase-defective Tip60 decreases axonal growth of the sLN_v in the fly model of Alzheimer’s disease [45]. These results suggest that DOM-A might interact with Tip60 to control the axonal projection and PDF expression of sLN_v.

Here we found that activation of PDFR signaling by t-PDF in tim-GAL4 expressing neurons not only restored the circadian rhythmicity but also the length of period and PER abundance in DOM knockdown (Fig 7). How does PDFR signaling affect PER? PDF is an important neuropeptide as both being an circadian output factor and adjusting the molecular clock [16]. After activation, PDFR signaling increases cAMP-PKA pathway and stabilized PER in PDF positive neurons [17]. This may explain why we saw a restoration of PER with co-expression of t-PDF and dom dsRNA. In addition to PER, we also observed a restoration of TIM abundance. Previous results on the effect of PDF signaling on TIM in non-PDF circadian cells are controversial [18, 46]. The exact mechanism of how TIM level is rescued by t-PDF here is still unclear.

Lastly, how does DOM control circadian period? This is probably through regulation the balance between H2Av and phosphor-H2Av at the promoter of per and tim via binding to CLK. Interestingly, compared to the decrease of per and tim mRNA in DOM downregulation, the master circadian transcription factor clk mRNA is not affected. Indeed, we found that from Drosophila S2 cell affinity pull-down, CLK interacts with DOM complex (Table 1). Furthermore, DOM-A and DOM-B binding is enriched in the E-box region of per and tim promoters. Even though no binding differences were observed in DOM-A and DOM-B at ZT4 and ZT16 (Fig 3), it is possible that other subunits in the DOM complex bind rhythmically, leading to rhythmic activity of the complex. Given that the conserved functions of DOM and human SRCAP/p400 in Notch signaling and histone variant exchange, it is possible that similar mechanisms are leveraged to control circadian rhythms in mammals.

Materials and methods
Fly stocks
All the flies were raised on cornmeal/agar medium at 25˚C under a LD cycle. The following strains were used: yw, w^{1118}, yw; tim-GAL4/CyO [36], yw;Pdf-GAL4/CyO [11]; yw;tim-GAL4/CyO;Pdf-GAL80/TM6B [47], tublin-GAL80^ts, UAS-cd8-GFP, clk^{out}. The following stocks were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/): UAS-dom^{RNAi#1} (BL31054), UAS-dom^{RNAi#2} (BL38941), UAS-domA (BL64261) and UAS-domB (BL64263). DomA^{RNAi} (sh-domA) and domB^{RNAi} (sh-domB) fly lines were generous gifts from Dr. Peter B. Becker. PdfTomato line was generated by Dr. Sebastian Kadener.

Behavioral experiments and analysis
For behavioral experiments, adult male flies (2–4 days old) were used for testing locomotor activity rhythms. Flies were entrained for 4 full days LD cycle at 25˚C, using about 500 lux light intensities, and then released into DD at 25˚C for at least 6 days. Locomotor activity was measured with TriKinetics Activity Monitors in 136-LL Percival Incubators. Locomotor activity was averaged over the 4 days entrainment for LD and 6 days for DD. Analyses of period
and power were carried out using FaasX software as previously described [48]. Actograms were generated using a signal-processing toolbox implemented in MATLAB (MathWorks) [49]. For GAL80ts experiments, flies were raised at 18°C and tested at 30°C. They were entrained for 5 days and then released in DD for at least 6 days. Morning anticipation was calculated by the ratio of activity counts between 2 hrs before light on and 6 hrs before light on. We first measured the single fly activity counts obtained in twelve 30-min bins between Zeitgeber Time (ZT) 17.5 and ZT24 (6 hr before lights on) and six 30-min bins between ZT22.5 and ZT24 (2 hrs before lights on). The first value is divided by the second to obtain the morning activity index. Morning anticipations of individual flies were then averaged and plotted on the graphs.

**Whole-Mount immunohistochemistry**

Whole-mount immunohistochemistry for fly brains were done as previously described [50]. Adult fly (3–6 days old) or L3 stage larval brains were dissected in chilled PBT (PBS with 0.1% Triton X-100) at the indicated time points and fixed in 4% formaldehyde diluted in PBS for 30 min at room temperature. For pupal brains dissection, *Drosophila* were fully developed and hatched 7 days after pupation in the same cross, pupa were collected 3 days after pupation using a small wet paintbrush and transfer to a dissection dish. After 2–3 times PBS wash, the pupal brains were dissected and fixed as previously described method. The brains were rinsed and washed with PBT three times (10 min each). Then, brains were incubated with 10% normal Goat serum diluted in PBT to block for 60 min at room temperature and incubated with primary antibodies at 4°C overnight. For PER, TIM and CLK staining, we used 1:1,500 rabbit anti-PER, 1:2,000 rat anti-TIM (gift from Dr. Rosbash) and guinea pig 1:2,500 anti-CLK (gift from Dr. Hardin), respectively. We used a 1:200 dilutions for mouse anti-PDF and 1:200 for rabbit anti-GFP (DSHB). After six washes with PBT, brains were incubated with relative secondary antibody at 4°C overnight, followed by another six washes with PBT. For *PdfTomato* staining, flies were directly dissected in chilled PBT at the indicated time points and mounted in the medium (VECTOR). All samples were imaged on a Leica Confocal SP8 system, with laser settings kept constant within each experiment. 10 to 12 fly brains for each genotype were dissected for imaging. Representative images are shown. Image-J software (National Institutes of Health [NIH]) was used for PER, PDF, TIM and CLK quantification in 15–30 sLNvs from at least seven brains. For quantification, signal intensity in each sLNv and average signals in three neighboring non-circadian neurons were measured, and the ratio between signals in sLNvs and non-circadian neurons was calculated.

**Chromatin immunoprecipitation (ChIP)**

Chromatin immunoprecipitation (ChIP) was performed based on published protocols [31]. Flies entrained in 12 hr light:12 hr dark (LD) conditions at 25°C for four days were collected at two time-points (ZT4 and ZT16) on the fifth day. Briefly, chromatin was isolated from 500 μl of fly heads homogenized with a dounce homogenizer (Kimble Chase) for 20 strokes using the loose “A” pestle. Homogenate was sieved by a 70 μm cell strainer (Falcon) then centrifuged to remove cell debris. Pellets were cross-linked using formaldehyde. Samples were sonicated using a focused-ultrasonicator (Covaris M220) on setting for 400–500 bp cDNA and then centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected in two 130 μl aliquots for IP and 26 μl was collected for input and frozen at -80°C for analysis. Sonicated chromatin was roughly 500 bp in length (<1000 bp). For each IP, 30 μl of a Protein G Dynabead slurry (Life Technologies) was washed then incubated along with the appropriate antibodies for 4 hours at 4°C with rotation. Amount of antibodies used for ChIP is as follows: anti-H2Av (10 ug/ml,
rabbit, Active Motif), anti-DOM-A (20 μg/ml, rabbit, GenScript) which was generated in our lab (antigen protein sequence designed according to 2008–2349 aa of DOM-A), anti-rabbit-IgG (20 μg/ml, Life Technologies), anti-DOM-B (10 μg/ml, mouse, from Dr. Peter B. Becker), anti-mouse-IgG (10 μg/ml, Life Technologies). Following incubation, beads were collected and incubated with chromatin overnight at 4°C with rotation. DNA was eluted using the Qia-gen PCR purification kit and subjected to qPCR. At least three technical replicates of qPCR were performed for each biological ChIP replicate and three biological replicates were performed for H2Av, DOM-A and DOM-B assays. Background binding to a nonspecific antibody (anti-IgG; Life Technologies) bound to Dynabeads was subtracted from input samples and results are presented as the percentage of the input samples. For each assay, at least three biological replicates were performed. The specific primers used for qPCR are described in S2 Table. The technical qPCR triplicates were averaged for each biological replicate as no significant differences were found between the technical replicates, and the error bars represent SEM calculated from variance between biological replicates. Two-tailed t-tests were used to determine statistical differences between control and experimental treatment at each ZT.

**Real time quantitative reverse transcription PCR**

Flies were collected at the indicated time points and isolated heads were stored at −80°C. Total RNA was extracted from 25–30 heads with TRIZol based on the manufactures protocol (Life Technologies, USA). A 2-μg quantity of RNA was reverse-transcribed with reverse transcription reagents (Invitrogen). For real-time PCR (qRT-PCR) of per, tim, clk, dom, domA, domB and actin, we used a qPCR detection kit (SYBR Select Master Mix For CFX) (Life Technologies, USA). The specific primers used for qPCR are described in S2 Table. All the experiments were performed in the CFX96 Real-Time System (BIO-RAD).

**Western blot analysis**

Fly heads were collected at the indicated time points and homogenized with pestles, protein extracts were prepared with HEPES-Triton lysis buffer (1X HEPES-Triton buffer, 1 mM DTT, 0.4%NP-40, 0.1% SDS, 10% glycerol, 1X tablet protease inhibitor). Proteins were quantified using BSA assay. For immunoblot analysis, proteins were transferred to PVDF membranes (Genesee Scientific) and incubated with anti-DOM-A (1:300, GenScript), anti-DOM-B (1:5, from Dr. Peter B. Becker) and anti-ACTIN (1:100, DSHB) in blocking solution. Band intensity was calculated and analysed with Image J.

**Statistics analysis**

Statistical analysis of two data points was performed with Student’s t-test. Statistical analysis of multiple data points was performed with one-way analysis of variance with Tukey post-hoc tests using GraphPad software.

**Supporting information**

S1 Fig. *Dom* RNAi efficiency. A. Schematic map of the *domA* and *domB* locus. Regions of the *domA* and *domB* mRNA targeted by the four specific RNAi lines. *domRNAi#1* and *domRNAi#2* are RNAi lines targeting different regions of *domA RNAi* and *domB RNAi* are short hairpin RNAi lines specifically targeting *domA* or *domB* isoform CDS regions. B. Quantitative RT-PCR showing the expression of *dom, domA* and *domB*. Flies were collected at ZT1. Downregulation of *dom* (*domRNAi#1* and *domRNAi#2*) in circadian neurons decreased *dom* mRNA levels, comparing to the controls. Downregulation of *domA* and *domB* (*domA RNAi* and *domB RNAi*).
and domB RNAi in circadian neurons specifically decreased domA and domB mRNA levels, comparing to the relative controls. Error bars correspond to SEM. n.s., nonsignificant; **p < 0.01; ***p < 0.001; ****p < 0.0001 as determined by t-test.

(TIF)

S2 Fig. PER expression pattern in other groups of circadian neurons. A-B. Whole mount immunostaining showing the expression pattern of PER in LNds and DN1s. Red is PER and green is PDF. Flies were entrained for 4 days in LD and transferred to DD and dissected every 4 h on the fifth day. Downregulation of DOM decreased PER levels at CT1-9 and CT17 in LNds, while reduced PER levels at CT1-5 and CT17-21 in DN1s (Scale bar: LNds, 50 um; DN1s, 150 um).

(TIF)

S3 Fig. DOM-A, but not DOM-B was clk dependent. A-B. Quantitative RT-PCR showing the expression patterns of domA and domB in w1118 and Clkout flies heads. Flies were collected at the indicated time points (ZT = Zeitgeber Time or CT = circadian time). Dom-A exhibited a strong oscillation pattern with a trough around ZT9 and CT9 while has a peak expression near ZT21 and CT21 in w1118 flies heads, while the oscillation of dom-A expression was abolished in Clkout. There is no obvious oscillation for dom-B expression in w1118 and Clkout flies heads both ZT and CT conditions.

C-D. Validation of specificity for domA and domB shRNA knockdowns and overexpression. UAS-domA, UAS-domB and UAS-shRNA of domA and domB males for domA and domB were crossed with tim-Gal4 females. F1 offspring adult brains are probed with anti-DOM-A and anti-DOM-B antibodies in western blot. ACTIN signals provided controls.

E-H. Western blot showing the expression patterns of DOM-A and DOM-B in w1118 and Clkout flies heads. Flies were collected at the indicated time points (ZT = Zeitgeber Time). DOM-A did not show a strong oscillation pattern in w1118 flies heads, while the expression levels of DOM-A were remarkably decreased in Clkout. There is no obvious change for DOM-B expression in w1118 and Clkout flies heads. Band intensity was calculated and analyzed with the Image J. Error bars correspond to SEM. *P < 0.05; **p < 0.01, ***p < 0.001 as determined by the t-test.

(TIF)

S4 Fig. DomA and domB locomotor activity. A. Average locomotor activity of flies of different genotypes under 3 days of 12:12 hr LD conditions. Dark activity bars represent the night, and white bars represent the day. Comparing to the control (left panel), morning anticipation was severely disrupted in domA shRNAi lines (middle panel), while morning anticipation was normal in domB downregulation flies (Right panel).

B. Morning anticipation was calculated following the method described before. Error bars correspond to SEM. n.s., nonsignificant; ****p < 0.0001 as determined by t-test.

(TIF)

S5 Fig. Downregulation of DOM in adult stage decreases the mRNA levels of per and tim. A-B. Quantitative RT-PCR showing the expression of per and tim. Flies were collected at the indicated time points (ZT = Zeitgeber Time). Downregulation of dom in adult stage decreased per and tim mRNA levels.

(TIF)

S6 Fig. Constitutively activation of PDFR signaling rescued the TIM expression in sLNvs. A. Quantitative RT-PCR showing the expression of dom. Flies were collected at ZT1. Downregulation of dom (domRNAi#1) in circadian neurons decreased dom mRNA levels (positive
control. Dom mRNA level is still reduced by dom RNAi even expressing the membrane-tethered PDF. Error bars correspond to SEM. ***p < 0.001; ****p < 0.0001 as determined by t-test.  

B. Representative confocal images of brains of dom RNAi flies expressing the membrane-tethered PDF or scrambled PDF. Flies were entrained for 4 days in LD 25˚C, and brains were dissected at ZT23 for anti-PDF antibody (green) and anti-TIM antibody (red). From top to bottom: (Top panel) fly brain expressing the membrane-tethered scrambled PDF; (middle panel) domRNAi#1 flies expressing a membrane-tethered scrambled PDF; and (bottom panel) domRNAi#1 flies expressing the membrane-tethered PDF. Confocal images are whole brain and soma of sLNvs from left to the right (Scale bar: whole brain, 500 um; sLNvs, 50 um).  

C. Quantification of the number and relative PDF and TIM levels of sLNvs. For each genotype, totally, 14–20 flies brains and 30–55 neurons were used for quantification of the staining. Error bars correspond to SEM. n.s., nonsignificant; ***p < 0.001, ****p < 0.0001 as determined by t-test.  

(TIF)  
S1 Table. DOM regulates drosophila circadian behavior.  
(PDF)  
S2 Table. Primers used in this study.  
(PDF)  

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