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Diurnal protein oscillation profiles in *Drosophila* head

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Abstract

Circadian clocks control daily rhythms in physiology, metabolism, and behavior in most organisms. Proteome-wide analysis of protein oscillations is still lacking in *Drosophila*. In this study, the total protein and phosphorylated protein in *Drosophila* heads in a 24-hour daily time-course were assayed by using the iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) method, and 10 and 7 oscillating proteins as well as 19 and 22 oscillating phosphoproteins in the w^{1118} wild type and Clk^{Jrk} mutant strains were separately identified. Lastly, we performed a mini screen to investigate the functions of some oscillating proteins in circadian locomotion rhythms. This study provides the first proteomic profiling of diurnally oscillating proteins in fly heads, thereby providing a basis for further mechanistic studies of these proteins in circadian rhythm.

Keywords

Circadian rhythm; clock; proteomics; *Drosophila*

Introduction

Daily rhythms are a universal phenomenon in many species. These rhythms are regulated by the circadian clock, a molecular machinery that involves a conserved transcriptional-translational feedback loop. In *Drosophila*, two transcriptional activators, CLOCK (CLK) and CYCLE (CYC), form a heterodimeric complex and activate rhythmic transcription of clock-controlled genes [1]. PERIOD (PER) and TIMELESS (TIM) are the main repressors, which first accumulate in the cytoplasm and then enter the nucleus to repress CLK/CYC activity [2]. Post-translational regulation, such as phosphorylation events, is also critical in circadian regulation. A series of kinases and phosphatases regulate the stability of pacemaker proteins and their nuclear localization. Nuclear localization or stability of PER and TIM is regulated by phosphorylation events mediated by DOUBLETIME (DBT), casein kinase II (CK2), or glycogen synthase kinase 3 (GSK3) [3–11]. Phosphorylation of PER by

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Conflict of interest

The authors declare no conflict of interest.

Nemo (NMO) controls its stability [12–13]. Another type of post-translational modification, glycosylation, regulates PER nuclear accumulation [14–15].

Many circadian related genes in *Drosophila* have been identified at the genome wide level using microarrays or RNA sequencing [16–19]. Furthermore, clock-controlled genes from different groups of clock neurons have been also identified by RNA-seq [20–22]. As CLK is a critical circadian trans-activator, targets of CLK have been characterized by chromatin immunoprecipitation [23]. Although these studies provide insights into circadian transcriptional regulation, much less is known about oscillations at the proteome level. Recently, proteomics and phospho-proteomics have been used to identify circadian changes in proteins in mouse and rat [24–25, 40–46]; however, a proteomic profile of oscillating proteins in the nervous system in *Drosophila* is still lacking.

In order to identify oscillating proteins that may play a role in regulation of circadian rhythms, we performed quantitative proteomics analysis of fly heads using a previously described approach named iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) [26]. Compared to other quantitative mass spectrometry (MS) methods, such as isotope-coded affinity tags (ICAT) and metabolic labeling or stable isotope labeling of amino acids in culture (SILAC), the design of this method enables more confident peptide identifications and processing of eight samples at the same time, thereby greatly reducing technical error [26].

The *Drosophila* CLK protein is at the center of multiple regulatory loops involved in circadian rhythm [27–31]. In this study, we identified total proteins as well as phosphorylated proteins that oscillate across the 24-hour day in both wild-type (w^{1118}) and Clk^{Jrk} mutant fly heads by using iTRAQ, and further identified their differentially expressed proteins and phosphoproteins. Lastly, we analyzed the behavior of flies with mutations in genes encoding several of the oscillating proteins identified in the screen and revealed potential roles for *α-catenin* in circadian rhythms.

Results

Ontological features of proteins expressed in the fly head

Clk^{Jrk} is a *clock* gene mutant that abolishes fly circadian rhythms [28]. Here we first verified that our Clk^{Jrk} mutant line exhibited arrhythmic locomotor behavior and lack of full length CLK protein. Results showed that the locomotor rhythm of the Clk^{Jrk} mutant in light: dark (LD) and constant darkness (DD) was disrupted compared to a w^{1118} control (Figure S1A), and the abundance of CLOCK protein was decreased in the Clk^{Jrk} mutant in an immunoblot experiment (Figure S1B). Thus, the Clk^{Jrk} and w^{1118} control lines were used for this study.

In order to identify diurnally oscillating proteins, total proteins from whole head in both wild-type (w^{1118}) and Clk^{Jrk} mutant flies were sampled at Zeitgeiber time (ZT) ZT2 (2 hours after lights on), ZT8, ZT14, ZT20 and analyzed using the iTRAQ-MS method (Figure S1C), in which 3,270 proteins in total were identified, of which 3,154 (96.5%) were detected in all three replicates.

Protein expression profiles in w^{1118} and Clk^{Jrk}

In this study, the total protein profiles at ZT2, ZT8, ZT14, and ZT20 between w^{1118} and Clk^{Jrk} lines were compared using Scaffold Q+ Quantitation Module. With coefficient greater than 0.6 (fold change>1.5 as $\log_2 1.5=0.6$) and $p < 0.05$, there were 45 differentially expressed proteins (Table 1). Pathway clustering revealed that the differentially expressed proteins were most often involved in cancer and organismal injury (Table S1). These proteins function in uridine-5'-phosphate biosynthesis and L-glutamine biosynthesis, PI3K/AKT signaling, RNA polymerase II processes, transcription activator BRG1 and MYC-centered gene expression and transcriptional regulation network, and mitogen-activated protein kinase 3 (MAPK3) and insulin-like receptor-centered network of tissue development and metabolism (Figure 1A, B).

In order to get a global view of the regulatory mechanisms involved in the differential protein expression in the Clk^{Jrk} line, we searched for the possible upstream regulatory factors that might regulate these differentially expressed proteins (Figure 1C). Results showed that three upstream regulatory factors were possibly involved in regulation of expression of these proteins at ZT8 and ZT20. At ZT8, four of one hundred and four downstream targets of X-box-binding protein 1 (XBP1) were enriched in the Clk^{Jrk} -specific differentially expressed proteins. At ZT20, four of one hundred and twenty-five downstream targets of nuclear factor erythroid 2-related factor 2 (NFE2L2) and rapamycin-insensitive companion of mTOR (RICTOR) were enriched in Clk^{Jrk} -specific differentially expressed proteins. These results suggest that the activity of these upstream regulatory factors may be altered more in Clk^{Jrk} than in w^{1118} flies.

Oscillating proteins in w^{1118} and Clk^{Jrk} mutants

To comprehensively look for the oscillating total proteins in w^{1118} and Clk^{Jrk} mutant flies, we applied both a JTK cycle for assay of the expression data [32] and assay of differential proteins at different time points. Results from JTK assay showed 10 and 7 oscillating proteins in w^{1118} and Clk^{Jrk} mutants, respectively, by applying criteria of ADJ ($P<0.05$). AMP (amplitude) and LAG (phase) were indicated for these detected oscillating proteins (Table 2). Interestingly, a transcription factor UPF1 was identified as an oscillating protein in both w^{1118} and Clk^{Jrk} (Table 2). Moreover, some proteins identified as oscillating proteins in both genotypes were ribosome structural components.

Phosphorylated proteins with altered expression in the Clk^{Jrk} mutant

As phosphoproteins play important roles in circadian regulation, we concentrated phosphorylated peptides using the TiO_2 method [33] and analyzed the phosphorylated peptides profiles of both w^{1118} and Clk^{Jrk} at ZT2, ZT8, ZT14, and ZT20. Totally, we identified 5961 phosphorylated peptides, of which 1345 were found in all three replicates. A full list of identified phosphorylated peptides was shown in Table S2.

In this study, 1,275 phosphorylated proteins were detected, of which 649 (51%) were found in all three replicates at ZT2, ZT8, ZT14 and ZT20 in both w^{1118} and Clk^{Jrk} mutant flies. Analysis in phosphorylated protein profiles at these four time points between the two

genotypes, by using One-way ANOVA analysis with coefficient greater than 0.6 and $p < 0.05$, we found that there were totally 55 differentially phosphorylated proteins (Table 3).

Phosphorylated oscillating peptides in w^{1118} and Clk^{Jrk} mutants

To comprehensively look for the oscillating phosphorylated proteins in w^{1118} and Clk^{Jrk} mutant flies, we applied the JTK cycle for both assay of the expression data and assay of differentially phosphorylated proteins at different time points. Results from the JTK assay showed that there were 19 and 22 phosphorylated oscillating proteins in w^{1118} and Clk^{Jrk}, respectively, by applying criteria of ADJ ($P<0.05$) (Table 4). Besides, for the oscillating phosphorylated proteins identified in this study, there were no significant changes in correspondence with total protein abundance at different time points, and JTK cycle analysis for these proteins also did not show significant oscillating. These data suggested that the oscillating phosphorylated protein only reflected changes in phosphorylation.

Functional analysis of proteins in oscillatory expression patterns

Importantly, many identified proteins in this study are known to be involved in circadian regulation such as SLMB, Brm, and ATX2 [34–36]. Here we focused on some proteins that have not been linked to circadian rhythms, so 15 proteins that showed oscillations or differences in expression between w^{1118} and Clk^{Jrk} were selected for further functional analysis (Table 5).

For most of these proteins, no mutants were available, so we used *tim*-GAL4 to express double-stranded RNAs (dsRNAs) targeting these genes in all circadian neurons, in which Dicer2 was introduced to enhance the RNAi efficiency [36, 37]. For most of the dsRNAs tested, there were no obvious defects in circadian behavior (Table 5), indicating that these genes are not involved in locomotor circadian behavior. However, expression of dsRNA designed to target the mRNA that encodes *α-catenin* (α-Cat) lengthened the period by about 1 hour (Figure 2). To determine which neuron requires α-Cat expression, we used *pdf-GAL4* to restrict the dsRNA expression to pacemaker neurons. This caused a phenotype similar to that of flies in which dsRNA expression was driven by *tim-GAL4*, indicating that α-Cat is required in pacemaker neurons for circadian behavior. To exclude the potential developmental defects of depletion of α-Cat, we restricted dsRNA expression only in adulthood, by using a temperature sensitive gal80 ($gal80^{0ts}$). A significant lengthened circadian period was still observed while knocking down α-Cat only during the adult stages (Figure 2). Together, these data indicate that α-Cat may play a role in regulation of circadian rhythms.

Materials and methods

Fly rears and strains

Fly lines used in this study were w^{1118} (Bloomington No. 5905) and Clk^{Jrk} (a gift from Michael Rosbash, Brandeis University). Flies were raised on standard *Drosophila* food. ZT (*Zeitgeber time*) was the time of collection relative to the light:dark cycle used in the experiments. ZT0 was defined as the time point of lights on, while ZT12 was the time point of lights off. The incubator conditions were: 25 °C, 60% humidity, 350 Lux light intensity,

and a 24-hour photoperiod (12L:12D; ZT0 was 6:30, ZT12 was 18:30). Newly eclosed male and female virgin flies were synchronized and collected at different time points after aging for 2 days.

Fly strains used to verify the proteins in this screen including *tim-Gal4*, UAS-Dicer (TD2-Gal4), *pdf*-Gal4, *UAS-Dicer* (PD2-Gal4), BL38987, BL38197, BL57488, BL51678, BL38987, BL38197, BL57488, BL26219, BL32034, BL56895, BL58353, BL6832, BL57695, BL20805, BL31957, BL51406, and BL53868 were from the Bloomington stock center. VDRC107276, VDRC40026, VDRC107276, and VDRC40026 were from Vienna Drosophila Resource center.

Experiment Design and Statistical Rationale

In order to determine the protein oscillation at different time points, we collected head tissue samples at ZT2, ZT8, ZT14, ZT20 of w^{1118} and Clk^{Jrk} flies. Eight samples were employed for each experiment, and the data from w^{1118} were used as controls. Three biological replicates were carried out for each sample. Eighty fly heads were collected for each sample. Statistical analysis was performed using Spss11.5.

T-test or one-way ANOVA was used for all variation analysis in which $p<0.05$ was regarded as significant, and $p<0.01$ was regarded as very significant. After aging for 2 days, flies from ZT2, ZT8, ZT14, and ZT20 were anesthetized with CO_2 and frozen in liquid nitrogen. Fly heads were cut off and lysed in STD buffer (2% SDS, 5 mM Tris-HCl, 10 mM DTT, pH 6.8) with phosphatase inhibitor complex from Sangon Biotech (No. C500019) and protease inhibitor from Roche (No. 04693132001). After grinding, boiling, sonicating (80 w, 10-s 15 times with 15-s intervals), and centrifuging (13000 rpm 4 °C for 25 min), the protein solution in the central layer was collected and stored at −80 °C. Concentration of the protein solution was determined by BCA assay. 200 μg protein was digested using a modified FASP method as described previously [38]. In short, the protein solution was loaded onto an ultrafltration device (30 K MWCO, 500 μL, Sartorius), washed with 50 mM NH_4HCO_3 (ABC), reduced with 200 mM DCEP at 56 °C, and alkylated with 200 mM MMTS in the dark. The protein was digested with trypsin at 37 °C overnight (enzyme: protein ratio, 1: 50). The digested peptides were desalted and lyophilized for iTRAQ labeling with iTRAQ 8-plex reagent (Sigma 4381663 and 4381664) according to manufacturer's instructions. Protein samples from w^{1118} flies were labeled with isobaric tags 113, 114, 115, 116 (ZT2, ZT8, ZT14 and ZT20), protein samples from Clk^{Jrk} were labeled with 117, 118, 119, 121 (ZT2, ZT8, ZT14 and ZT20). Three biological replicates were performed.

For phosphopeptide enrichment, the labeled peptides were dissolved in 100 μL loading buffer (1 mL lactic acid, 2 mL 80% ACN, 0.4% TFA), and phosphopeptides were enriched using the Titansphere Phos-TiO kit (GL Sciences, No. 5010-21514) according to manufacturer's protocol. Labeled peptides were fractionated with strong cation exchange on an Acquity HPLC system (Waters) using SCX column (Protein-Pak Hi Res SP, 7 μm, 4.6×100 mm, non-porous; Waters). Fifteen fractions were collected and analyzed using a Q-Exactive high-resolution mass spectrometer (Thermo Fisher Scientific) coupled with Acquity nano-HPLC system (Waters). Peptides were eluted at a flow rate of 0.2 μl/min with a 95-min gradient on a 75 μm×100 mm column filled with 3 μm C_{18} stationary phase (AQ

C18, Phenomenex). The mass resolutions of the mass analyzer were set at 70,000 and 17,500 for full MS and MS/MS analyses, respectively, and the scan range was 300 to 1800 m/z. The 10 most intensive precursor ions were selected for MS/MS fragmentation with NCE at 30. Phosphopeptides were analyzed with a 120-min nano-LC gradient without SCX fractionation.

Data analysis and quality control

Raw data were processed with Mascot Distiller 2.5 for peak picking and searched using Mascot 2.5.1 software (Matrix Science) against flybase protein database r5.4 (30362 entries) with the following parameters: enzyme, trypsin, 2 missed cleavages. Methylthio (C) was set as fixed modification and phospho (STY) was set as variable modification. Mass tolerance for precursor ions was 20ppm. Mass tolerance for fragment ions was 0.02Da. Known contaminants was excluded. The search results were imported into Scaffold (version 4.4.5, Proteome Software Inc.) for further analysis. The peptides were filtered with a false discovery rate (FDR) of 1.0%. We required that at least two peptides be identified for each protein and a Mascot ion score ≥ 20. Quantification of the peptides was performed using Scaffold Q+ (version Scaffold_4.4.5, Proteome Software Inc.). Normalization was performed iteratively (across samples and spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA. Medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. We identified 96.5% (3154/3270) of proteins in all three replicates. To ensure accuracy of quantitative results, coefficient of variation (CV) < 20% were applied.

For analysis of the proteins differentially expressed in w^{1118} and Clk^{Jrk}, we used Scaffold Q + Quantitation Module. We used the Log2 Fold Change method to measure differential abundance of proteins between two genotypes (detailed method described scaffold Q+ USERS GUIDE P58-59 from http://www.proteomesoftware.com/). We chose the proteins as significantly changed between w^{1118} and Clk^{Jrk} with a mean value fold change greater than 1.5 (coefficient $>\log_2 1.5 = 0.6$) for further analysis.

Bioinformatics analysis

The original data was uploaded to website http://www.iprox.org/page/DSV021.html;?url=1506131721592IQ9Y with password XOCS. The Gene Ontology (GO) analysis was performed at http://www.geneontology.org and http://www.pantherdb.org/. The pathway analysis was done using Ingenuity Pathway Analysis according to a detailed protocol on website: https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/ and http://celldesigner.org/.

Behavior experiments and analysis

Adult male flies (2-5 days old) were used for testing circadian locomotor activity. Flies were entrained for 3 days to LD cycles at 25 °C and then released into DD for at least 6 days. Locomotor activity was recorded with *Drosophila* Activity Monitors from Trikenetics in Percival I36-LL incubators. Data analysis was performed with the FAAS-X software

provided by the F. Rouyer lab [34]. For actograms, a signal-processing toolbox implemented in MATLAB (MathWorks) was used [39].

Discussion

There are advantages and disadvantages of the proteomic approach applied in this study. The advantage of this study was that capacity to show a number of novel oscillating proteins and phosphorylated proteins, and the locomotion behavior analysis was used to confirm any roles of these in circadian rhythm. But one of the potential caveats for this proteomic analysis is that proteomic methods may miss some specific proteins due to low abundance. This may explain why we were not able to get differential signal for PER or TIM in this study. In this study, a significant number of differentially expressed proteins was acquired. However, another limitation of this study was that any spatial resolution of these differentially expressed proteins was not determined because we used head samples, in which the potential change in phase in one brain area was not captured. Further studies with more spatial resolutions are needed in the future.

The phosphorylated proteins are very important for maintaining function of protein activity. Although studies of core clock proteins have demonstrated the importance of phosphorylated proteins in circadian and sleep regulation [1, 2], a systematic study of protein phosphorylation in circadian regulation has not been performed. This study provides a proteome-wide view of phosphorylated proteins that are potentially important in circadian regulation. Most of the oscillatory phosphorylated proteins in w^{1118} are not disrupted by mutation of a critical clock gene, indicating that these time-dependent expressions of phosphorylated proteins may be independent of *clock*.

Importantly, some oscillatory phosphorylated proteins found in Clk^{Jrk} flies do not show the same pattern in w^{1118} flies. These phosphoproteins in Clk^{Jrk} flies might be involved in daily rhythmic loss. The oscillatory phosphorylated proteins identified also do not overlap with the total oscillatory proteins (both phosphorylated and non-phosphorylated proteins), thereby implying that some proteins have an oscillation only in a phosphorylated form. Thus, further investigation into the role of post-translational regulation in the circadian rhythm may lead to new insights into how the daily rhythms are regulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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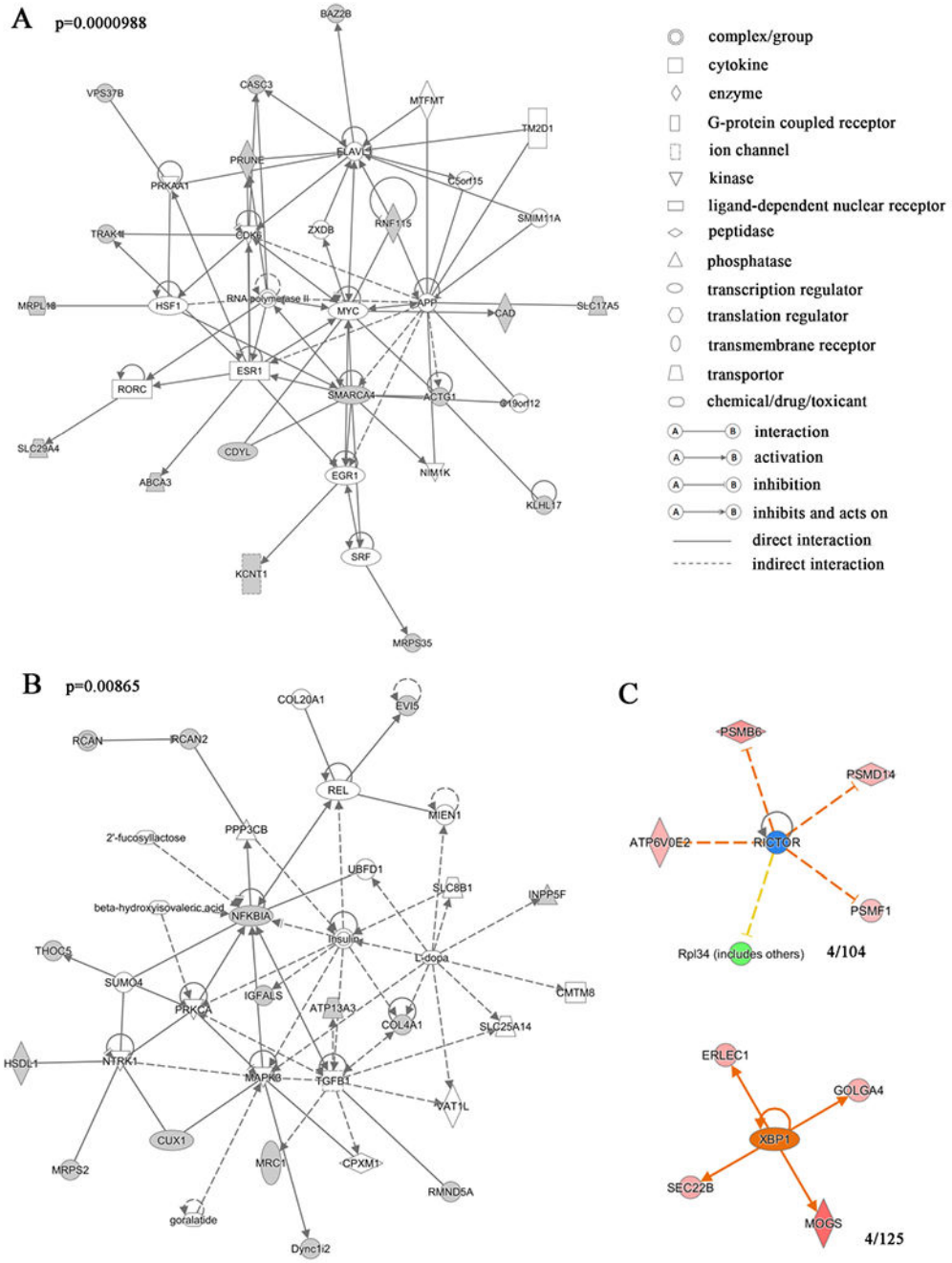

Figure 1.

(A, B) Gene network analysis of the proteins differentially expressed in w1118 and ClkJrk flies revealed that these proteins are involved in the RNA polymerase II complex, transcription activator BRG1 and MYC-centered gene expression and transcriptional regulation network, and MAPK3 and insulin-like receptor-centered network of tissue development and metabolism. Variation coefficient > 0.6 and p value <0.05. The proteins shaded were presented in the differentially expressed proteins. The p value of the gene network was 0.0000988 for figure 1A and 0.00865 for figure 1B. The statistical information

for the other gene networks identified is in Table S1. (C) Transcription factors that may regulate expression of differentially expressed proteins. 4/104 and 4/125 proteins were found to have common upstream factors XBP1 and RICTOR, respectively. The red to pink colors indicate up-regulation, and greens indicate downregulation. Darker colors indicate more extreme up or down regulation. Yellow indicates predicted activation, and blue predicted inhibition with darker hue indicative of more confidence in the prediction. Gray line indicates that effects are not predicted. Different types of the molecules are indicated by shapes as shown.

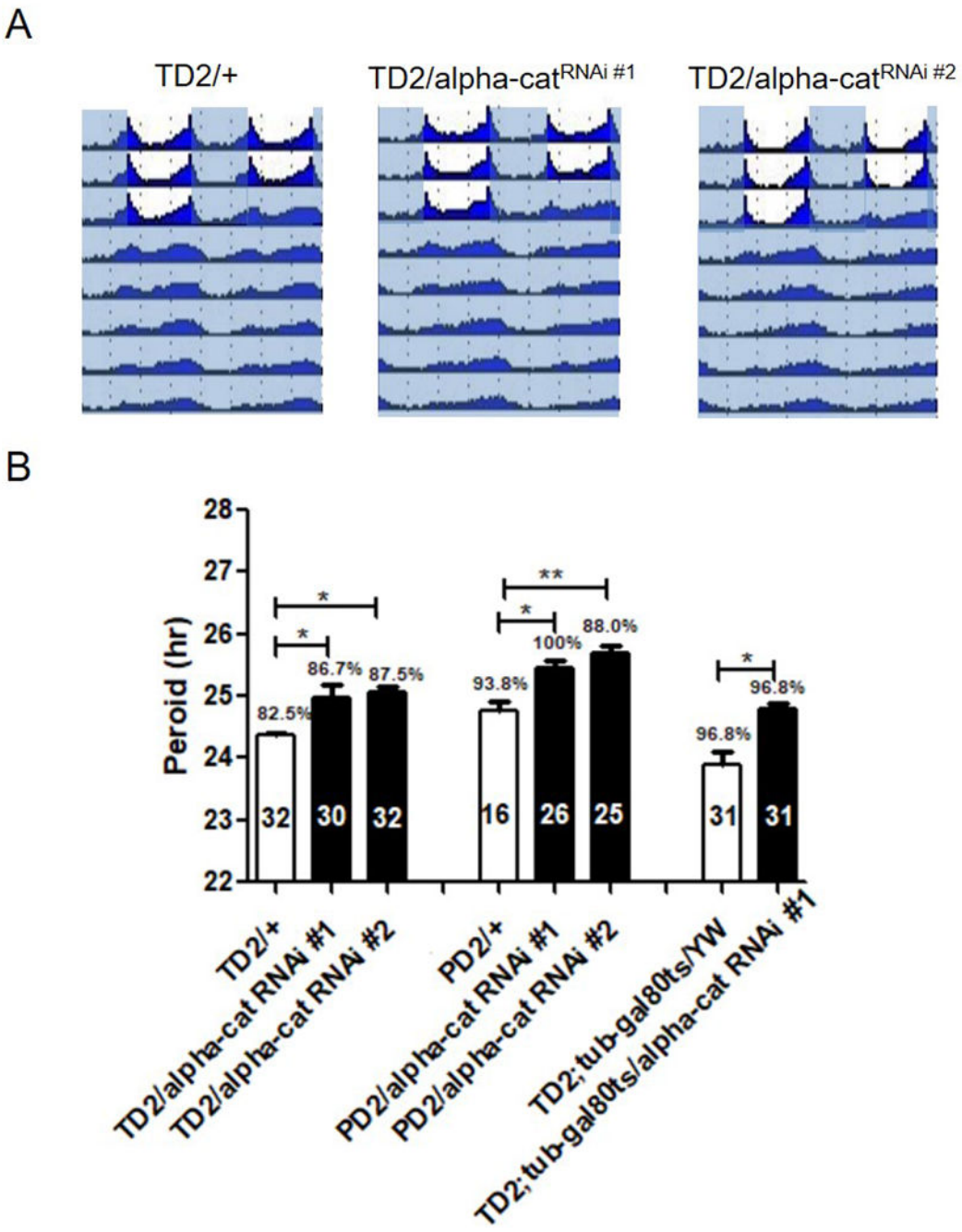

Figure 2.

α-Cat is potentially required for circadian locomotor rhythm. (A) Locomotion behavior under light dark (LD) cycles and constant darkness (DD). Representative double plotted actograms showing last 3 days of LD and 5 days of DD for controls, flies that express dsRNA targeting α-Cat (TD2/+; α-Cat RNAi#2/+flies). White indicates the light phase, and gray indicates the dark phase. (B) Effects of α-Cat downregulation in circadian neurons on period length of circadian locomotion behavior. Bar graph showing period length (y axis), percentage of rhythmic flies, and number of flies tested (in the bars). Error bars indicate

SEM. Each genotype was compared to appropriate genotype control or driver control. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant at the 0.05 level, tested using Student's t test.

Table 1.

Differential expressed total proteins in w^{1118} and Clk^{Irk}

Accession Number	Molecular Weight	Protein Grouping Ambiguity	P-Value	Symbol	Feature type
FBpp0086494	25 kDa	TRUE	< 0.0001	Dmel\igl-PA	myosin light chain binding
FBpp0086695 (+2)	96 kDa		< 0.0001	Dmel\CG8485-PA	ATP binding; protein serine/threonine kinase activity; SAP kinase activity
FBpp0081636 (+1)	4 kDa		0.00055	Dmel\MtnA-PA	metal ion binding
FBpp0087193 (+2)	322 kDa	TRUE	0.0011	Dmel\tou-PA	chromatin binding; DNA binding; zinc ion binding
FBpp0070136	25 kDa		0.0024	Dmel\Sec22-PA	SNAP receptor activity
FBpp0076085 (+2)	29 kDa		0.0029	Dmel\CG6707-PA	phosphatidylinositol-4,5-bisphosphate 4-phosphatase activity
FBpp0083464	59 kDa	TRUE	0.0033	Dmel\mod(mdg4)-PF	phosphatidate phosphatase activity
FBpp0070743 (+1)	62 kDa		0.022	Dmel\CG15784-PA	unknown
FBpp0289513	33 kDa	TRUE	0.027	Dmel\Eb1-PF	microtubule binding
FBpp0291330	73 kDa	TRUE	0.029	Dmel\drongo-PF	GTPase activator activity
FBpp0085710 (+1)	98 kDa	TRUE	0.03	Dmel\CG10062-PA	unknown
FBpp0076837 (+2)	70 kDa		0.031	Dmel\Msr-110-PA	unknown
FBpp0088293 (+3)	114 kDa	TRUE	< 0.0001	Dmel\4E-T-PB	unknown
FBpp0078708 (+1)	15 kDa		< 0.0001	Dmel\TotM-PA	unknown
FBpp0070468	76 kDa		< 0.0001	Dmel\w-PA	ATP binding; ATPase activity, coupled to transmembrane movement of substances; eye pigment precursor transporter activity; transmembrane signaling receptor activity
FBpp0078534 (+2)	46 kDa		0.00012	Dmel\CG9775-PA	ubiquitin-protein transferase activity; zinc ion binding
FBpp0081445	40 kDa		0.00025	Dmel\CG11982-PA	ubiquitin-protein transferase activity; zinc ion binding
FBpp0079168 (+1)	13 kDa		0.00028	Dmel\RpL36A-PA	structural constituent of ribosome
FBpp0081192	21 kDa	TRUE	0.00028	Dmel\Ccp84Ae-PA	structural constituent of chitin-based cuticle; structural constituent of chitin-based larval cuticle
FBpp0291858	51 kDa		0.00044	Dmel\Cpr47Ef-PC	structural constituent of chitin-based cuticle; structural constituent of chitin-based larval cuticle
FBpp0071423 (+3)	58 kDa		0.00054	Dmel\ras-PC	IMP dehydrogenase activity
FBpp0073330	10 kDa		0.00078	Dmel\ssp7-PA	unknown
FBpp0112233	59 kDa	TRUE	0.00079	Dmel\Ugt86Dd-PB	glucuronosyltransferase activity
FBpp0292247	157 kDa	TRUE	0.00084	Dmel\Ptp99A-PF	protein tyrosine phosphatase activity; transmembrane receptor protein tyrosine phosphatase activity
FBpp0080757 (+3)	77 kDa		0.0023	Dmel\CG17544-PA	acyl-CoA dehydrogenase activity; acyl-CoA oxidase activity; flavin adenine dinucleotide binding; pristanoyl-CoA oxidase activity
FBpp0290840	38 kDa	TRUE	0.0025	Dmel\mRpS35-PA	structural constituent of ribosome
FBpp0072560	41 kDa		0.0052	Dmel\CG9149-PA	acetyl-CoA C-acyltransferase activity
FBpp0081187	19 kDa		0.013	Dmel\Ccp84Ag-PA	structural constituent of chitin-based cuticle; structural constituent of chitin-based larval cuticle

Accession Number	Molecular Weight	Protein Grouping Ambiguity	P-Value	Symbol	Feature type
FBpp0082709 (+1)	31 kDa		0.017	Dmel\sra-PA	protein binding kinesin-associated
FBpp0297341 (+1)	135 kDa	TRUE	0.017	Dmel\milt-PD	mitochondrial adaptor activity
FBpp0081725	26 kDa	TRUE	0.019	Dmel\Rrp46-PA	3′-5′-exoribonuclease activity
FBpp0071590 (+3)	13 kDa	TRUE	0.022	Dmel\HmgZ-PB	DNA binding
FBpp0073789 (+3)	66 kDa		0.032	Dmel\pdgy-PA	long-chain fatty acid-CoA ligase activity
FBpp0078071 (+1)	31 kDa		0.036	Dmel\CG9391-PA	inositol monophosphate 1-phosphatase activity
FBpp0077099	30 kDa		0.05	Dmel\mRpS2-PA	structural constituent of ribosome
FBpp0297622	28 kDa	TRUE	0.051	Dmel\CG8757-PB	oxidoreductase activity, acting on CH-OH group of donors
FBpp0072365	93 kDa	TRUE	< 0.0001	Dmel\Lsp1γ-PA	nutrient reservoir activity ATP binding; ATPase activity, coupled to transmembrane movement of substances; transporter activity
FBpp0290160	190 kDa		< 0.0001	Dmel\CG1801-PC	
FBpp0086581 (+1)	58 kDa	TRUE	0.00017	Dmel\Cyp6a17-PA	electron carrier activity; heme binding; iron ion binding; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
FBpp0070533 (+1)	26 kDa		0.00024	Dmel\CG3603-PA	oxidoreductase activity, acting on CH-OH group of donors
FBpp0079279 (+3)	79 kDa	TRUE	0.00074	Dmel\Akap200-PA	protein kinase A binding
FBpp0081851	58 kDa	TRUE	0.0014	Dmel\Ugt35b-PA	transferase activity, transferring hexosyl groups; UDP-glycosyltransferase activity
FBpp0078854 (+1)	70 kDa	TRUE	0.0016	Dmel\mtm-PA	protein tyrosine phosphatase activity; protein tyrosine/ serine/ threonine phosphatase activity
FBpp0071095 (+1)	43 kDa	TRUE	0.0017	Dmel\CG10932-PA	acetyl-CoA C-acetyltransferase activity
FBpp0080957 (+1)	16 kDa		0.028	Dmel\CG9338-PA	unknown

Table 2.

Oscillating total proteins in w^{1118} and Clk^{Jrk}

	Accession Number	Gene Name	Biological Process	ADJ.P	PER	LAG	AMP
w^{1118}	FBpp0073433 (+1)	Upf1	ATP binding; DNA binding; helicase activity; zinc ion binding	0.005595	24	12	0.307591
	FBpp0081815 (+1)	CG4757	carboxylic ester hydrolase activity	0.005595	24	9	0.143189
	FBpp0075445	CG17839	Unknown	0.005595	24	12	0.077782
	FBpp0078854 (+1)	mtm	phosphatidylinositol-3-phosphatase activity; phorsphortase activity	0.005595	24	12	0.070711
	FBpp0080049	CG9302	protein disulfide isomerase activity	0.007424	24	12	0.106066
	FBpp0086965	mRpL18	structural constituent of ribosome	0.01368	24	12	0.091924
	FBpp0079790	hgo	homogentisate 1,2-dioxygenase activity	0.018788	24	15	0.116673
	FBpp0289679 (+1)	CG31205	serine-type endopeptidase activity	0.018788	24	12	0.067175
	FBpp0078424 (+2)	Vps37B	ESCRT assembly domain	0.023896	24	12	0.144957
	FBpp0084730	CG11841	serine-type endopeptidase activity	0.039848	24	12	0.152028
Clk^{Jrk}	FBpp0079614	CG5188	Proteolysis	0.003766	24	9	0.258094
	FBpp0073433 (+1)	Upf1	ATP binding; DNA binding; helicase activity; zinc ion binding	0.010552	24	9	0.212132
	FBpp0083560 (+3)	CASK	ATP binding; protein kinase activity	0.018788	24	6	0.13435
	FBpp0075743 (+1)	Pbgs	porphobilinogen synthase activity; zinc ion binding	0.018788	24	9	0.120208
	FBpp0072925	Drsl4	antifungus	0.031872	24	3	0.26163
	FBpp0072056	thoc5	mRNA binding	0.031872	24	6	0.219203
	FBpp0077099	mRpS2	structural constituent of ribosome	0.031872	24	6	0.127279

Table 3.

Differential phosphorylated proteins in w^{1118} and Clk^{Jrk}

Accession Number	MW (kDa)	Protein Grouping Ambiguity	P-Value	Symbol	Feature type
FBpp0292500	6		0.002	Dmel\CG31808-PC	
FBpp0080074	11		0.005	Dmel\CG9928-PA	
FBpp0077716	12		0.024	Dmel\RpLP1-PA	structural constituent of ribosome
FBpp0088837	12		0.001	Dmel\SC35-PA	mRNA binding; nucleotide binding
FBpp0071590	13	TRUE	0.00096	Dmel\HmgZ-PB	DNA binding
FBpp0071308	23		< 0.0001	Dmel\CG32700-PA	
FBpp0088688	24		0.00025	Dmel\Mlc2-PA	calcium ion binding; microfilament motor activity
FBpp0086860	25		< 0.0001	Dmel\CG4716-PA	methylenetetrahydrofolate dehydrogenase [NAD(P)+] activity
FBpp0082724	28		< 0.0001	Dmel\SF2-PA	mRNA binding; nucleotide binding
FBpp0082987	30	TRUE	< 0.0001	Dmel\14-3-3ε-PA	protein domain specific binding; protein kinase C inhibitor activity
FBpp0071307	33		< 0.0001	Dmel\l(1)G0320-PA	signal sequence binding
FBpp0074056	35		0.001	Dmel\Rbp2-PB	mRNA binding; nucleic acid binding; nucleotide binding; translation initiation factor activity
FBpp0074530	35		0.002	Dmel\CG12237-PA	phosphatase activity; pyrophosphatase activity
FBpp0077186	37		< 0.0001	Dmel\Reph-PA	
FBpp0076552	40		0.057	Dmel\lark-PA	mRNA binding; nucleic acid binding; nucleotide binding; RNA binding; zinc ion binding
FBpp0078321	40		0.0016	Dmel\CG2082-PC	
FBpp0073344	41		< 0.0001	Dmel\Gs2-PB	glutamate-ammonia ligase activity
FBpp0083266	41		0.042	Dmel\ninaE-PA	G-protein coupled photoreceptor activity; G-protein coupled receptor activity
FBpp0084457	41		< 0.0001	Dmel\CG6330-PA	uridine phosphorylase activity
FBpp0070787	42	TRUE	< 0.0001	Dmel\Act5C-PA	structural constituent of cytoskeleton
FBpp0081544	42		< 0.0001	Dmel\RnpS1-PA	Rab GTPase binding; transporter activity
FBpp0076326	45	TRUE	< 0.0001	Dmel\Arr2-PA	opsin binding
FBpp0078393	49		< 0.0001	Dmel\kra-PA	protein binding; ribosome binding; translation initiation factor binding
FBpp0077367	51		0.019	Dmel\NTPase-PA	guanosine-diphosphatase activity; nucleoside-diphosphatase activity; nucleoside-triphosphatase activity
FBpp0080277	53	TRUE	0.031	Dmel\vig-PD	mRNA binding
FBpp0087631	56		0.0001	Dmel\ced-6-PB	PTB/PI domain; Pleckstrin-homology domain (PH domain)/ Phosphotyrosine-binding domain (PTB)
FBpp0077872	57		< 0.0001	Dmel\CG4825-PA	CDP-diacylglycerol-serine O-phosphatidyltransferase activity
FBpp0073173	58		< 0.0001	Dmel\HDAC1-PA	histone deacetylase activity; transcription corepressor activity
FBpp0079471	58		< 0.0001	Dmel\Srp54-PA	mRNA binding; nucleotide binding
FBpp0078561	60		0.00083	Dmel\lost-PA	RNA binding
FBpp0112089	62		0.024	Dmel\Cpr73D-PB	structural constituent of chitin-based cuticle; structural constituent of chitin-based larval cuticle
FBpp0081881	64		< 0.0001	Dmel\CG6723-PA	sodium:iodide symporter activity
FBpp0088565	64		0.003	Dmel\eIF-3p66-PB	translation initiation factor activity
FBpp0086941	66		0.0003	Dmel\Amph-PA	phospholipid binding

Accession Number	MW (kDa)	Protein Grouping Ambiguity	P-Value	Symbol	Feature type
FBpp0079999	68	TRUE	< 0.0001	Dmel\Vha68-2-PA	ATP binding; proton-transporting ATPase activity, rotational mechanism; contributes_to proton-transporting ATPase activity, rotational mechanism
FBpp0070336	75		< 0.0001	Dmel\eIF2B-ε-PA	nucleotidyltransferase activity; translation initiation factor activity
FBpp0307811	76		< 0.0001	Dmel\CG44195-PA	ATP binding; calcium-dependent protein kinase C
FBpp0086196	80		0.023	Dmel\inaC-PA	activity; diacylglycerol binding; protein kinase C activity; protein serine/threonine kinase activity; zinc ion binding
FBpp0074557	88		0.028	Dmel\CG12531-PA	amino acid transmembrane transporter activity; basic amino acid transmembrane transporter activity
FBpp0087933	89		< 0.0001	Dmel\nito-PA	mRNA binding; nucleic acid binding; nucleotide binding G-protein coupled receptor activity; receptor
FBpp0084323	100		0.045	Dmel\boss-PA	binding; sevenless binding; transmembrane signaling receptor activity
FBpp0075617	108		< 0.0001	Dmel\SRm160-PA	
FBpp0088653	109		< 0.0001	Dmel\CG1677-PA	metal ion binding
FBpp0084655	113		< 0.0001	Dmel\Moca-cyp-PA	peptidyl-prolyl cis-trans isomerase activity
FBpp0290385	122		0.00098	Dmel\CG5549-PB	glycine:sodium symporter activity; neurotransmitter transporter activity; neurotransmitter:sodium symporter activity
FBpp0289455	134		0.001	Dmel\CG42450-PA	signal transducer activity
FBpp0078930	135		< 0.0001	Dmel\Liprin-α-PA	protein binding
FBpp0080638	152		< 0.0001	Dmel\ncm-PA	RNA binding ATP binding; platelet-derived growth factor-activated receptor activity; protein tyrosine kinase activity; transmembrane receptor protein tyrosine kinase activity; vascular endothelial growth factor-activated receptor activity
FBpp0079244	170		0.001	Dmel\Pvr-PA	
FBpp0079064	174	TRUE	< 0.0001	Dmel\ninaC-PB	ATP binding; ATPase activity, coupled; calmodulin binding; motor activity; protein kinase activity; protein serine/ threonine kinase activity; receptor signaling protein serine/ threonine kinase activity
FBpp0074228	266		< 0.0001	Dmel\β-Spec-PA	actin binding; actin filament binding; calmodulin binding; cytoskeletal protein binding; structural constituent of cytoskeleton
FBpp0304412	311		0.00011	Dmel\E(bx)-PH	DNA binding; contributes_to nucleosome-dependent ATPase activity; zinc ion binding
FBpp0311334	367		< 0.0001	Dmel\CG45186-PAB	actin binding
FBpp0111616	380	TRUE	< 0.0001	Dmel\CG34417-PD	actin binding; structural constituent of cytoskeleton
FBpp0306407	405		0.011	Dmel\btsz-PI	Rab GTPase binding; transporter activity

Table 4

Oscillating phosphorylated proteins

	Accession Number	Gene Name	Biological Process	ADJ.P	PER	LAG	AMP
	FBpp0099563 (+3)	Dop2R	Dopamine receptor	0.001775	24	0	0.208597
	FBpp0099855 (+2)	Dek	chromatin binding	0.003766	24	0	0.144957
	FBpp0071590 (+3)	HMG protein Z	sequence-specific DNA binding proteins	0.005595	24	0	0.254558
	FBpp0075278 (+4)	brahma	chromatin-remodeling	0.005595	24	0	0.144957
	FBpp0071448	Actin 57B	structural constituent of cytoskeleton	0.005595	24	3	0.083085
	FBpp0076320 (+1)	Paramyosin	Myosin tail	0.007424	24	3	0.152028
	FBpp0078393 (+6)	krasavietz	translational regulator	0.010552	24	0	0.19799
	FBpp0088690 (+2)	Hrs	endocytosis	0.010552	24	3	0.10253
	FBpp0288578	CG42235	Sodium/solute symporter	0.01368	24	15	0.137886
	FBpp0072338 (+2)	Eps-15	calcium ion binding	0.023896	24	0	0.084853
w^{1118}	FBpp0078674 (+1)	Reticulon-like1	endoplasmic reticulum organization; endoplasmic reticulum tubular network membrane organization	0.031872	24	3	0.123744
	FBpp0087678 (+3)	DNA fragmentation factor-related protein 2	regulation of apoptotic process; negative regulation of neuron death	0.031872	24	0	0.070711
	FBpp0086860 (+1)	CG4716	methylenetetrahydrofolate dehydrogenase [NAD(P)+] activity	0.039848	24	0	0.275772
	FBpp0089041	Proteasome α7 subunit	Proteasome alpha-type subunit	0.039848	24	0	0.171473
	FBpp0081539	CG8149	SAP domain superfamily	0.039848	24	21	0.164402
	FBpp0085043 (+1)	jdp	protein folding	0.039848	24	0	0.146725
	FBpp0082687-DECOY (+1)	Myosin heavy chain-like	Class XVIII myosin	0.039848	24	12	0.137886
	FBpp0082692	moira	DNA binding; protein binding	0.039848	24	18	0.109602
	FBpp0080825 (+1)	Topoisomerase 2	DNA topoisomerase	0.039848	24	0	0.106066
	FBpp0086013 (+1)	eIF3c	Proteasome component	0.000519	24	9	0.127279
	FBpp0070618 (+5)	norpA	phosphatidylinositol phospholipase C activity	0.000758	24	9	0.279307
	FBpp0084566 (+1)	Myosin alkali light chain 1	microfilament motor activity; calcium ion binding	0.003766	24	6	0.127279
	FBpp0292500	CG31808	unknown	0.007424	24	9	0.309359
	FBpp0309308 (+4)	radish	GTPase activator activity	0.007424	24	15	0.176777
	FBpp0079420 (+1)	numb	Notch binding	0.010552	24	15	0.183848
Clk^{Jrk}	FBpp0085139 (+4)	chaoptin	unknown	0.010552	24	15	0.084853
	FBpp0086252	Ribosomal protein LP2	structural constituent of ribosome	0.01368	24	9	0.38007
	FBpp0074415 (+1)	CG32544	unknown	0.018788	24	15	0.160867
	FBpp0082514 (+6)	Heat shock protein cognate 4	chaperone binding; unfolded protein binding	0.018788	24	12	0.084853
	FBpp0078775	blue cheese	metal ion binding	0.018788	24	12	0.042426
	FBpp0081881	CG6723	transmembrane transporter activity	0.023896	24	6	0.387141
	FBpp0073309 (+2)	CG1737	unknown	0.031872	24	15	0.139654

Accession Number	Gene Name	Biological Process	ADJ.P	PER	LAG	AMP
FBpp0075023 (+5)	Nc73EF	thiamine pyrophosphate binding; oxoglutarate dehydrogenase (succinyl-transferring) activity	0.031872	24	9	0.083085
FBpp0074825	Catalase	antioxidant activity; heme binding; catalase activity	0.031872	24	12	0.06364
FBpp0112048	ensconsin	structural molecule activity; microtubule binding	0.039848	24	6	2.506694
FBpp0081470 (+4)	pumilio	mRNA 3′-UTR binding; translation repressor activity, nucleic acid binding; RNA binding	0.039848	24	9	0.228042
FBpp0099901	Synapsin	unknown	0.039848	24	18	0.16617
FBpp0087502 (+4)	14-3-3ζ	protein kinase C inhibitor activity	0.039848	24	12	0.127279
FBpp0071757 (+4)	Liprin-γ	protein binding	0.039848	24	21	0.120208
FBpp0293740 (+2)	rhea	integrin binding; actin binding; protein binding; structural constituent of cytoskeleton; actin filament binding	0.039848	24	15	0.106066
FBpp0076320 (+1)	Paramyosin	motor activity	0.039848	24	12	0.095459

Table 5.

Behavior results for circadian locomotion screen.

Gene	Genotype	Period (h)	Rhythmicity	N number
	TD2/+	24.3±0.21	82.50%	32
alpha-cat	TD2/BL38987	25.1±0.20	87.50%	32
alpha-cat	TD2/BL38197	25.1±0.12	86.70%	30
Tps1	TD2/BL57488	24.4±0.13	30.40%	23
Dsor1	TD2/VDRC107276	25.0±0.17	93.80%	16
Dsor1	TD2/VDRC40026	24.9±0.15	100%	8
CG13117	TD2/BL51678	25.2±0.22	100%	15
	PD2/+	24.5±0.22	93.80%	16
alpha-cat	PD2/BL38987	25.6±0.12	88.00%	25
alpha-cat	PD2/BL38197	25.5±0.08	100%	26
Tps1	PD2/BL57488	24.8±0.05	93.80%	16
Dsor1	PD2/VDRC107276	24.7±0.15	100%	8
Dsor1	PD2/VDRC40026	25.0±0.13	100%	8
HmgZ	TD2/BL26219	23.8±0.13	59.10%	22
SLO2	TD2/BL32034	24.4±0.20	87.50%	24
CG5641	TD2/BL56895	24.7±0.32	69.20%	13