An RNAi Screen To Identify Protein Phosphatases That Function Within the Drosophila Circadian Clock

Parul Agrawal* and Paul E. Hardin*†,1
*Department of Biology and †Center for Biological Clocks Research, Texas A&M University, College Station, Texas 77845-3258

ABSTRACT  Circadian clocks in eukaryotes keep time via cell-autonomous transcriptional feedback loops. A well-characterized example of such a transcriptional feedback loop is in Drosophila, where CLOCK-CYCLE (CLK-CYC) complexes activate transcription of period (per) and timeless (tim) genes, rising levels of PER-TIM complexes feed-back to repress CLK-CYC activity, and degradation of PER and TIM permits the next cycle of CLK-CYC transcription. The timing of CLK-CYC activation and PER-TIM repression is regulated posttranslationally, in part through rhythmic phosphorylation of CLK, PER, and TIM. Previous behavioral screens identified several kinases that control CLK, PER, and TIM levels, subcellular localization, and/or activity, but two phosphatases that function within the clock were identified through the analysis of candidate genes from other pathways or model systems. To identify phosphatases that play a role in the clock, we screened clock cell-specific RNA interference (RNAi) knockdowns of all annotated protein phosphatases and protein phosphatase regulators in Drosophila for altered activity rhythms. This screen identified 19 protein phosphatases that lengthened or shortened the circadian period by ≥1 hr (p ≤ 0.05 compared to controls) or were arrhythmic. Additional RNAi lines, transposon inserts, overexpression, and loss-of-function mutants were tested to independently confirm these RNAi phenotypes. Based on genetic validation and molecular analysis, 15 viable protein phosphatases remain for future studies. These candidates are expected to reveal novel features of the circadian timekeeping mechanism in Drosophila that are likely to be conserved in all animals including humans.

A diverse array of organisms use circadian (~24 hr) clocks to regulate daily rhythms in physiology, metabolism, and behavior. These clocks keep time via self-sustained transcriptional feedback loops that are synchronized to daily environmental cycles and drive daily rhythms in gene expression. As in other animals, the Drosophila core timekeeping loop is activated by two bHLH-PAS transcription factors, CLOCK and CYCLE (CLK-CYC), and repressed by PERIOD-TIMELESS (PER-TIM) complexes. The generation of self-sustaining oscillations depends on posttranslational regulation of clock proteins, which modulates their stability, activity, and/or subcellular localization during the daily cycle. Multiple levels of posttranslational controls are built into this oscillatory system to produce a ~24 hr period, support a robust cycling amplitude, and enable resetting by environmental inputs.

The best characterized posttranslational modification of clock proteins is phosphorylation. In Drosophila, nuclear localization of the PER-TIM repressor complex is regulated by CASEIN KINASE II (CK2) and SHAGGY (SGG) phosphorylation (Martinek et al. 2001; Lin et al. 2002; Akten et al. 2003, 2009), whereas PER degradation in the nucleus is regulated by DOUBLE-TIME (DBT) and NEMO (Kloss et al. 1998, 2001; Price et al. 1998; Chiu et al. 2008, 2011). Phosphorylation also controls CLK-CYC function where NEMO, DBT, and CK2-dependent phosphorylation control CLK stability and activity (Kim and Edery 2006; Yu et al. 2006; Szabo et al. 2013). The phosphorylation state of a protein is controlled dynamically by protein kinases and phosphatases. However, few phosphatases have been identified that function in the Drosophila circadian clock; Protein Phosphatase 2a (PP2a) and Protein Phosphatase 1 (PP1), control PER-TIM repressor stability and nuclear localization (Sathyarayanan et al. 2004; Fang et al. 2007), and the PP2a-STRIPAK complex dephosphorylates CLK to promote CLK-CYC transcription (Andrezza et al. 2015). Despite our understanding of how phosphorylation controls nuclear localization and degradation of PER-TIM repressor complexes and activity of

KEYWORDS  Drosophila melanogaster activity rhythms protein phosphatases circadian clocks
CLK-CYC activator complexes, the role phosphorylation plays in controlling cytoplasmic PER-TIM accumulation and the light-dependent TIM degradation are not well understood. These events control progression through the feedback loop, and are therefore critical for controlling the period, phase, and amplitude of rhythmic transcription.

To determine how dephosphorylation regulates rhythmic transcription within the Drosophila clock, we used RNAi knockdown to screen all annotated protein phosphatases in Drosophila for defects in locomotor activity rhythms. Of 86 protein phosphatase or protein phosphatase regulator genes screened, 19 showing period alterations or motor activity rhythms. Of 86 protein phosphatase or protein phosphatase regulator genes screened, 19 showing period alterations or motor activity rhythms (Agrawal and Hardin 2016). Additional genetic agents were obtained or generated to validate the RNAi phenotypes. The validated phosphatases identified here may contribute to clock cell development or the circadian timekeeping mechanism, and represent potential genetic links to clock-associated disorders in humans and novel targets for the development of drugs to treat such disorders.

MATERIALS AND METHODS

Fly stocks

The w1118 and w1118, CyO/Sev TM6B/TM3 strains were used as wild-type controls for activity rhythms and as balancers to generate lines used for screening and analysis, respectively. The following Gal4 strains were used to drive RNAi expression in clock cells: w1118, timGal4[65], w1118, ptfGal4, and w1118, timGal4[64]. The following RNAi strains from the Vienna Drosophila RNAi Center (VDRC) were used to knockdown phosphatase/regulator expression in clock cells, listed as the gene name and abbreviation or CG number followed by the VDRC line number in parenthesis: pucked, puc (GD3018); Protein tyrosine phosphatase 52F, Ptp52F (GD3116); TbcM46, (GD17123); string, sgi (GD17760); CG17124 (GD19078); Calcineurin B, CanB (GD2161); Protein phosphatase 19C, Pp4-19C (GD25317); Protein tyrosine phosphatase 4E, Pp4E (GD27232); Calciumurin A1, CanA1 (GD32283); CG17598 (GD32956); Protein phosphatase 1 at 87B, Pp1-87B (GD35025); Phosphophorylholphatase activator, Ppta (GD41912); microtubule star, mts (GD41924); sbs22 (GD42051); Mitogen-activated protein kinase phosphatase 3, Mkp3 (GD45415); Protein phosphatase 2A at 29B, Ppa2-29B (GD94671); CG6380 (KK100121); CG7746 (KK100178); CG2104 (KK100216); Mapmodulin (KK100283); Glycogen binding subunit 70E, Gbs-70E (KK100593); CG4327 (KK100914); Ppl1 (KK101257); CG10376 (KK101335); widebar, wdb (KK101406); Calcium and integrin binding family member 2, Cib2 (KK101474); Inhibitor-2, I-2 (KK101547); Protein phosphatase V, PpV (KK101997); Protein phosphatase Y at 55A, Ppy-55A (KK102021); Protein phosphatases N at 58A, Ppn-58A (KK102060); CG14297 (KK102071); Protein tyrosine phosphatase 36E, Ptp36E (KK102397); CG31469 (KK102474); Glycogen binding subunit 76A, Gbs-76A (KK103044); Protein phosphatase 2B at 14D, Pp2B-14D (KK103144); CG32568 (KK103173); CG7115 (KK103354); Cep97 (KK103357); cell division cycle 14, cdc14 (KK103627); Protein tyrosine phosphatase Meg, Ptpmeg (KK103740); CG32812 (KK104081); twins, tws (KK104167); Protein phosphatase D6, PpD6 (KK104211); MAP kinase-specific phosphatase, Mkp (KK104374); Protein tyrosine phosphatase Meg2, Ptpmeg2 (KK104427); Protein phosphatase D5, PpD5 (KK104452); flapwing, fww (KK104677); CG11597 (KK104729); Protein tyrosine phosphatase 69D, Ptp69D (KK104761); Protein Tyrosine Phosphatase monochtza- drial 1, PTPMT1 (KK104774); Dullard, Dd (KK104785); myopic, mop (KK104860); MAPK Phosphatase 4, MKP-4 (KK104884); CG13197 (KK105122); Protein phosphatase 2C, Pp2C (KK105249); Protein phosphatase 4 regulatory subunit 2-related protein, PP4PR2 (KK105399); alfabet, alph (KK105483); CG15528 (KK105484); Protein phosphatase 1 at 96A, Pp1a-96A (KK105525); inhibitor-4, I-4 (KK105565); CG6036 (KK105566); CG5026 (KK105674); CG3737 (KK106098); CG10417 (KK106180); TFIH-interacting CTD phosphatase, Fcp1 (KK106253); P2A-B (KK107097); Protein phosphatase D3, PpD3 (KK107386); CG4733 (KK107621); Protein phosphatase 1 at 9C, Pp1-9C (KK107770); Leucocyte-antigen-related-like, Lar (KK107966); singlet, ssh (KK107998); eyes absent, eya (KK108071); carkers, csw (KK108352); Protein tyrosine phosphatase 99A, Ppp9A (KK108050); CG10089 (KK108744); CG8509 (KK108802); Nuclear inhibitor of Protein phosphatase 1, NPP1 (KK108859); Protein tyrosine phosphatase 61F, Ptp61F (KK108888); Protein phosphatase 1, Y-linked 2, Pp1-Y2 (KK109147); CG14411 (KK109622); Calciumurin A at 14F, CanA-14F (KK109858); CG3632 (KK110167); PH domain leucine-rich repeat protein phosphatase, Phlp (KK110360); Protein tyrosine phosphatase 10D, Ptp10D (KK110443); IA-2 protein tyrosine phosphatase, IA-2 (KK110595); and CG3530 (KK110786). The following strains were used to characterize clock candidate protein phosphatases in UAS-mRNAi (GD35171), w1118, UAS-mts, w1118, P[RP4]Ppa-29B, Pp2A-29B, Pp4E, PpD3 (GD35233), PpD5 (GD35383), P[RP3]Pp2A-29B, Pp4E, PpD3 (GD35493), P[RP1]Pp2A-29B, Pp4E, PpD3 (GD35541), P[RP2]Pp2A-29B, Pp4E, PpD3 (GD35571). The following strains were used for screening and analysis, respectively. The following Gal4 strains were used to drive RNAi knockdown to screen phosphatase regulator genes screened, 19 showing period alterations or motor activity rhythms. Of 86 protein phosphatase or protein phosphatase regulator genes screened, 19 showing period alterations or motor activity rhythms (Agrawal and Hardin 2016). Additional genetic agents were obtained or generated to validate the RNAi phenotypes. The validated phosphatases identified here may contribute to clock cell development or the circadian timekeeping mechanism, and represent potential genetic links to clock-associated disorders in humans and novel targets for the development of drugs to treat such disorders.

Drosophila activity monitoring and behavior analysis

One to three d old male flies were entrained for 3 d in 12:12 light-dark (LD) and transferred to constant darkness (DD) for 7 d at 25°C. The screen employed testing of each UAS-RNAi alone (as a control), driver alone (as a control), and a combination of UAS-RNAi line with timGal4 or ptfGal4 (as the RNAi knockdown). Locomotor activity was monitored using the Drosophila Activity Monitor (DAM) system (Trikinetics). Analyses of period, power and rhythm strength during DD was carried out using ClockLab (Actimetrics) software as previously described (Pfeifferberger et al. 2010). UAS-RNAi lines that produce consistent period lengthening or shortening of ≥1 hr with p ≤ 0.05 compared to UAS-RNAi and Gal4 driver controls or >50% arrhythmicity were analyzed further. Different genetic backgrounds may show small differences in circadian period, therefore a period change of ≤1 hr due to RNAi knockdown was not considered significant.

Generation of Pp1α-96A CRISPR mutants

The CRISPR-Cas9 system was used to generate deletions in Pp1α-96A (Gratz et al. 2015). Two guide RNAs (gRNAs) targeting the Pp1α-96A
Testing for the presence of perSLIH mutation

To determine whether flies carry the perSLIH allele, a DNA fragment containing the perSLIH mutation site was amplified from genomic DNA using the Per-F (5' GCCGTGCTCTGAATGATCCGG-3') and Per-R (5'-ATTGGCTACGTCTGTCAGACC-3') primers via PCR. These DNA fragments were sequenced using the primer 5'-TCCGGCAGCAGTGCGCGGTGCAC-3' to determine whether they contained a C to A nucleotide change at position 3035 corresponding to the perSLIH mutation (Hamblen et al. 1998).

Isogenizing phosphatase alleles to w1118

The w+ Ptp69D1 and CanA14F-KO null mutants are both marked with w+, and were isogenized by backcrossing w+ male progeny to the w1118 reference strain (BDSC) for seven generations.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

To identify phosphatases that mediate circadian clock function, clock cell-specific RNAi knockdowns of all annotated Drosophila protein phosphatases were screened for altered locomotor activity rhythms. An RNAi screening strategy is advantageous because: (1) lethality can be avoided by targeting RNAi to clock cells via the Gal4/UAS system, (2) RNAi is efficient method for knocking down target gene expression, and (3) transgenic UAS-RNAi lines are available for all annotated protein phosphatase/regulator from either the VDRC, Transgenic RNAi Project at the Harvard Medical School plans (TRIP), or the National Institute of Genetics RNAi Stocks (NIG-Fly). RNAi lines from VDRC were used in this screen because the vast majority are inserted at a single genomic site (e.g., KD lines) whereas some lines were inserted at random genomic sites (e.g., GD lines) because KK lines for some protein phosphatases/regulators were not available. A total of 94 annotated protein phosphatase catalytic and regulatory subunits are present in the Drosophila genome (Drosophila RNAi Screening Center, DRSC). When this screen was initiated in 2010, we were able to obtain UAS-RNAi lines for 67 of these protein phosphatase catalytic and regulatory subunits and an additional 19 protein phosphatase regulators. Thus, a total of 86 protein phosphatases/regulators were screened to identify phosphatases that disrupt circadian activity rhythms.

Flies bearing UAS-RNAi and the w1118;timGal462 driver or controls bearing UAS-RNAi alone and Gal4 driver alone were placed into Drosophila Activity Monitors, entrained for 3 d in LD cycles, and then released into DD for 7 d. Locomotor activity rhythms recorded during DD were analyzed for circadian rhythmicity and period via ClockLab software. This initial screen identified a total of 26 candidate phosphatases, all of which lengthened or shortened circadian period by ≥1 h or were ≥50% arrhythmic (Table 1). Since many RNAi lines with a lengthened period also had a low proportion of rhythmic flies, we were concerned that the widespread RNAi knockdown of these phosphatases or ectopic RNAi expression from the w1118;timGal462 driver impaired fly health. Consequently, we used w1118;pdfGal4 to restrict RNAi expression to PDF neuron-expressing ventral lateral pacemaker neurons (LNv+s) and another timGal4 insert, w1118;timGal461, to drive protein phosphatase/regulator RNAi expression. Out of the 86 lines screened with all three Gal4 drivers, 19 RNAi lines with a significantly (p ≤ 0.05 compared to controls) different period or ≥50% arrhythmic were identified (Table 1).

Based on these criteria, candidate phosphatases that mediate Drosophila circadian clock function include: mts (GD41924), Pp2a-29B (GD49671), CG6380 (KK100121), CG17746 (KK100178), Gbs-70E (KK100593), Ppm1 (KK101257), L-2 (KK101547), CG7115 (KK103354), Cep97 (KK103357), PpD6 (KK104211), Ptpmeg2 (KK104427), Ptp69D (KK104761), MPK4 (KK104848), Pp1a-96A (KK105525), CG14017 (KK106180), Lar (KK107996), Pp1-Y2 (KK109147), CanA-14F (KK109858), and CG3530 (KK110786). Next, the efficacy and specificity of RNAi-mediated knockdown of these candidate “clock phosphatases” was validated by testing RNAi lines that target another region of the mRNA and/or other genetic reagents (i.e., transposon insert, Gal4/UAS system driven overexpression, and existing loss-of-function mutants) for activity rhythm defects. For each candidate clock phosphatase, we provide a description of the protein, its known functions, and the results of the genetic reagents used to verify the RNAi phenotype below.

Microtubule star (mts)

MTS is the catalytic subunit of a protein phosphatase 2a (Pp2a) that dephosphorylates proteins at serine and threonine residues. It functions in many cellular process including the mitotic cell cycle, cell surface receptor signaling, and cell adhesion (Janssens and Goris 2001; Chen
Table 1 Activity rhythms of clock cell-specific phosphatase RNAi knockdowns in Drosophila

| Genotype | N  | % Rhythmic | Period | Mean ± SEM |
|----------|----|-------------|--------|------------|
| w1118/+;GD3018 (puc) | 13 | 100 | 23.57 ± 0.08 |
| w1118/+;timGal4/GD3018 | 16 | 100 | 24.40 ± 0.05 |
| w1118;GD 3018/+;timGal4/+ | 15 | 93 | 24.21 ± 0.16 |
| w1118;GD3018/+;pdfGal4/+ | 15 | 100 | 24.13 ± 0.06 |
| w1118;+/GD3116 (Psp52F) | 13 | 92 | 23.92 ± 0.15 |
| w1118;timGal4/GD3116 | 16 | 94 | 24.50 ± 0.09 |
| w1118;GD3116/+;timGal4/+ | 9 | 78 | 23.46 ± 0.22 |
| w1118;GD3116/+;pdfGal4/+ | 8 | 75 | 23.92 ± 0.13 |
| w1118;+/GD17123 (TbCMF46) | 16 | 94 | 23.5 |
| w1118;timGal4/GD17123 | 16 | 81 | 24.08 ± 0.14 |
| w1118;GD17123/+;timGal4/+ | 15 | 100 | 24.00 ± 0.12 |
| w1118;GD17123/+;pdfGal4/+ | 16 | 88 | 23.89 ± 0.13 |
| w1118;+/GD17760 (stg) | 16 | 100 | 23.56 ± 0.04 |
| w1118;timGal4/GD17760 | 16 | 88 | 24.10 ± 0.11 |
| w1118;GD17760/+;timGal4/+ | 14 | 100 | 24.11 ± 0.14 |
| w1118;GD17760/+;pdfGal4/+ | 13 | 85 | 23.86 ± 0.15 |
| w1118;+/GD19078 (CG17124) | 16 | 100 | 23.50 ± 0.10 |
| w1118;timGal4/GD19078 | 16 | 100 | 23.81 ± 0.10 |
| w1118;GD19078/+;timGal4/+ | 16 | 88 | 24.36 ± 0.09 |
| w1118;GD19078/+;pdfGal4/+ | 12 | 100 | 24.04 ± 0.11 |
| w1118;+/GD21611 (Carb) | 16 | 100 | 24.03 ± 0.03 |
| w1118;timGal4/GD21611 | 15 | 93 | 24.64 ± 0.14 |
| w1118;GD21611/+;timGal4/+ | 14 | 100 | 24.14 ± 0.16 |
| w1118;GD21611/+;pdfGal4/+ | 16 | 100 | 23.66 ± 0.09 |
| w1118;+/GD25317 (Pp4-19C) | 14 | 100 | 24.37 ± 0.01 |
| w1118;timGal4/GD25317 | 16 | 100 | 23.50 ± 0.11 |
| w1118;GD25317/+;timGal4/+ | 15 | 93 | 24.39 ± 0.15 |
| w1118;GD25317/+;pdfGal4/+ | 16 | 100 | 23.72 ± 0.09 |
| w1118;+/GD27232 (Ptp4E) | 8 | 75 | 24.41 ± 0.05 |
| w1118;timGal4/GD27232 | 16 | 100 | 23.50 ± 0.08 |
| w1118;GD27232/+;timGal4/+ | 16 | 100 | 24.50 ± 0.20 |
| w1118;GD27232/+;pdfGal4/+ | 13 | 100 | 25.44 ± 0.88 |
| w1118;+/GD32283 (CanA1) | 16 | 100 | 23.50 ± 0.04 |
| w1118;timGal4/GD32283 | 14 | 93 | 24.39 ± 0.23 |
| w1118;GD32283/+;timGal4/+ | 16 | 94 | 24.53 ± 0.10 |
| w1118;GD32283/+;pdfGal4/+ | 14 | 93 | 24.90 ± 0.13 |
| w1118;+/GD32956 (CG17598) | 16 | 94 | 23.54 ± 0.09 |
| w1118;timGal4/GD32956 | 15 | 93 | 24.58 ± 0.08 |
| w1118;GD32956/+;timGal4/+ | 14 | 43 | 24.20 ± 0.08 |
| w1118;GD32956/+;pdfGal4/+ | 16 | 94 | 23.86 ± 0.10 |
| w1118;+/GD35025 (Psp-l-87F) | 15 | 100 | 24.63 ± 0.08 |
| w1118;timGal4/GD35025 | 13 | 100 | 24.23 ± 0.12 |
| w1118;GD35025/+;timGal4/+ | 16 | 100 | 24.34 ± 0.12 |
| w1118;GD35025/+;pdfGal4/+ | 16 | 100 | 24.69 ± 0.12 |
| w1118;+/GD41912 (Ppca) | 16 | 100 | 24.57 ± 0.04 |
| w1118;timGal4/GD41912 | 15 | 80 | 24.04 ± 0.15 |
| w1118;GD41912/+;timGal4/+ | 15 | 100 | 24.23 ± 0.16 |
| w1118;GD41912/+;pdfGal4/+ | 11 | 100 | 25.53 ± 0.03 |
| w1118;+/GD41924 (mts) | 16 | 100 | 25.14 ± 0.15 |
| w1118;timGal4/GD41924 | 16 | 88 | 24.73 ± 0.08 |
| w1118;GD41924/+;timGal4/+ | 14 | 93 | 24.73 ± 0.08 |
| w1118;GD41924/+;pdfGal4/+ | 14 | 93 | 24.81 ± 0.06 |
| w1118;+/GD42051 (sds22) | 14 | 86 | 23.71 ± 0.09 |
| w1118;timGal4/GD42051 | 16 | 100 | 24.87 ± 0.11 |
| w1118;GD42051/+;timGal4/+ | 16 | 94 | 24.14 ± 0.12 |
| w1118;GD42051/+;pdfGal4/+ | 14 | 100 | 23.78 ± 0.09 |
| w1118;+/GD45415 (Mkp3) | 15 | 93 | 24.67 ± 0.08 |
| w1118;timGal4/GD45415 | 15 | 100 | 24.33 ± 0.15 |
| w1118;GD45415/+;timGal4/+ | 14 | 93 | 24.38 ± 0.10 |
| w1118;GD45415/+;pdfGal4/+ | 14 | 93 | 24.38 ± 0.10 |

(continued)
### Table 1, continued

| Genotype                       | N  | % Rhythmic | Period  | Mean ± SEM |
|--------------------------------|----|------------|---------|------------|
| w¹¹第八;KK10452/+;timGal4/+    | 15 | 67         | 24.35 ± 0.21 |
| w¹¹第八;KK10452/+;pdfGal4/+    | 13 | 15         | 23.25 ± 0.18 |
| w¹¹第八;+;KK104677 (fly)       | 16 | 94         | 24.5     |
| w¹¹第八;timGal4/KK104677      | 16 | 100        | 24.15 ± 0.13 |
| w¹¹第八;KK104677;+timGal4/+    | 16 | 100        | 23.97 ± 0.03 |
| w¹¹第八;KK104677;+pdfGal4/+    | 16 | 88         | 23.91 ± 0.07 |
| w¹¹第八;+;KK104729 (CG111397)| 13 | 69         | 23.61 ± 0.07 |
| w¹¹第八;timGal4/KK104729      | 11 | 91         | 23.60 ± 0.07 |
| w¹¹第八;KK104729;+timGal4/+    | 16 | 94         | 23.80 ± 0.12 |
| w¹¹第八;KK104729;+pdfGal4/+    | 15 | 100        | 23.53 ± 0.03 |
| w¹¹第八;KK10761 (Ptpe69D)     | 12 | 17         | 24.75 ± 0.18 |
| w¹¹第八;timGal4/KK10761       | 13 | 54         | 25.43 ± 0.34 |
| w¹¹第八;KK10761;+timGal4/+    | 8  | 0          | AR       |
| w¹¹第八;KK10761;+pdfGal4/+     | 15 | 100        | 23.47 ± 0.03 |
| w¹¹第八;KK107474 (PtPMT1)     | 15 | 93         | 24.18 ± 0.14 |
| w¹¹第八;KK107474;+timGal4/+   | 14 | 86         | 24.63 ± 0.24 |
| w¹¹第八;KK107474;+pdfGal4/+    | 8  | 88         | 23.36 ± 0.09 |
| w¹¹第八;+;KK104785 (Dd)       | 16 | 100        | 23.03 ± 0.03 |
| w¹¹第八;timGal4/KK104785      | 16 | 100        | 23.94 ± 0.13 |
| w¹¹第八;KK104785;+timGal4/+   | 16 | 100        | 23.97 ± 0.11 |
| w¹¹第八;KK104785;+pdfGal4/+    | 16 | 94         | 23.70 ± 0.15 |
| w¹¹第八;+;KK104860 (map)      | 16 | 100        | 23.5      |
| w¹¹第八;timGal4/KK104860      | 13 | 92         | 24.25 ± 0.14 |
| w¹¹第八;KK104860;+timGal4/+    | 15 | 80         | 24.83 ± 0.31 |
| w¹¹第八;KK104860;+pdfGal4/+     | 16 | 81         | 23.62 ± 0.12 |
| w¹¹第八;+;KK104884 (Mbp-4)    | 16 | 90         | 23.37 ± 0.04 |
| w¹¹第八;timGal4/KK104884      | 15 | 33         | 24.40 ± 0.40 |
| w¹¹第八;KK104884;+timGal4/+    | 12 | 67         | 25.44 ± 0.18 |
| w¹¹第八;KK104884;+pdfGal4/+    | 11 | 27         | 24.00 ± 0.41 |
| w¹¹第八;KK105122 (CG13197)    | 16 | 88         | 23.46 ± 0.03 |
| w¹¹第八;timGal4/KK105122      | 16 | 94         | 23.83 ± 0.12 |
| w¹¹第八;KK105122;+timGal4/+   | 16 | 100        | 23.69 ± 0.12 |
| w¹¹第八;KK105122;+pdfGal4/+    | 15 | 100        | 24.07 ± 0.16 |
| w¹¹第八;+;KK105249 (P2C)      | 16 | 100        | 23.33 ± 0.16 |
| w¹¹第八;timGal4/KK105249      | 16 | 31         | 24.20 ± 0.30 |
| w¹¹第八;KK105249;+timGal4/+   | 13 | 62         | 25.56 ± 0.20 |
| w¹¹第八;KK105249;+pdfGal4/+    | 16 | 100        | 23.78 ± 0.08 |
| w¹¹第八;KK105399 (PPP42r)     | 13 | 100        | 23.65 ± 0.05 |
| w¹¹第八;timGal4/KK105399      | 12 | 25         | 23.17 ± 0.17 |
| w¹¹第八;KK105399;+timGal4/+    | 7  | 0          | AR       |
| w¹¹第八;KK105399;+pdfGal4/+     | 9  | 78         | 23.00 ± 0.25 |
| w¹¹第八;+;KK105483 (alph)     | 16 | 100        | 23.5      |
| w¹¹第八;timGal4/KK105483      | 16 | 88         | 23.89 ± 0.10 |
| w¹¹第八;KK105483;+timGal4/+    | 16 | 100        | 23.73 ± 0.13 |
| w¹¹第八;KK105483;+pdfGal4/+    | 13 | 100        | 24.50 ± 0.13 |
| w¹¹第八;KK105484 (CG115528)   | 13 | 77         | 23.25 ± 0.08 |
| w¹¹第八;timGal4/KK105484      | 16 | 50         | 23.69 ± 0.26 |
| w¹¹第八;KK105484;+timGal4/+    | 14 | 29         | 23.63 ± 0.27 |
| w¹¹第八;KK105484;+pdfGal4/+    | 15 | 100        | 23.57 ± 0.05 |
| w¹¹第八;+;KK105525 (Pp1a-96A)| 10 | 50         | 25.20 ± 0.34 |
| w¹¹第八;timGal4/KK105525      | 15 | 53         | 23.63 ± 0.08 |
| w¹¹第八;KK105525;+timGal4/+    | 16 | 56         | 23.56 ± 0.05 |
| w¹¹第八;KK105525;+pdfGal4/+    | 11 | 55         | 23.47 ± 0.06 |
| w¹¹第八;+;KK105568 (CG6036)   | 16 | 100        | 23.5      |
| w¹¹第八;timGal4/KK105568      | 16 | 56         | 24.28 ± 0.12 |
| w¹¹第八;KK105568;+timGal4/+    | 14 | 71         | 23.90 ± 0.19 |
| w¹¹第八;timGal4/KK104374 (Mkp)                      | 14 | 86         | 23.5 ± 0.05 |
| w¹¹第八;timGal4/KK104374        | 16 | 88         | 23.46 ± 0.04 |
| w¹¹第八;KK104374;+timGal4/+    | 16 | 94         | 24.21 ± 0.14 |
| w¹¹第八;KK104374;+pdfGal4/+    | 15 | 100        | 25.34 ± 0.09 |
| w¹¹第八;KK104427 (Ptpmeg2)     | 16 | 94         | 23.57 ± 0.12 |
| w¹¹第八;timGal4/KK104427       | 16 | 100        | 25.49 ± 0.12 |
| w¹¹第八;KK104427;+timGal4/+    | 13 | 54         | 25.00 ± 0.12 |
| w¹¹第八;KK104427;+pdfGal4/+    | 10 | 10         | 23.5      |
| w¹¹第八;+;KK104452 (PpDS)     | 16 | 100        | 23.30 ± 0.04 |
| w¹¹第八;timGal4/KK104452       | 16 | 44         | 25.29 ± 0.33 |
| Genotype   | N  | % Rhythmic | Period Mean ± SEM |
|-----------|----|------------|------------------|
| w1118;KK105568/+;pdfGal4/+ | 12 | 92 | 23.68 ± 0.10 |
| w1118;+/KK105674 (CG5026) | 16 | 100 | 23.47 ± 0.03 |
| w1118;timGal4/KK105674 | 16 | 100 | 24.19 ± 0.12 |
| w1118;KK105674/+;timGal4/+ | 16 | 100 | 23.97 ± 0.13 |
| w1118;KK105674/+;pdfGal4/+ | 16 | 100 | 23.5 |
| w1118;+/KK106098 (CG7378) | 16 | 100 | 23.53 ± 0.03 |
| w1118;timGal4/KK106098 | 16 | 69 | 24.14 ± 0.27 |
| w1118;KK106098/+;timGal4/+ | 16 | 94 | 25.07 ± 0.15 |
| w1118;KK106098/+;pdfGal4/+ | 13 | 54 | 20.00 ± 0.49 |
| w1118;+/KK106180 (CG10417) | 16 | 94 | 23.50 ± 0.05 |
| w1118;timGal4/KK106180 | 14 | 14 | 24.75 ± 0.18 |
| w1118;KK106180/+;timGal4/+ | 15 | 73 | 24.64 ± 0.27 |
| w1118;KK106180/+;pdfGal4/+ | 16 | 13 | 23.25 ± 0.18 |
| w1118;+/KK106253 (Fcp1) | 16 | 100 | 23.53 ± 0.03 |
| w1118;timGal4/KK106253 | 11 | 100 | 24.36 ± 0.15 |
| w1118;KK106253/+;timGal4/+ | 4 | 100 | 23.75 ± 0.22 |
| w1118;KK106253/+;pdfGal4/+ | 14 | 87 | 23.47 ± 0.03 |
| w1118;+/KK107057 (P2PA-B') | 16 | 50 | 22.25 ± 0.34 |
| w1118;timGal4/KK107057 | 16 | 42 | 22.30 ± 0.34 |
| w1118;KK107057/+;timGal4/+ | 12 | 81 | 23.65 ± 0.14 |
| w1118;+/KK107386 (PpdD3) | 8 | 100 | 23.5 |
| w1118;timGal4/KK107386 | 12 | 100 | 24.04 ± 0.12 |
| w1118;KK107386/+;timGal4/+ | 11 | 100 | 23.68 ± 0.16 |
| w1118;KK107386/+;pdfGal4/+ | 15 | 100 | 23.57 ± 0.04 |
| w1118;+/KK107621 (CG4733) | 16 | 100 | 23.56 ± 0.04 |
| w1118;timGal4/KK107621 | 16 | 81 | 23.65 ± 0.09 |
| w1118;KK107621/+;timGal4/+ | 16 | 100 | 23.67 ± 0.06 |
| w1118;KK107621/+;pdfGal4/+ | 15 | 100 | 23.63 ± 0.10 |
| w1118;+/KK107770 (Pt-1-1C) | 15 | 100 | 23.43 ± 0.04 |
| w1118;timGal4/KK107770 | 16 | 100 | 23.44 ± 0.08 |
| w1118;KK107770/+;timGal4/+ | 16 | 94 | 23.63 ± 0.07 |
| w1118;KK107770/+;pdfGal4/+ | 15 | 93 | 23.43 ± 0.05 |
| w1118;+/KK107996 (Lar) | 16 | 100 | 23.53 ± 0.03 |
| w1118;timGal4/KK107996 | 12 | 25 | 24.17 ± 0.27 |
| w1118;KK107996/+;timGal4/+ | 13 | 0 | AR |
| w1118;KK107996/+;pdfGal4/+ | 13 | 0 | AR |
| w1118;+/KK107998 (ssh) | 16 | 100 | 23.5 |
| w1118;timGal4/KK107998 | 15 | 93 | 24.07 ± 0.10 |
| w1118;KK107998/+;timGal4/+ | 15 | 100 | 24.30 ± 0.15 |
| w1118;KK107998/+;pdfGal4/+ | 16 | 100 | 23.44 ± 0.04 |
| w1118;+/KK108071 (eya) | 16 | 100 | 23.5 |
| w1118;timGal4/KK108071 | 16 | 88 | 24.11 ± 0.13 |
| w1118;KK108071/+;timGal4/+ | 16 | 100 | 23.66 ± 0.09 |
| w1118;KK108071/+;pdfGal4/+ | 16 | 100 | 23.5 |
| w1118;+/KK108352 (Csw) | 15 | 100 | 23.60 ± 0.05 |
| w1118;timGal4/KK108352 | 16 | 100 | 24.41 ± 0.08 |
| w1118;KK108352/+;timGal4/+ | 16 | 94 | 24.23 ± 0.09 |
| w1118;KK108352/+;pdfGal4/+ | 15 | 87 | 23.62 ± 0.08 |
| w1118;+/KK108505 (Ptp99A) | 16 | 100 | 23.5 |
| w1118;timGal4/KK108505 | 13 | 92 | 23.71 ± 0.11 |
| w1118;KK108505/+;timGal4/+ | 16 | 100 | 23.56 ± 0.04 |
| w1118;KK108505/+;pdfGal4/+ | 16 | 100 | 23.75 ± 0.09 |
| w1118;+/KK108744 (CG10089) | 15 | 87 | 23.38 ± 0.10 |
| w1118;timGal4/KK108744 | 14 | 86 | 24.00 ± 0.12 |
| w1118;KK108744/+;timGal4/+ | 15 | 47 | 24.29 ± 0.14 |
| w1118;KK108744/+;pdfGal4/+ | 16 | 81 | 23.73 ± 0.14 |
| w1118;+/KK108802 (CG8509) | 14 | 100 | 23.54 ± 0.03 |
| w1118;timGal4/KK108802 | 14 | 93 | 24.00 ± 0.09 |
| w1118;KK108802/+;timGal4/+ | 16 | 81 | 24.15 ± 0.14 |
| w1118;KK108802/+;pdfGal4/+ | 15 | 93 | 23.79 ± 0.07 |

*Table 1, continued*
Table 2 Activity rhythms of strains used to validate candidate clock phosphatases

| Genotype                        | n  | % Rhythmic | Period Mean ± SEM |
|---------------------------------|----|------------|-------------------|
| w^{116+}/+;GD35171              | 43 | 100        | 23.73 ± 0.04      |
| w^{116+}timGal4/GD35171         | 16 | 94         | 24.43 ± 0.08      |
| w^{116+}GD35171/+;timGal4/+     | 11 | 91         | 24.50 ± 0.19      |
| w^{116+}GD35171/+;pdfGal4/+     | 15 | 93         | 23.82 ± 0.07      |
| w^{116+};UAS-mts/+              | 12 | 100        | 23.5             |
| w^{116+};timGal4/+;UAS-mts/+    | 8  | 0          | AR               |
| w^{116+};UAS-mts/pdfGal4        | 14 | 43         | 23.30 ± 0.18      |
| w^{116+}P[EP]P2A-298B/+          | 16 | 100        | 23.63 ± 0.05      |
| w^{116+}P[EP]P2A-298B/timGal4   | 15 | 100        | 23.90 ± 0.13      |
| w^{116+}P[R3]P2A-298B/C3426-3   | 16 | 88         | 23.57 ± 0.05      |
| w^{116+}P{WH}CG17746/0541       | 11 | 91         | 23.30 ± 0.08      |
| y^{1}w^{118+}P{EP}CG17746G4827  | 16 | 94         | 23.53 ± 0.06      |
| y^{1}w^{118+}P{BPac(HPy)}-Z/0426| 14 | 100        | 23.75 ± 0.13      |
| w^{116+};P{UAS-i-2.HA}G/+;P{UAS-Pp1-87B.HA}H/+ | 15 | 100 | 23.67 ± 0.11 |
| w^{116+};P{UAS-i-2.HA}G/timGal4;P{UAS-Pp1-87B.HA}H/ | 15 | 87 | 24.15 ± 0.15 |
| w^{116+};P{UAS-i-2.HA}G/+;P{UAS-Pp1-87B.HA}H/pdfGal4 | 9  | 100 | 24.22 ± 0.11 |
| y^{1}w^{172+}P{SUPor-P}CG1715K02655 | 12 | 75 | 23.50 ± 0.14 |
| w^{116+}UAS-Cep977/+            | 15 | 100        | 24.27 ± 0.14      |
| w^{116+}UAS-Cep977/timGal4      | 9  | 100        | 24.28 ± 0.08      |
| w^{116+}UAS-Cep977/+;pdfGal4/+  | 13 | 100        | 24.67 ± 0.54      |
| UAS-Cep977/Y                    | 14 | 93         | 23.38 ± 0.06      |
| UAS-Cep977/Y;timGal4/+         | 15 | 60         | 23.83 ± 0.11      |
| UAS-Cep977/Y;pdfGal4/+         | 13 | 85         | 23.59 ± 0.06      |
| w^{116+}UAS-Cep977/+;timGal4    | 16 | 100        | 23.5               |
| w^{116+}UAS-Cep977/+;PDFgal4    | 16 | 100        | 23.41 ± 0.10      |
| y^{1}w^{172+}P{SUPor-P}CG089899P6D6K02655 | 11 | 100 | 23.68 ± 0.12 |
| y^{1}w^{172+}P{M{IC}Ptptmem2c403011/Y | 15 | 100 | 23.73 ± 0.08 |
| w^{67c23}P{lacW}Ptptmem2c40302/Y | 16 | 63 | 24.65 ± 0.21 |
| w^{172+}P{Mae-UAS.6.11/P}Ptptmem2c403012/Y | 16 | 94 | 23.57 ± 0.07 |
| y^{1}w^{172+}P{Mae-UAS.6.11/P}Ptptmem2c403012/Y,timGal4/+ | 10 | 90 | 24.22 ± 0.19 |
| y^{1}w^{172+}P{Mae-UAS.6.11/P}Ptptmem2c403012/Y,;pdfGal4/+ | 15 | 100 | 23.80 ± 0.09 |
| w^{116+}P{BPac(WH)Ptptmem2c403012/Y | 13 | 100 | 23.62 ± 0.11 |
| w^{116+};Ptpt69D/+              | 8  | 88         | 26.57 ± 0.21      |
| w^{116+};Df(3)B8ex25            | 10 | 60         | 26.5               |
| w^{116+};Ptpt69D1               | 15 | 93         | 26.96 ± 0.14      |
| w^{116+};Ptpt69D1               | 16 | 50         | 27.19 ± 0.15      |
| w^{116+};Ptpt69D2               | 10 | 100        | 23.60 ± 0.06      |
| w^{116+};Ptpt69D2               | 16 | 100        | 23.53 ± 0.30      |
| w^{116+};UAS-Ptpt69D/+          | 9  | 89         | 24.19 ± 0.22      |
| w^{116+};UAS-Ptpt69D/pdfGal4    | 12 | 67         | 24.13 ± 0.19      |
| w^{116+};timGal4/+;UAS-Ptpt69D/+| 12 | 92         | 27.82 ± 0.28      |
| w^{116+};UAS-DNpt69D/+          | 15 | 100        | 23.47 ± 0.00      |
| w^{116+};UAS-DNpt69D/pdfGal4    | 8  | 63         | 27.20 ± 0.18      |
| w^{116+};timGal4/+;UAS-DNpt69D/+| 15 | 100        | 23.50 ± 0.05      |
| w^{116+};Ptpt69D/+              | 8  | 100        | 23.44 ± 0.06      |
| y^{1}w^{172+}P{SUPor-P}MK4P/+KG3420 | 13 | 100 | 24.23 ± 0.12 |
| w^{116+};P{GSV6}Pp1-x/96A<GS1179/+ | 16 | 100 | 23.59 ± 0.08 |
| w^{116+}timGal4/+;P{GSV6}Pp1-x/96A<GS1179/+ | 16 | 25 | 24.13 ± 0.21 |
| w^{116+};P{GSV6}Pp1-x/96A<GS1179/pdfGal4 | 9  | 67 | 23.67 ± 0.15 |
| w^{116+};Pp1-x/96A/+            | 16 | 94         | 23.77 ± 0.12      |
| w^{116+};UAS-Pp1-x/96A.HA/+     | 16 | 100        | 23.38 ± 0.05      |
| w^{116+}timGal4/+;UAS-Pp1-x/96A.HA/+ | 16 | 94 | 23.87 ± 0.11 |
| w^{116+}timGal4/+;UAS-Pp1-x/96A.HA/pdfGal4 | 16 | 94 | 23.33 ± 0.08 |
| w^{116+};Pp1-x/96A-CRISPmutant-1/+ | 17 | 82 | 23.52 ± 0.07 |
| w^{116+};Pp1-x/96A-CRISPmutant-2/+ | 13 | 92 | 23.58 ± 0.05 |
| w^{116+};Pp1-x/96A-CRISPmutant-3/+ | 9  | 100 | 23.44 ± 0.05 |
| w^{116+};UAS-CG10417/+          | 16 | 100        | 23.44 ± 0.04      |
| w^{116+};UAS-CG10417/timGal4    | 14 | 100        | 24.56 ± 0.03      |
| w^{116+};UAS-CG10417/+;pdfGal4/+ | 16 | 100        | 24.56 ± 0.03      |
| w^{116+};La1-2/+                | 14 | 93         | 23.54 ± 0.04      |
| Df(2L)Tr138.2/+                 | 14 | 86         | 23.71 ± 0.11      |

(continued)
CanA-14F is a cation binding, PPM-type phosphatase, part of the protein phosphatase 2C family that dephosphorylates proteins at serine and threonine residues. There are no known cellular functions described for this phosphatase. We tested the only available P element transposon insert for this gene, but activity rhythms in this strain were not altered (Table 2).

CG6380

CG6380 is related to Protein phosphatase inhibitor 2, IPP-2. Based on association of InterPro records with GO terms, it functions to regulate signal transduction and phosphoprotein phosphatase pathways. Although the phenotype of this RNAi knockdown was strongly arrhythmic, no other genetic reagents were available to confirm the RNAi phenotype.

CG17746

CG17746 is a member of the protein phosphatase 2C family that has cation binding domains and dephosphorylates proteins at serine and threonine residues. We tested additional P element transposon inserts for activity rhythms; however, these reagents did not validate the RNAi phenotype (Table 2).

Gbs-70E

Gbs-70E is a protein phosphatase 1 regulatory subunit with a carbohydrate binding type-21 (CBM21) domain. It functions in regulation of glycogen metabolic process (Kerkes et al. 2014). However, there are no additional genetic reagents available for this gene to validate the arrhythmic RNAi phenotype.

Ppm1

Ppm1 is a protein phosphatase, Mg\(^{2+}\)/Mn\(^{2+}\)-dependent (PPM type), member of the protein phosphatase 2C family that dephosphorylates proteins at serine and threonine residues. There are no known cellular functions described for this phosphatase, and no additional genetic reagents available to validate the RNAi phenotype.

Table 2, continued

| Genotype                                      | n  | % Rhythmic | Period Mean ± SEM |
|-----------------------------------------------|----|------------|-------------------|
| Df(2L)E55,ro1;hook1LarE55pr1/+                | 16 | 88         | 24.32 ± 0.08      |
| w\(^1118\)+/UAS-Lar/+                        | 14 | 100        | 23.57 ± 0.04      |
| w\(^1118\)timGal4/+;UAS-Lar/+                | 15 | 93         | 24.06 ± 0.07      |
| w\(^1118\)UAS-Lar/pdfGal4                    | 16 | 75         | 24.17 ± 0.07      |
| w\(^1118\)Lar\(Df(2L)E55\)Lar13.2;+          | 14 | 0          | AR                |
| w\(^1118\)UAS-CanA-14Fmyc/+                 | 16 | 75         | 24.50 ± 0.20      |
| w\(^1118\)UAS-CanA-14Fmyc/timGal4           | 16 | 63         | 23.60 ± 0.10      |
| w\(^1118\)UAS-CanA-14Fmyc/+;pdfGal4/+       | 15 | 100        | 24.03 ± 0.10      |
| w\(^1118\)UAS-CanA-14Fact-myc/+             | 9  | 100        | 23.94 ± 0.11      |
| w\(^1118\)timGal4/+;UAS-CanA-14Fact-myc/+   | 15 | 0          | AR                |
| w\(^1118\)UAS-CanA-14Fact-myc/pdfGal4       | 16 | 94         | 25.03 ± 0.15      |
| CanA-14F-KO/Y                                 | 15 | 13         | 25.0              |
| CanA-14F-KO/Y                                 | 16 | 94         | 23.67 ± 0.08      |
| w\(^1118\)GD26216/+                         | 12 | 100        | 23.63 ± 0.13      |
| w\(^1118\)GD26216/timGal4                   | 15 | 100        | 24.90 ± 0.13      |
| w\(^1118\)GD26216/+;pdfGal4/+               | 13 | 85         | 23.68 ± 0.12      |
| w\(^1118\)GD26216/+;pdfGal4/+               | 15 | 100        | 24.30 ± 0.18      |

Adult males were entrained in LD for 3 d and transferred to DD for at least 7 d. Analysis of activity rhythms in DD and fly genotypes are as described in Methods and Materials; n, number of animals tested; % Rhythmic, percentage of rhythmic animals; Period ± SEM, rhythm period in hours ± SEM. Bold “% Rhythmic” values signify < 50% rhythmicity and bold “Period ± SEM” values are significantly different (p ≤ 0.05) from their respective UAS-RNAi/+ control flies. AR, arrhythmic; LD, 1h:2:12h light-dark cycle; DD, complete darkness; RNAi, RNA interference.

Pp2A-29B

Pp2A-29B is a protein phosphatase type 2A regulatory subunit. It functions in many cellular processes including chromosome segregation, centriole assembly, and phagocytosis (Stroschein-Stevenson et al. 2006; Dobbelaere et al. 2008). We tested additional P element transposon inserts to independently validate the RNAi phenotype, but activity rhythms were not altered (Table 2).

CG7115

CG7115 is a cation binding, PPM-type phosphatase, part of the protein phosphatase 2C family that dephosphorylates proteins at serine and threonine residues. It functions in many cellular processes including cell adhesion and regulation of cell shape (Sopko et al. 2014). We tested the available P element insert to independently validate the long period and/or arrhythmicity associated with RNAi, but did not observe any alteration in activity rhythms (Table 2).

Cep97

Cep97 is a protein phosphatase type 1 regulator with a characteristic leucine-rich repeat domain. It is known to function in centriole replication (Dobbelaere et al. 2008). Behavioral analysis of Cep97 overexpression using clock cell-specific Gal4 drivers did not alter activity rhythms (Table 2).

PpD6

PpD6 is a protein phosphatase that dephosphorylates proteins at serine and threonine residues. There are no known cellular functions described for this phosphatase. We tested the only available P element transposon insert for this gene, but activity rhythms in this strain were not altered (Table 2).

Ptpmeg2

Ptpmeg2, also known as lethal-1-G0232, is a nonmembrane spanning protein tyrosine phosphatase. It functions in many cellular processes including phagocytosis, neurogenesis, and cell migration (Stroschein-Stevenson et al. 2006; Chen et al. 2012). We tested the available Ptpmeg2 P element transposon insert lines and clock cell-specific
_Ptpmeg2_ overexpression flies, but none of these genetic reagents altered activity rhythms (Table 2).

**Ptp69D**

Ptp69D is a protein tyrosine phosphatase with characteristic Fibronectin type III, Immunoglobulin subtype, and tyrosine-specific protein phosphatase domains. It is a transmembrane RPTP that dephosphorylates protein’s tyrosine residues. It functions in many cellular processes including dendrite morphogenesis, axon guidance, and fasciculation-deascellulation of neuron axons (Desai et al. 1996; Desai and Purdy 2003). Interestingly, when we tested additional _Ptp69D_ reagents including loss-of-function mutants and a dominant negative UAS strain, many showed an even longer period phenotype compared to the RNAi while some showed no phenotype (Table 2). When _Ptp69D_ mutants that showed a long period were isogenized to the wild-type (w^{1118}) reference strain or paired with a wild-type X chromosome (data not shown), the long period phenotype was lost (Table 2). Upon further analysis (see Methods and Materials), we confirmed that the long period (~26.5 hr) phenotype was due to _per^A118_, a naturally occurring _per_ mutant (Hamblen et al. 1998).

**MKP-4**

MKP-4 is a dual specificity protein phosphatase that dephosphorylates proteins at tyrosine, serine, and threonine residues. Its known cellular function is in negative regulation of JUN kinase activity (Sun et al. 2008). We tested an additional _P_ element transposon insert to independently validate the RNAi phenotype but activity rhythms were not altered (Table 2).

**Pp1α-96A**

Pp1α-96A is a protein phosphatase, part of the PP1 subfamily, which dephosphorylates proteins at serine and threonine residues. It functions in many cellular processes including positive regulation of the canonical Wnt signaling pathway and innate immune response (Schertel et al. 2013). Importantly, the PP1 subfamily is proposed to regulate clock function in _Drosophila_ by maintaining rhythms in PER-TIM abundance (Fang et al. 2007). None of the available _Pp1α-96A_ _P_ element inserts or clock cell-specific _Pp1α-96A_ overexpression altered activity rhythms (Table 2). Given the involvement of PP1 in _Drosophila_ circadian clocks, we wanted to test loss-of-function mutants. Therefore, we used CRISPR technology to generate three _Pp1α-96A_ deletion mutants (See Methods and Materials). However, none of these mutants were homozygous viable as adults, and heterozygotes did not display altered activity rhythms (Table 2).

**CG10417**

CG10417 is a PPM-type phosphatase, a member of the protein phosphatase 2C family that dephosphorylates proteins at the serine and threonine residues (Sopko et al. 2014). No other genetic reagents were available for this gene. However, given its association with the PP2 family known to be involved in _Drosophila_ circadian clock function (Sathyanarayanan et al. 2004), we generated a UAS-CG10417 strain to overexpress this phosphatase in clock cells. Interestingly, CG10417 overexpression in clock cells resulted in a long period phenotype (Table 2). This phenotype is similar to that of the RNAi knockdown, demonstrating that increasing or decreasing the dephosphorylation of CG10417 targets slows the pace of the clock. This is not unprecedented, since RNAi knockdown and overexpression of the PP2A subunit WDB also leads to long period rhythms (Sathyanarayanan et al. 2004; Andreazza et al. 2015). Since CG10417 RNAi knockdown and overexpression both lengthened period, we investigated whether either manipulation altered CLK, PER, or TIM protein levels or phosphorylation, but no obvious change in either parameter was detected (data not shown).

**LAR**

LAR is a transmembrane RPTP bearing Fibronectin type III, Immunoglobulin-like, and tyrosine-specific protein phosphatase domains. It is a transmembrane receptor protein tyrosine phosphatase that dephosphorylates proteins tyrosine residues (Streuli et al. 1989). It functions in many cellular processes including cell adhesion, axon guidance, and regulation of nervous system development (Krueger et al. 1996, 2003; Kiger et al. 2003). Although multiple loss-of-function mutants were available for this phosphatase, none were homozygous viable. However, one heterozygous combination of _Lar_ loss-of-function alleles survived and phenocopied the arrhythmicity seen in _Lar_ RNAi knockdown flies (Table 2). Further analysis showed that _Lar_ is required for the development of circadian pacemaker neuron processes required for activity rhythms during constant darkness but not light:dark cycles (Agrawal and Hardin 2016).

**Pp1-Y2**

Pp1-Y2 is a protein phosphatase that dephosphorylates proteins at the serine and threonine residues. The RNAi shows a strong arrhythmic and/or long period phenotype, but no additional genetic reagents were available to validate the RNAi phenotype.

**CanA-14F**

CanA-14F is a protein phosphatase that dephosphorylates proteins at serine and threonine residues. It functions in many cellular processes including positive regulation of nuclear factor of activated T cells (NFAT) protein import into nucleus (Shibasaki et al. 1996) and sleep (Nakai et al. 2011). A _CanA-14F_ knockout (KO) and a constitutively active _CanA-14F_ form (expressed via Gal4/UAS) were generated previously (Nakai et al. 2011), and showed strong arrhythmic and long period activity phenotypes, respectively (Table 2). However, the arrhythmicity associated with _CanA-14F_ KOs was lost when this allele was isogenized to a wild-type (w^{1118}) reference strain (Table 2).

**CG3530**

CG3530 is a protein phosphatase that dephosphorylates proteins at the tyrosine residues. Its known cellular function is in the mitotic cell cycle (Chen et al. 2007). Behavioral analysis of an additional RNAi line that targeted another region of the mRNA did not validate the initial screen phenotype (Table 2).

**DISCUSSION**

An in vivo screen of 86 RNAi lines, representing the majority of annotated _Drosophila_ phosphatases/regulators, for altered activity rhythms was carried out. The screen identified a total of 19 candidate genes (Table 1) that altered clock function upon RNAi knockdown in _Drosophila_ clock cells. Further genetic validation of one candidate showed that the RPTP _Lar_ is required for the development of axonal projections from circadian pacemaker neurons that support rhythmic activity in constant darkness but not during light:dark cycles (Agrawal and Hardin 2016).

As expected, a majority of these candidates were not validated upon further analysis of independent genetic reagents (Table 2). However, these reagents consisted of additional _P_ element inserts, where the _P_ element insertion site may not interfere with gene function, or strains...
that could be used for overexpression, which also may not impact the function of a protein that is already at saturating levels. Therefore, a lack of validation with P element inserts and overexpression for these candidate clock phosphatases does not eliminate them from the list of viable candidates. However, for two candidate phosphatases, Ptp69D and CanA14Ff, loss-of-function mutants following isogenization did not alter activity rhythms (Table 2), therefore these can be eliminated from the list of viable candidates.

Previous studies show that PP1 and PP2a both function within the Drosophila clock (Sathyanarayanan et al. 2004; Fang et al. 2007; Andreazza et al. 2015). PP1 is comprised of a catalytic subunit that engages with one of dozens of regulatory subunits to select substrates and control enzymatic activity (Petti et al. 2011). PP1 function in the Drosophila clock was assessed previously by overexpressing the nuclear inhibitor of PP1 (NIPP1), which reduced TIM levels and lengthened circadian period, indicating that PP1 dephosphorylates and stabilizes TIM to maintain circadian period (Fang et al. 2011). Since these circadian phenotypes were produced by generically inhibiting PP1, the clock-relevant catalytic and regulatory subunits involved were not identified. Five PP1 catalytic subunit genes (flw, Pp1-13C, Pp1-87B, Pp1α-96A, and Pp1-Y2) and eight PP1 regulator genes (sds22, NIPP1, 1-2, Pp1-13C, Pp1-87B, Pp1α-96A, and Pp1-Y2) were tested in our screen, and Pp1α-96A, Pp1-Y2, Gbs-70E, I-2, and Cep97 showed aberrant circadian phenotypes (Table 1). Further analysis of Pp1α-96A showed that none of the available Pp1α-96A P element inserts or overexpression of Pp1α-96A in clock cells altered activity rhythms (Table 2). Thus, we used the CRISPR/Cas9 system to generate three Pp1α-96A deletion mutants expected to disrupt Pp1α-96A protein expression and/or

| Candidate | RNAi Phenotype | Genetic Reagents Tested | Validation | Spatial Expressiona | Clock-Related Expression |
|-----------|----------------|-------------------------|------------|-----------------|------------------------|
| mtsb      | Long           | Additional RNAi, overexpression | Yes | Brain, eye, tubule, carcass, ovary, heart, spermatheca, gut, fat body, head | CLK target and cyclingc |
| Pp2A-29Bb | Long           | P element inserts       | No         | Head, brain, tubule, ovary, testis, fat body, gut, salivary gland | CLK target and noncyclingc; cycling mRNA in large PDF neuronsd |
| IPP-2     | AR             | No reagents             | —          | Tests           | CLK target and cyclingc; cycling mRNA in large PDF neuronsd |
| CG17746   | AR             | P element inserts       | No         | Tubule, ovary   | CLK target and noncyclingc; cycling mRNA in large PDF neuronsd |
| Gbs-70E   | Long           | No reagents             | —          | Head, carcass, ovary, heart, fat body, eye, crop, salivary gland, spermatheca | CLK target and cyclingc; cycling mRNA in large PDF neuronsd |
| Ppm1      | AR             | No reagents             | —          | Testis         | Cycling mRNA in large PDF neuronsd |
| I-2       | AR             | P element insert, overexpression | No         | Head, tubule, carcass, ovary, testis, spermatheca, ganglion | mRNA enriched in s-LN,sdf |
| CG7115    | Long           | P element insert        | No         | Carcass, ovary  | mRNA enriched in s-LN,sdf |
| Cep97     | AR             | Overexpression strains  | No         | Testis         | mRNA enriched in s-LN,sdf |
| PpD6      | Long           | P element insert        | No         | Head           | mRNA enriched in s-LN,sdf |
| Ptpmeg2   | Long           | Loss-of-function mutants, Dominant negative strain | Noe | Ovary | Cycling mRNA in large PDF neuronsd |
| Ptp69D    | Long           | Loss-of-function mutants, overexpression | —          | —              | Cycling mRNA in large PDF neuronsd |
| MKP-4     | Long           | P element insert        | No         | —              | Cycling mRNA in large PDF neuronsd |
| Pp1a-96Aa | Long           | P element inserts, CRISPR heterozygous mutants | —          | Head, carcass, ovary, testis, crop, fat body, gut, salivary gland, spermatheca, accessory gland | mRNA enriched in s-LN,sdf |
| CG10417   | Long           | Overexpression          | Yes        | Carcass, ovary  | mRNA enriched in s-LN,sdf |
| Lar       | AR             | Deficiency over point mutant heterozygote | Yes | — | mRNA enriched in s-LN,sdf |
| Pp1-Y2    | Long           | No reagents             | —          | Head, carcass, ovary | mRNA enriched in s-LN,sdf |
| CanA-14Ff | AR             | Knockout, expression of constitutively active form | No | Brain, head, ganglion | mRNA enriched in s-LN,sdf |

Bold denotes the remaining candidate clock protein phosphatases. RNAi, RNA interference; CLK, CLOCK; AR, arrhythmic; mRNA, messenger RNA; PDF, pigment dispersing factor; CRISPR, clustered regularly interspaced short palindromic repeats.

aHigh/very high expression level in adult fly tissues based on Celniker et al. (2009) and Chintapalli et al. (2007).
bClock-related based on Sathyanarayanan et al. (2004).
cDirect CLK binding target and cycling or noncycling mRNA expression based on Abuzui et al. (2011).
dDifferential mRNA expression in pacemaker neurons based on Kula-Eversole et al. (2010).
eAll genetic reagents used to verify the RNAi phenotype that produced long period rhythms were due to peGAL4H.
fClock-related based on Fang et al. (2007).
gSleep-related based on previously analyzed for circadian phenotype Nakai et al. (2011).
hKnockout allele had a long period rhythm which was lost upon isogenization, but constitutively active form produces a long period rhythm upon overexpression.
function (see Methods and Materials; Figure 1). However, none of these Pp1α-96A deletions were homozygous viable as adults, and heterozygotes did not display altered activity rhythms (Table 2). Further characterization of Pp1α-96A function in the clock will benefit from targeted loss of Pp1α-96A in clock cells. The Pp1-Y2 catalytic subunit and the Gbs-70E and I-2 regulators could not be tested further due to lack of genetic reagents and Cep97 overexpression did not alter circadian rhythms (Table 2), but each of these genes remain as viable candidate clock protein phosphatases until additional loss-of-function reagents are available to test. Although inhibiting PPI via NIPP1 overexpression lengthened circadian period (Sathyanarayanan et al. 2004), RNAi knockdown of NIPP1 did not alter activity rhythms (Table 1). These results suggest that NIPP1 RNAi is either ineffective, NIPP1 is not involved in suppressing PPI activity in clock cells, or increased PPI activity does not disrupt the circadian clock.

The Pp2α holoenzyme contains a structural subunit Pp2A-29B, a catalytic subunit MTS, and regulatory subunits TWS, WDB, Pp2A-B, CG4733, and Connector of kinase to AP-1 (CKA) (Andreazza et al. 2015). Previous work shows that mts, tws, and wdb overexpression, hypomorphic mutants, and/or RNAi knockdown, alter the levels and localization of PER and disrupt activity rhythms (Sathyanarayanan et al. 2004; Andreazza et al. 2015), whereas a hypomorphic cka mutant and cka RNAi knockdown reduces CLK activity and lengthens period (Andreazza et al. 2015). We tested RNAi knockdowns of all Pp2α components except cka, but only mts and Pp2A-29B produced circadian phenotypes (Table 1). Our inability to generate circadian phenotypes for tms and wdb may be due to inefficient RNAi knockdown, since expressing a different wdb RNAi along with Dicer2 (to enhance RNAi potency) produced a long period phenotype (Andreazza et al. 2015). We did not test cka because it is not annotated as a Pp2α subunit.

Genetic reagents for affecting a loss- or gain-function were not available for four additional candidate phosphatases, which can be characterized further when such reagents are available. For example, P element inserts are now available for Gbs-70E and CG3530. Another method that can be used to follow up on these candidates is to generate mutants using CRISPR technology. Since loss of most phosphatases is lethal, it is likely that generating conditional null mutants via CRISPR will provide the best opportunity to assess loss-of-function phenotypes in adults (Gratz et al. 2014).

Overall, we identified 19 protein phosphatases that may function within the Drosophila circadian clock (Table 3). Lar and mts functions have now been characterized, and they are shown to be important for dephosphorylation events that regulate fly clock development or function (Andreazza et al. 2015; Agrawal and Hardin 2016). Pp69D and CanA-14F loss-of-function mutants do not alter activity rhythms upon isogenization, thus, identification of these genes may have been due to off-target effects of RNAi. No loss-of-function mutants are currently available for the remaining 15 candidates, and four of these candidates could not be validated due to the lack of independent genetic reagents (Table 3). We analyzed previous mRNA expression and CLK binding data that may further support a possible role for these candidates in the clock (Chintapalli et al. 2007; Celniker et al. 2009; Kula-Eversole et al. 2010; Abruzzi et al. 2011). Of the remaining 15 candidates, one is a rhythmic and two are nonrhythmic CLK binding targets, three are enriched in small LN5a, five are cycling in large LN5a, and five are highly or very highly expressed in pacemaker neuron-containing tissues (i.e., brain and head) that could account for the altered activity rhythms due to candidate gene RNAi knockdown (Table 3). The other nine candidates were not detected as highly or very highly expressed transcripts in brains and heads, but moderate levels of transcripts were found in heads or brains for five of these candidates, leaving four candidates with low or no expression in the head (Table 3). One candidate (i.e., Ppm1) that was detected in ILN, pacemaker neurons showed low or no expression in the head (Table 3), consistent with there being only eight ILNvs per head (Helfrich-Forster 2014). The strong behavioral phenotypes displayed by RNAi knockdowns of these 15 candidates suggest they are viable candidate clock phosphatases, a possibility that is further supported by data showing that nine of these candidates are either CLK binding targets or produce cycling mRNAs in clock neurons (Table 3). Additional characterization of the remaining candidates may reveal novel features of the circadian timekeeping mechanism that are conserved in all animals including humans.

ACKNOWLEDGMENTS
This work was supported by National Institutes of Health grant NS052894.

LITERATURE CITED
Abruzzi, K. C., J. Rodriguez, J. S. Menet, J. Desrochers, A. Zadina et al., 2011 Drosophila CLOCK target gene characterization: implications for circadian tissue-specific gene expression. Genes Dev. 25: 2374–2386.
Agrawal, P., and P. E. Hardin, 2016 The Drosophila receptor protein tyrosine phosphatase LAR is required for development of circadian pacemaker neuron processes that support rhythmic activity in constant darkness but not during light/dark cycles. J. Neurosci. 36: 3860–3870.
Akten, B., E. Jauch, G. K. Genova, E. Y. Kim, I. Edery et al., 2003 A role for CK2 in the Drosophila circadian oscillator. Nat. Neurosci. 6: 251–257.
Akten, B., M. M. Tangedi, E. Jauch, M. A. Roberts, F. Ng et al., 2009 Ribosomal s6 kinase cooperates with casin kinase 2 to modulate the Drosophila circadian molecular oscillator. J. Neurosci. 29: 466–475.
Andreazza, S., S. Bouleau, B. Martin, A. Lamouroux, P. Ponien et al., 2015 Daytime CLOCK dephosphorylation is controlled by STRIPAK complexes in Drosophila. Cell Reports 11: 1266–1279.
Celniker, S. E., L. A. Dillon, M. B. Gerstein, K. C. Gunsalus, S. Henikoff et al., 2004 Unlocking the secrets of the genome. Nature 459: 927–930.
Chen, D. Y., M. Y. Li, S. Y. Wu, Y. L. Lin, S. P. Tsai et al., 2012 The Brol-domain-containing protein Myopic/HDFPT coordinates with Rab4 to regulate cell adhesion and migration. J. Cell Sci. 125: 4841–4852.
Chen, F., V. Archambault, A. Kap, P. Lio, P. P. D’Avino et al., 2007 Multiple plant phosphatases are required for miosis in Drosophila. Curr. Biol. 17: 293–303.
Chintapalli, V. R., J. Wang, and J. A. Dow, 2007 Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat. Genet. 39: 715–720.
Chiu, J. C., T. Vanselow, A. Kramer, and I. Edery, 2008 The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. Genes Dev. 22: 1758–1772.
Chiu, J. C., H. W. Ko, and I. Edery, 2011 NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. Cell 145: 357–370.
Desai, C., and J. Purdy, 2003 The neural receptor protein tyrosine phosphatase DPTP69D is required during periods of axon outgrowth in Drosophila. Genes Dev. 164: 575–588.
Desai, C., I. J. G. Gindhart, Jr, L. S. Goldstein, and K. Zinn, 1996 Receptor tyrosine phosphatases are required for motor axon guidance in the Drosophila embryo. Cell 84: 599–609.
Dietzl, G., D. Chen, F. Schnorrer, K. C. Su, Y. Barinova et al., 2007 A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448: 151–156.
Dobbelare, J., F. Josue, S. Suijkerbuijk, B. Baum, N. Tapon et al., 2008 A genome-wide RNAi screen to dissect centriole duplication and centro-some maturation in Drosophila. PLoS Biol. 6: e224.
Fang, Y., S. Sathyanarayanan, and A. Sehgal, 2007 Post-translational regulation of the Drosophila circadian clock requires protein phosphatase 1 (PP1). Genes Dev. 21: 1506–1518.
Gratz, S. J., F. P. Ukken, C. D. Rubinstein, G. Thiede, L. K. Donohue et al., 2014 Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in Drosophila. Genetics 196: 961–971.

Gratz, S. J., C. D. Rubinstein, M. M. Harrison, J. Wildonger, and K. M. O’Connor-Giles, 2015 CRISPR-Cas9 genome editing in Drosophila. Curr. Protoc. Mol. Biol. 111: 31.2.1–31.2.20.

Hamblen, M. J., N. E. White, P. T. Emery, K. Kaiser, and J. C. Hall, 1998 Molecular and behavioral analysis of four period mutants in Drosophila melanogaster encompassing extreme short, novel long, and unorthodox rhythmic types. Genetics 149: 165–178.

Helfrich-Forster, C., 2014 From neurogenetic studies in the fly brain to a concept in circadian biology. J. Neurogenet. 28: 329–347.

Janssens, V., and J. Goris, 2001 Protein phosphatase 2A: a highly regulated protein phosphatase activities regulate phosphorylation and stability of Drosophila CLOCK protein. Proc. Natl. Acad. Sci. USA 103: 6178–6183.

Kloss, B., J. L. Price, L. Saez, J. Blau, A. Rothen et al., 2013 CRISPR-Cas9 genome editing in Drosophila. Curr. Protoc. Mol. Biol. 111: 31.2.1–31.2.20.

Krupp, N. X., D. Van Vactor, H. I. Wan, W. M. Gelbart, C. S. Goodman et al., 2014 Functional analysis of the glycogen binding subunit CG9238/Gbs-70E of protein phosphatase 1 in Drosophila melanogaster. Insect Biochem. Mol. Biol. 49: 70–79.

Kiger, A. A., B. Baum, S. Jones, M. R. Jones, A. Coulson et al., 2003 A functional genomic analysis of cell morphology using RNA interference. J. Biol. 2: 27.

Kim, E. Y., and I. Edery, 2006 Balance between DBT/CKI kinase and protein phosphatase activities regulate phosphorylation and stability of Drosophila CLOCK protein. Proc. Natl. Acad. Sci. USA 103: 6178–6183.

Kloss, B., J. L. Price, L. Saez, J. Blau, A. Rothenfluh et al., 1998 The Drosophila clock gene double-time encodes a protein closely related to human casein kinase 1ε. Cell 94: 97–107.

Kloss, B., A. Rothenfluh, M. W. Young, and L. Saez, 2001 Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the Drosophila clock. Neuron 30: 699–706.

Krueger, N. X., D. Van Vactor, H. I. Wan, W. M. Gelbart, C. S. Goodman et al., 1996 The transmembrane tyrosine phosphatase Dlar controls motor axon guidance in Drosophila. Cell 84: 611–622.

Krueger, N. X., R. S. Reddy, K. Johnson, J. Bateman, N. Kaufmann et al., 2003 Functions of the ectodomain and cytoplasmic tyrosine phosphatase domains of receptor protein tyrosine phosphatase Dlar in vivo. Mol. Cell. Biol. 23: 6909–6921.

Kula-Eversole, E., E. Nagoshi, Y. Shang, J. Rodriguez, R. Allada et al., 2010 Surprising gene expression patterns within and between PDF-containing circadian neurons in Drosophila. Proc. Natl. Acad. Sci. USA 107: 13497–13502.

Lin, J. M., V. L. Kilman, K. Keegan, B. Paddock, M. Emery-Le et al., 2002 A role for casein kinase 2alpha in the Drosophila circadian clock. Nature 420: 816–820.

Martinek, S., S. Inonog, A. S. Manoukian, and M. W. Young, 2001 A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. Cell 105: 769–779.

Nakai, Y., J. Hortuchi, M. Tsuda, S. Takeo, S. Akahori et al., 2011 Calcineurin and its regulator adaptor S/DSCR1 are essential for sleep in Drosophila. J. Neurosci. 31: 12759–12766.

Peti, W., A. C. Nairn, and R. Page, 2013 Structural basis for protein phosphatase 1 regulation and specificity. FEBS J. 280: 596–611.

Pfeifferberger, C., B. C. Lear, K. P. Keegan, and R. Allada, 2010 Sleep and circadian behavior monitoring in Drosophila, pp. 483–504 in Drosophila Neurobiology: A Laboratory Manual, edited by Zhang, B., M. R. Freeman, and S. Waddell. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Price, J. L., J. Blau, A. Rothenfluh, M. Abodeely, B. Kloss et al., 1998 Double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell 94: 83–95.

Ren, X., J. Sun, B. E. Housden, Y. Hu, C. Roesel et al., 2013 Optimized gene editing technology for Drosophila melanogaster using germ line-specific Cas9. Proc. Natl. Acad. Sci. USA 110: 19012–19017.

Sam, F., C. Smet-Noccia, M. Khan, I. Landrieu, G. Lippens et al., 2011 Molecular basis for an ancient partnership between prolyl isomerase Pin1 and phosphatase inhibitor-2. Biochemistry 50: 6567–6578.

Sathyarayanan, S., X. Zheng, R. Xiao, and A. Sehgal, 2004 Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. Cell 116: 603–615.

Schertel, C., D. Huang, M. Bjorklund, J. Bischof, D. Yin et al., 2013 Systematic screening of a Drosophila ORF library in vivo uncovers Wnt/Wg pathway components. Dev. Cell 25: 207–219.

Shibasaki, F., E. R. Price, D. Milan, and F. McKeon, 1996 Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature 382: 370–373.

Sopko, R., M. Foos, A. Vinayagam, B. Zhai, R. Binari et al., 2014 Combining genetic perturbations and proteomics to examine kinase-phosphatase networks in Drosophila embryos. Dev. Cell 31: 114–127.

Streuli, M., N. X. Krueger, A. Y. Tsai, and H. Saito, 1989 A family of receptor-linked protein tyrosine phosphatases in humans and Drosophila. Proc. Natl. Acad. Sci. USA 86: 8698–8702.

Stroscio-Stevenson, S. L., E. Foley, P. H. O’Farrell, and A. D. Johnson, 2006 Identification of Drosophila gene products required for phagecysis of Candida albicans. PLoS Biol. 4: e4.

Sun, L., M. C. Yu, L. Kong, Z. H. Zhuang, J. H. Hu et al., 2008 Molecular identification and functional characterization of a Drosophila dual-specificity phosphatase DMKP-4 which is involved in PGN-induced activation of the JNK pathway. Cell. Signal. 20: 1329–1337.

Szabo, A., C. Papin, D. Zorn, P. Ponien, F. Weber et al., 2013 The CK2 kinase stabilizes CLOCK and represses its activity in the Drosophila circadian oscillator. PLoS Biol. 11: e1001645.

Yu, W., H. Zheng, J. H. Hou, B. Dauwalder, and P. E. Hardin, 2006 PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev. 20: 723–733.

Communicating editor: J. C. Dunlap