Supporting Information

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SI Materials and Methods

Molecular Constructs. P*sur-5::luc* was constructed by ligation of a 1,673-bp PCR-amplified *P. pyralis* luciferase ORF from the pGL3-Basic Vector (Promega) between the KpnI and EcoRI sites of pPD158.87 (Addgene), replacing the *gfp* coding sequence (CDS). The luciferase ORF was amplified using the primers forward, 5′-CATCGGGGTACCATGGAAGACGCCAAAAA-C-3′ and reverse, 5′- GGAATTCTACACGGCGATCTTTCC-GCCCTTC-3′.

P*sur-5::luc* was constructed by ligation of a 1,673-bp PCR-amplified *P. pyralis* luciferase ORF from the pGL3-Basic Vector (Promega) between the KpnI and EcoRI sites of pPD158.87 (Addgene), replacing the *gfp* CDS. The luciferase ORF was amplified using the primers forward, 5′-CATCGGGGTAC-CATGGAAGACGCCAAAAAC-3′ and reverse, 5′- GGAAT-TCTACACGGCGATCTTTCCGCCCTTC-3′.

P*sur-5::AI::luc* was constructed by ligation of a 1,673-bp PCR-amplified *P. pyralis* luciferase ORF from the pGL3-Basic Vector (Promega) between the KpnI and EcoRI sites of pPD95.75 (Addgene), replacing the *gfp* CDS. Then, the *AI::luc* was cut with BamHI and EcoRI and cloned into pPD158.87 (Addgene) under the *sur-5* promoter. The luciferase ORF was amplified using the primers used for the P*sur-5::luc*.

For the P*sur-5::luc::icr::gfp*, we first synthetized the intercistronic region (icr) sequence described by Lee et al. (58) in GenScript with KpnI and BglII sites in the 5′ and XhoI and EcoRI in the 3′ extreme (pGEM-T Easy Vector; Promega). Then we amplified the luciferase ORF from the pGL3-Basic Vector (Promega) and ligated the 1,679-bp fragment between the KpnI and BglII sites of pGEMT*::icr* with primers forward, 5′-CATCGGGGTACCATGGAAGACGCCAAAAAC-3′ and reverse, 5′- CATGAGAAGATCTTTTACACGGCGATCTTT-CCGC-3′. Next, we amplified a 900-bp fragment of the *gfp* ORF from the pPD158.87 (Addgene) and cloned it into the pGEMT*::luc::icr* between SalI (XhoI compatible end) and EcoRI sites using the primers forward, 5′-CAACGCGTCGACATGAG-TAAAGGAGAAGAAC-3′ and reverse, 5′- CAGCGCGGAT-CCGAATTCCTATTTGTATAGTTCATCC-3′. Finally, we cut and ligated the *luc::icr::gfp* construct between KpnI and EcoRI with pPD158.87 (Addgene) under the *sur-5* promoter.

Bioluminescence Recordings and Treatments.

Assays of FR luminescence (dish plate setup). For experiments in the FR conditions, eight populations of 100 L4-stage nematodes (each population was considered an independent biological replicate) were selected as described previously, washed twice with M9 buffer to remove all traces of bacteria, and transferred to a 35-mm plate dish (Greiner CELLSTAR) with 1 mL of the luminescence medium; then the dish was sealed. Nematodes were placed in the incubator at ZT2 and were left there under entrainment conditions for 2 d. Then, they were transferred at ZT12 to an AB-2550 Kronos Dio luminometer (ATTO) in which luminescence was monitored for 7 d in DD/WW (20 °C, which is the minimal temperature allowed in this luminometer). The signal was integrated for 1 min, and readings were taken every 10 min.

For single-nematode measurements, P*sur-5::luc::gfp* nematodes at the L4 stage were selected as before but starting at ZT10, at a constant temperature of 18 °C, washed, and then transferred directly to the liquid luminescence medium. A small, square piece of a transparent 96-well plate containing three × three wells was inserted inside a 35-mm dish plate to reduce the total volume and limit the nematode movement to the center of the plate. One nematode was placed in the center well with 200 μL of the luminescence medium, and water was placed in the other wells to avoid evaporation. The plate was sealed with a Microseal 'B' Adhesive Seal (Bio-Rad) to avoid evaporation and contamination, and the seal over each well was perforated twice to avoid condensation. At ZT12, the plates were transferred to an AB-2550 Kronos Dio luminometer, and luminescence was monitored in DD/WW (20 °C) for 7 d. Single-nematode recordings were taken every 37 min with an integration time of 4 min.

For treatment with the CKIδ/ε inhibitor PF-670462 (Abcam), 5 or 10 μM of drug dissolved in water was added to the medium just before the start of the luminescence recording at ZT12, and the same volume of vehicle was used as control. Single-nematode recordings were taken every 37 min with an integration time of 4 min.

Assay CKI ε/δ inhibitor toxicity. The toxicity of PF-670467 was tested by recording the global motility of populations of wild-type adult nematodes (N2) over time under different concentrations of PF-670462, using an infrared tracking device (WMicroTracker, PhylumTech) previously used for toxicity screenings (59). We tested wild-type nematodes instead of the transgenic P*sur-5::luc::gfp* lines, because the transgenic lines have a roller marker and are not able to swim correctly. Nematodes were grown on an LD/CW cycle as described above. At the L4 stage, synchronized populations (40 nematodes each) were transferred by pipetting to a flat-bottomed, 96-well plate (Greiner) according to the previously described protocol (10, 11) but using the luminescence assay medium without D-luciferin. Plates were covered with an optic film to avoid evaporation, and the film over each well was perforated twice to avoid condensation. Nematode population recordings are preferred to single-worm recordings because of the higher amplitude of activity resulting from a higher number of infrared beam crosses. Individual recordings exhibit a low signal-to-noise ratio and therefore are not suitable for accurate recordings of long-term activity. The assay was performed under 17 °C and constant darkness in an I-291PF incubator (Ingelab), and temperature was monitored using DS1921H-F5 Thermochron iButtons (Maxim Integrated). The activity was recorded every min and binned in 5-min blocks. The experiment was performed for a period equivalent to the duration of a typical luminescence assay.

Assays of luminescence in entrainment followed by FR conditions (multiwell setup). For these experiments, 48 populations each comprised of 100 stage-L4 larvae (each population was considered an independent biological replicate) were selected manually as described above starting at ZT1, washed twice with M9 buffer to remove all traces of bacteria, and resuspended in 200 μL of luminescence medium. Nematodes were transferred to a white, flat-bottomed, 96-well plate (Greiner). The plate was sealed with optic film with two small perforations per well to prevent condensation. Performing the manual selection required around 5 h. The nematodes were left in the new LD/CW cycle until ZT10, and luminescence was registered using a Berthold Centro LB 960 microplate luminometer (Berthold Technologies) stationed inside an E-30B incubator (Percival) to allow tight control of the light and temperature in each experiment. Microwin 2000 software version 4.43 (Mikrotek-Laborsysteme) was programed to leave the plate outside the luminometer after each recording was performed to expose nematodes to the environmental cues. Only 48 of the 96 wells were used to avoid background contamination between contiguous wells; water was placed in the empty wells to avoid evaporation. The luminescence of each well was integrated for 10 s every 30 min. Every experiment was repeated at least twice.

The general entrainment conditions used for most experiments were 3.5 d at a 12-h/12-h LD/CW cycle (400/0 lx; 15.5/17 °C;

ZT0, lights on and onset of the cold-temperature phase) and 4 d at the FR condition (DD/WW, 17 °C). This protocol was performed to analyze synchronization of the N2 (wild-type) strain under LD/CW, LD/WC, and CW conditions, temperature compensation, phase-shift assays, and ATP treatment and to analyze the rhythms of the mutants strains TQ1101 *lite-1(xu7)*, MT21793 *lite-1(ce314);gur-3(ok2245)*, PR671 *tax-2(p671)*, and the PR671 full-rescue strain. We choose the LD/CW (15.5/17 °C) cycle for most recordings because 15.5 °C is the minimal temperature allowed in the Berthold Centro LB 960 microplate luminometer and because the populations have a longer life span under this condition than at 18.5/20 °C, resulting in a sustained luminescence signal for more days than at higher temperatures.

For the in vivo ATP treatment in the N2 strain, 1 mM of filter-sterilized ATP (GE Healthcare) dissolved in water was added to the medium at ZT10. Vehicle was used as control.

For temperature-compensation assays, the results of the general entrainment protocol were compared with the results of a similar assay performed for 3.5 d in LD/CW (400/0 lx; 19.5/21 °C) and for 4 d at DD/WW (21 °C). The period of the luminescence rhythms at 20 °C under DD was obtained from the AB-2550 Kronos Dio luminometer experiments as explained before.

For phase-shift assays, nematode populations were entrained for 3.5 d under a 12-h/12-h LD/CW cycle (400/0 lx; 15.5 °C/17 °C) and then were subjected to a phase shift caused by a 6-h night extension. After four more days, the nematodes were released into FR conditions (DD/WW, 17 °C) for 2 d.

For inverted zeitgeber cycle assays, nematode populations were entrained for 3.5 d under a 12-h/12-h regime of LD/WC (400/0 lx; 17/15.5 °C) and then were released into FR conditions (DD/CC, 15.5 °C) for 4 d.

For temperature-entrainment assays, nematode populations were entrained for 3.5 d in a 12-h/12-h DD/CW (15.5/17 °C) cycle and then were released into FR conditions with constant warm temperature (WW, 17 °C) for 4 d.

Finally, for the constant-light assays, nematode populations were entrained for 3.5 d under a 12-h/12-h LD/CW cycle (400/0 lx; 15.5/C/17 °C) and then were released into constant light and cold temperature (LL/CC, 15.5 °C) for 4 d.

Quantitative Real-Time PCR. The results for *sur-5* and *luc::gfp* expression were normalized to the mRNA levels of three selected references genes: *cdc-42*, *pmp-3*, and *Y45F10D.4* (60) by the standard curve method and were normalized to the sample with the highest mRNA level. Primers for amplifying each target were *sur-5* forward, 5′-CACCCCAAGGTTTTGTTCAC-3′; *sur-5* reverse, 5′-TGAAGGTGTCGGATACAACG-3′; *luc::gfp* forward, 5′-CTAGCCGGCCATACAAGTAATC-3′; *luc::gfp* reverse, 5′-CGGAATACGAATTGGGAGAC-3′; *cdc-42* forward, 5′-CTGCTGGACAGGAAGATTACG-3′; *cdc-42* reverse, 5′-CTCGGACATTCTCGAATGAAG-3′; *Y45F10D.4* forward, 5′-GTCGCTTCAAATCAGTTCAGC-3′; *Y45F10D.4* reverse, 5′-GTTCTTGTCAAGTGATCCGACA-3′; *pmp-3* forward, 5′-GTTCCCGTGTTCATCACTCAT-3′; *pmp-3* reverse, 5′-ACACCGTCGAGAAGCTGTAGA-3′. All primers were purchased from Thermo Fisher Scientific.

Data Analysis and Statistics. The circadian period was calculated by autocorrelation, fast Fourier nonlinear least square algorithm (FFT-NLLS), Maximum Entropy Spectral Analysis (MESA) (61) and the LS periodogram (62), evaluated inside a period range between 20 and 32 h. We chose to inform only the results of the LS algorithm because it produced the most accurate fits and also because it was reported to be the best method for period determination in short-term circadian recordings (63). With the period obtained from the LS algorithm we then estimated phase and amplitude using a cosinor algorithm, and a least-square regression fit using these parameters was calculated to evaluate the R^2 of the adjustment. Any luminescent signal of nematode populations with a period of 24 h and an R^2 least-square fit $\geq$0.7 was considered to be synchronized under the entrainment conditions. For FR conditions, any luminescent signal of a nematode population or of a single-nematode assay with a period close to 24 h and an R^2 less-square fit $\geq$0.5 was considered to be rhythmic. We considered entrained populations to be those whose acrophases in FR conditions had a difference of less than 3 h with respect to that of the entrainment conditions. To avoid confusion, the following nomenclature is used in this work: "percentage of synchronized" means the number of populations with a period and phase set by the zeitgeber conditions over the total number of populations tested; "percentage of entrained" means the number of populations that retain their circadian phase when placed under constant conditions over the number of populations set by the zeitgeber conditions; and "percentage of rhythmic under FR" means the number of rhythmic populations under FR over the total number of populations tested.

Rayleigh tests were used to determine the statistical significance of the mean phases, and a multisample test for equal median directions (a circular analog to the Kruskal–Wallis test) was performed to compare the mean phases under entrainment and FR conditions. In the phase-shift assay, Rayleigh tests followed by one-way ANOVA and Tukey's multiple comparisons test were used to assess significant differences between mean phases under the LD/CW condition before and after the phase shift and to compare the mean phases of the second entrainment condition (LD/CW2) with the FR condition.

Fisher's exact test was performed to compare the proportions of synchronized, entrained, and rhythmic populations in all experiments. One-way ANOVA was performed to identify statistically significant differences between control and PF-670462–treated samples, and an unpaired Student's *t* test was performed to compare the amplitude of the rhythms in ATP-addition experiments.

A cross-correlation test was applied to evaluate the phase differences between the average rhythmic patterns of luminescence, mRNA expression data, and endogenous ATP levels.

Bioinformatics Analysis. The *sur-5* minimal promoter portion of the P*sur-5::luc::gfp* construct was analyzed to search for the circadian regulatory elements with the next International Union of Pure and Applied Chemistry syntaxis: E-box (CACGTG) and E1–E2 box (CACGTG-[7- to 12-nt]-CAAGTG) (46, 64, 65). Analysis of regulatory elements was performed with the jPREdictor v1.23 with default parameters and one error allowed (66).

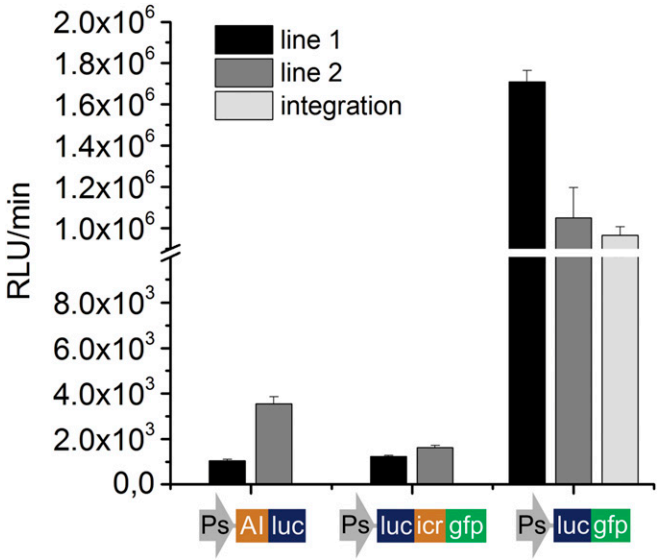

Fig. S1. The *luc::gfp* fusion enhances reporter luminescence activity. Average relative luminescence units (RLU) per minute are shown for transgenic nematode populations with three different luciferase constructs: transgenic line 1, black bars; transgenic line 2, dark gray bars; and integrated line, light gray bars ($n = 8$ for each population). Below each set of bars a schematic of each construct is shown representing a vector with a fusion between an artificial intron (AI) and the *luc* CDS, a bicistronic vector containing the *luc* and the *gfp* S65C variant CDS (with three artificial introns) separated by an icr region [from Lee et al. (58)], and a vector containing a translational fusion between the *luc* and the *gfp* S65C variant CDS. All constructs contained the same *sur-5* promoter (Ps) and the 3′ UTR region of pPD158.87. Under the same conditions, the luminescence signal from nematodes carrying *Psur-5::luc* alone was undetectable.

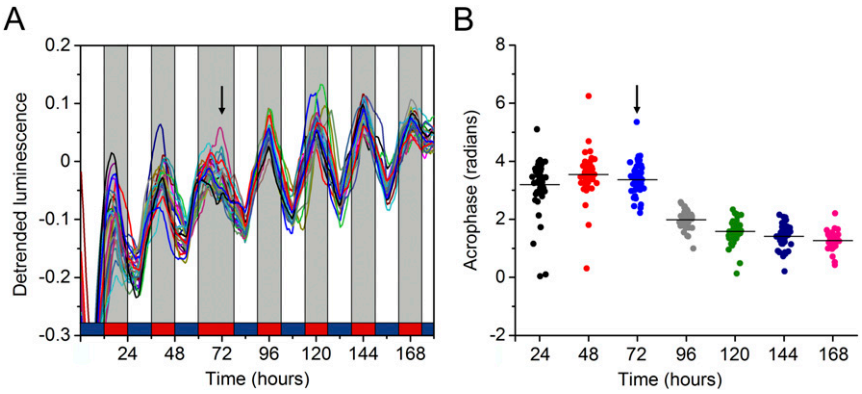

Fig. S2. Bioluminescent rhythms resynchronize after a phase shift. (*A*) Individual luminescence plots of worm populations during a 6-h phase-shift experiment ($n = 43$). The arrow indicates the time of the phase shift. Black/white bars indicate dark/light, respectively; blue/red bars indicate cold/warm, respectively. (*B*) Representation of acrophase changes throughout the entrainment days showing the gradual phase adjustment in the days after the shift. For comparison purposes, the *x* axis corresponds to the temporal scale shown in *A*. Each population consisted of 100 adult nematodes and was considered an independent biological replicate.

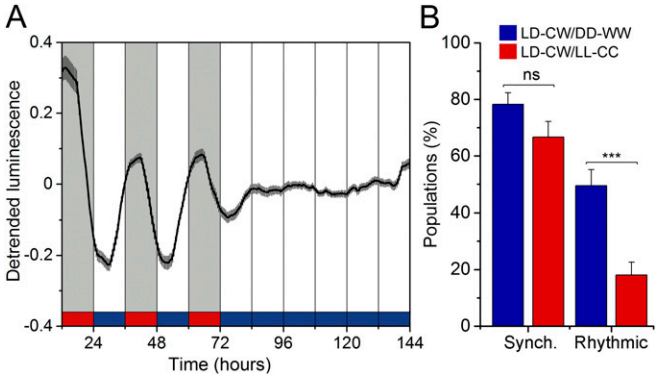

Fig. S3. Constant light conditions severely affect luminescence rhythms. (*A*) Average reporter activity of adult populations under LD/CW and then LL/CC conditions (15.5 °C, $n = 36$). (*B*) Proportion of synchronized (i.e., with their phase set by the LD/CW zeitgeber) and rhythmic (circadian under constant conditions) populations. Constant light and cold temperature increased the proportion of arrhythmic populations (LD/CW–DD/WW, $n = 101$; LD/CW–LL/CC $n = 72$; ***$P < 0.001$, two-tailed Fisher's exact test). Luminescence signals are shown as mean ± SEM. Each population consisted of 100 adult nematodes and was considered an independent biological replicate.

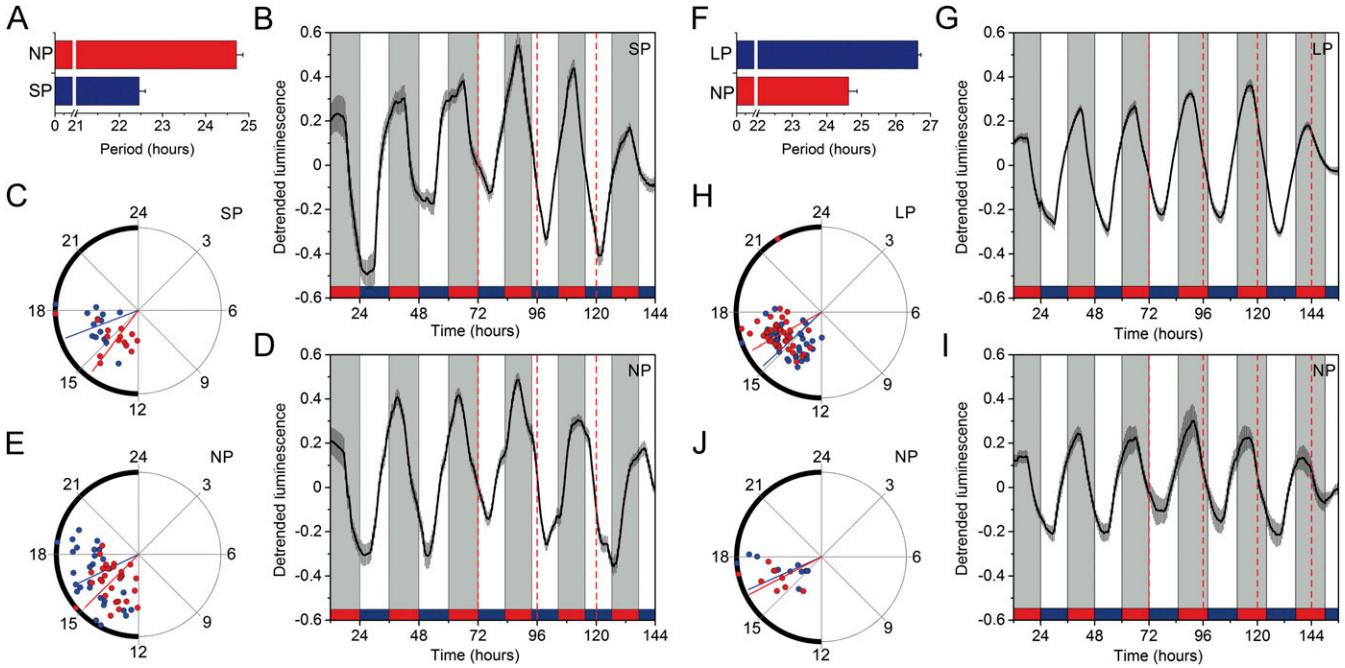

Fig. S4. Luminescence rhythms can be entrained to T22 and T26 cycles. Nematode populations were kept for 4 d under normal 12-h:12-h LD/CW (15.5/17 °C) conditions and for the following 4 d under a short (11-h:11-h) LD/CW (15.5/17 °C) or a long (13-h:13-h) LD/CW (15.5/17 °C) cycle. (*A*) Period length of nematode populations kept under T22 cycles. The reporter activity of populations ($n = 60$) exhibited either a short period (SP; 22.5 ± 0.1 h; blue bar, $n = 14$) or a normal period (NP, 24.7 ± 0.1 h; red bar, $n = 29$). The remaining 17 populations were arrhythmic. (*B* and *C*) Average luminescence signal (*B*) and Rayleigh plot (*C*) showing the acrophases of luminescence emission of the SP group under T24 (red dots) and T22 (blue dots) conditions (Rayleigh test, $P < 0.001$; LD/CW 24 h ZT14.5 ± 0.4 h vs. LD/CW 22 h ZT16.7 ± 0.3 h, $P < 0.001$; multisample test for equal median directions). (*D* and *E*) Average luminescence signal (*D*) and Rayleigh plot (*E*) showing the acrophases of luminescence emission of the NP group under T24 (red dots) and T22 (blue dots) conditions (Rayleigh test, $P < 0.001$; LD/CW 24 h ZT15 ± 0.4 h vs. LD/CW 22 h ZT16.3 ± 0.5 h, ns; multisample test for equal median directions). (*F*) Period length of nematodes kept under T26 cycles. The reporter activity of populations ($n = 60$) exhibited either a long period (LP, 26.6 ± 0.1, blue bar, $n = 37$) or a normal period (NP, 24.6 ± 0.2 h, red bar, $n = 10$). The remaining 13 populations were arrhythmic. (*G* and *H*) Average luminescence signal (*G*) and Rayleigh plot (*H*) showing the acrophases of luminescence emission of the LP group under T24 (red dots) and T26 (blue dots) conditions (Rayleigh test, $P < 0.01$; LD/CW 24 h ZT16.3 ± 0.4 h vs. LD/CW 26 h ZT15.5 ± 0.4 h, $P < 0.05$; multisample test for equal median directions). (*I* and *J*) Average luminescence signal (*I*) and Rayleigh plot (*J*) showing the acrophases of luminescence emission of the NP group under T24 (red dots) and T26 (blue dots) cycles (Rayleigh test $P < 0.001$; LD/CW 24 h ZT16.3 ± 0.3 h vs. LD/CW 26 h ZT16.5 ± 0.3 h, ns; multisample test for equal median directions). Luminescence signals are shown as mean ± SEM. Each population consisted of 100 adult nematodes and was considered an independent biological replicate. Red dashed lines in *B*, *D*, *G*, and *I* indicate a time lapse of 24 h in the T22 and T26 cycles.

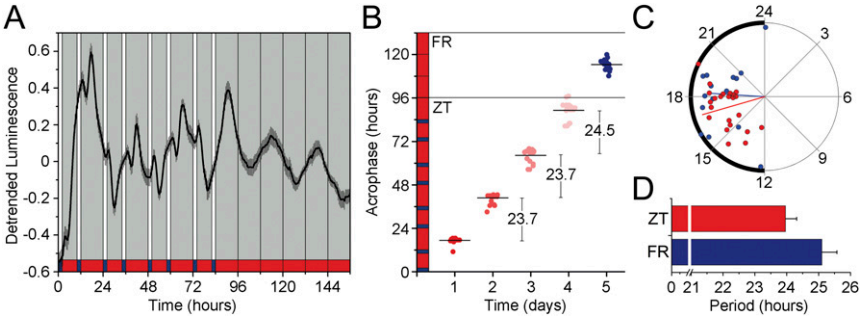

Fig. S5. Luminescence rhythms can be entrained to skeleton photo/thermo-cycles. Nematode populations (n = 48) were entrained to a classical skeleton photo/thermo-cycle for 4 d [ZT, 2-h L/C pulses (light and 15.5 °C) at ZT0–2 and ZT10–12) followed by 3 d of FR conditions (DD/WW, 17 °C). (*A*) Average reporter activity of the entrained populations (n = 15). (*B*) Dot plot of daily acrophases under ZT (daily periods were calculated as a mean of the distance between the acrophases with a moving average of 24 h are shown) and the first day of FR conditions (n = 15). (*C*) Rayleigh plot showing the acrophases of luminescence emission of populations under ZT (red dots) and FR (blue dots) conditions (Rayleigh test, $P < 0.001$; ZT16.9 ± 0.5 h vs. CT18.24 ± 0.7 h, ns; multisample test for equal median directions). (*D*) Average period of the luminescence activity under ZT conditions (red bar, n = 24; ZT LS period 23.9 h ± 0.3 h) or FR conditions (blue bar, n = 19; LS period 25.1 h ± 0.5 h). The remaining populations were arrhythmic. Luminescence signals are shown as mean ± SEM. Each population consisted of 100 adult nematodes and was considered an independent biological replicate.

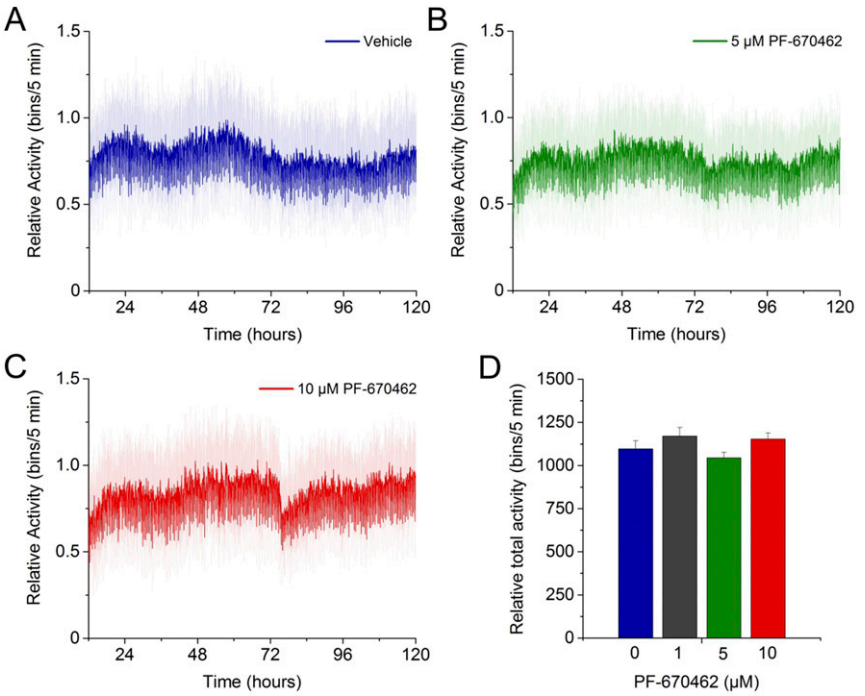

Fig. S6. PF-670462 is not toxic at the concentrations used in the luminescence assays. (*A–D*) Results from a toxicity text that recorded the global motility of populations of wild-type adult nematodes (N2) over time under different concentrations of the PF-670462, using an infrared tracking device (WMicroTracker) previously reported for toxicity screenings (59). Plots show the mean relative activity pattern ± SEM of nematodes treated with vehicle (*A*) or with 5 µM (*B*) or 10 µM of PF-670462 (*C*) under DD/WW (18 °C) conditions (n = 24 populations per treatment, 50 nematodes per well). The assay was performed for a period equivalent to the duration of a typical single-nematode luminescence assay. (*D*) No significant differences were found in the total activity of the control group treated with vehicle (water) and that of groups treated with different concentrations of PF-670462 (1 µM, 5 µM, and 10 µM) (one-way ANOVA with Dunnett's post hoc test, ns).

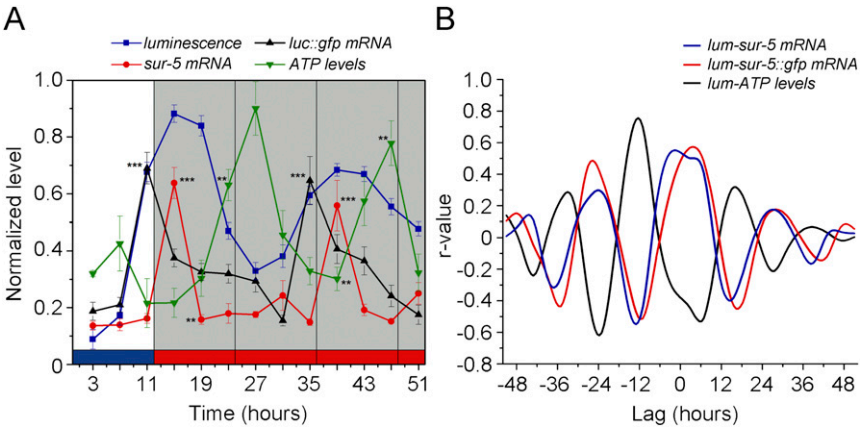

Fig. S7. Luminescence rhythms correlate with transcriptional activity but do not reflect endogenous ATP levels. (*A*) The *sur-5* and *luc::gfp* mRNA circadian patterns are in phase with the average luminescence pattern of nematode populations, whereas the daily variations in total ATP level are in antiphase with bioluminescence. (*B*) Cross-correlation analysis shows that the acrophase of the luminescence rhythms exhibits a 3-h phase advance with respect to the *sur-5* expression pattern (cross-correlation test; luminescence vs. *sur-5* mRNA, blue line; $n = 26$ and $n = 4$, respectively), a 3-h phase delay with respect to *luc::gfp* reporter mRNA (cross-correlation test; luminescence vs. *luc::gfp* mRNA, red line; $n = 26$ and $n = 4$, respectively), and an 11-h phase delay with respect to total ATP levels (cross-correlation test; luminescence vs. ATP, black line; $n = 26$ and $n = 4$, respectively).

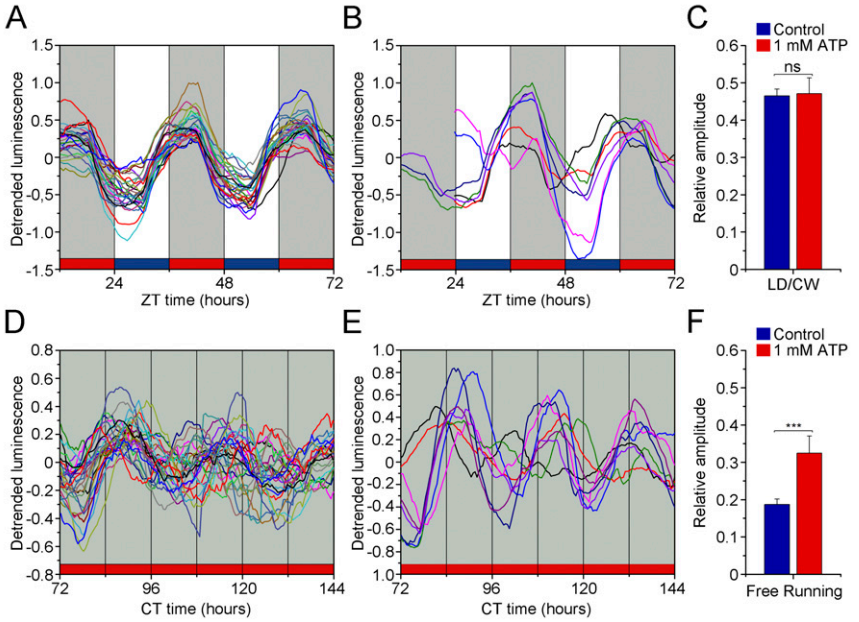

Fig. S8. The addition of exogenous ATP improves the amplitude of the general pattern of the luminescence oscillations under FR conditions. (*A* and *B*) Individual luminescence plots of nematode populations under LD/WC conditions without ATP treatment (*A*, $n = 26$) and with 1 mM ATP (*B*, $n = 8$). (*C*) The exogenous addition of 1 mM ATP does not change the general pattern of luminescence oscillations under LD/CW (ns, *t* test). (*D* and *E*) Individual luminescence plots of nematode populations under FR conditions without ATP treatment (*D*, $n = 26$) and with 1 mM ATP (*E*, $n = 8$). (*F*) Treatment with 1 mM ATP generates a significantly higher amplitude in circadian oscillations with respect to basal conditions only in the FR condition (***$P < 0.001$, *t* test). Each population consisted of 100 adult nematodes and was considered an independent biological replicate.

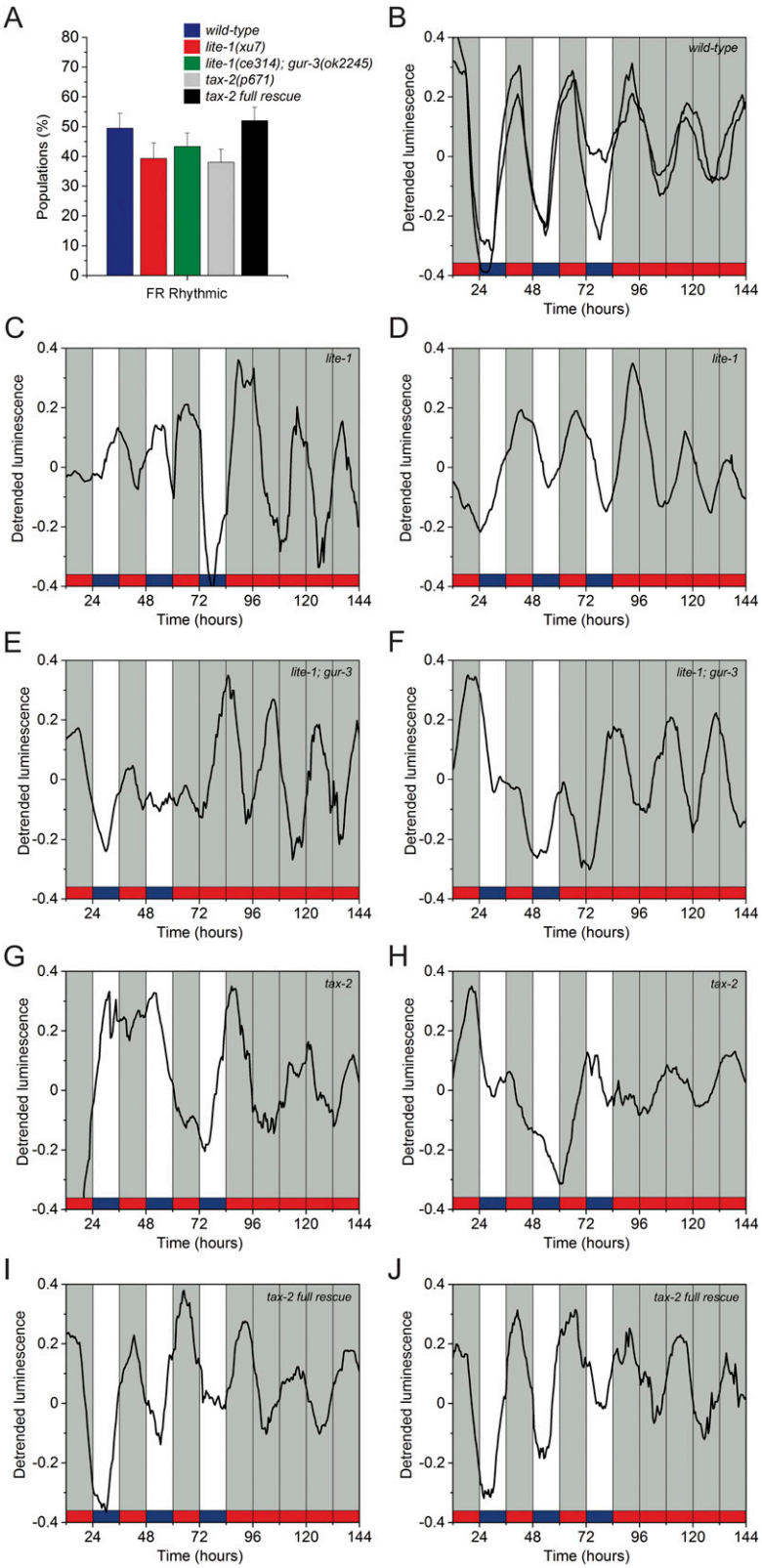

Fig. S9. Light and temperature sensing requires LITE-1, GUR-3, and TAX-2 proteins. (*A*) photoreception- and thermoreception-mutant nematode populations exhibit an FR phenotype similar to the wild-type strain. Average percentage of rhythmic nematode populations under FR conditions [wild-type, $n = 101$; *lite-1 (xu7)*, $n = 89$; *lite-1(ce314); gur-3(ok2245)*, $n = 120$; *tax-2 (p671)*, $n = 121$; *tax-2 (p671)* full rescue, $n = 121$; two-tailed Fisher's exact test, all vs. wild-type, ns]. (*B*) Representative luciferase activity rhythms of two individual wild-type populations under LD/CW and DD/WW conditions from the data recorded in Fig. 5*E*. (*C–J*) Representative luciferase activity rhythms of two individual mutant populations under LD/CW and DD/WW conditions from the data recorded in Fig. 5 *F–K*. (*C* and *D*) *lite-1(xu7)* mutants. (*E* and *F*) *lite-1(ce314)* mutants; *gur-3(ok2245)* mutants. (*G* and *H*) *tax-2 (p671)* mutants. (*I* and *J*) The *tax-2 (p671)* full rescue strain. Each population consisted of 100 adult nematodes and was considered an independent biological replicate.