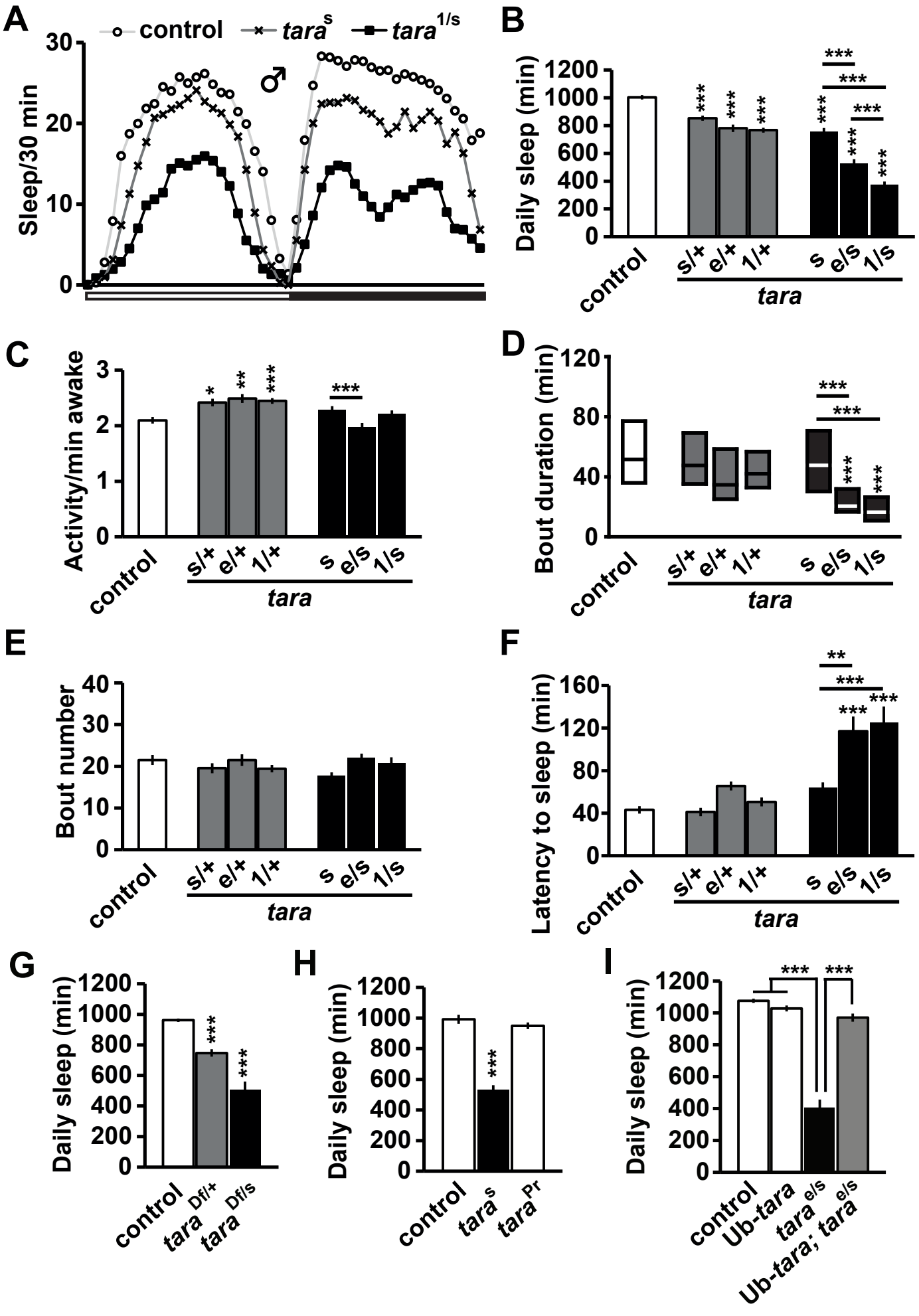
A
control
tara^s
tara^1/s
Sleep/30 min
♂
B
Daily sleep (min)
control
s/+
e/+
1/+
s
e/s
1/s
tara
C
Activity/min awake
D
Bout duration (min)
E
Bout number
F
Latency to sleep (min)
G
Daily sleep (min)
control
tara Df/+
tara Df/s
H
Daily sleep (min)
control
tara^s
tara^Pr
I
Daily sleep (min)
control
Ub-tara e/s
tara e/s
Ub-tara; tara e/s

Figure S1. Sleep Phenotypes and Genetic Analysis of *tara*: Male Data. (A) Sleep profile of homozygous *tara*^{s132} (black X's), transheterozygous *tara*$^{1/s132}$ (black squares), and their background control males (white circles) (n=60-73). The white and black bars below the X-axis represent light and dark periods, respectively. **(B)** Control and *tara* mutant males of the indicated genotypes (n=41-73). **(C)** Waking activity, **(D)** sleep bout duration, **(E)** sleep bout number, and **(F)** sleep latency at lights off for the same male flies shown in (B). Sleep bout duration is shown in simplified box plots, where the median and interquartile range are represented. **(G)** Total daily sleep of control and Df(3R)Exel7329 male heterozygotes in trans to either a wild type (Df/+) or *tara*^{s132} (Df/s) allele (n=35-70). **(H)** Total daily sleep of control, *tara*^{s132}, and precise excision male flies (n=15-34). **(I)** Total daily sleep of male flies of the indicated genotypes (n=35-42). Ubiquitous expression of *tara*-B (Ub-*tara*) rescued the *tara* sleep phenotypes. Mean ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test (G, H); Tukey post hoc test (B, C, E, F, I); Kruskal-Wallis test (D). For simplicity, only significant differences between each mutant and the control and those between *tara*^{s132}, *tara*$^{e01264/s132}$, and *tara*$^{1/s132}$ mutants are indicated. Related to Figure 1.

A

Genotype	N	% R	% WR	% AR	tau (h) ± SEM	Power ± SEM
control	155	100	0	0	23.59 ± 0.02	133.9 ± 2.76
tara$^{s/+}$	48	89.6	6.3	4.2	23.24 ± 0.26	129.4 ± 6.60
tara$^{e/+}$	59	94.9	1.7	3.4	23.59 ± 0.03	142.2 ± 5.11
tara$^{1/+}$	62	90.3	8.1	1.6	23.51 ± 0.03	129.9 ± 5.98
tara^{s}	76	77.6	9.2	13.2	23.51 ± 0.03	95.1 ± 6.45
tara$^{e/s}$	86	64.0	9.3	26.7	23.44 ± 0.26	65.0 ± 5.99
tara$^{1/s}$	55	18.2	16.4	65.5	23.57 ± 0.06	18.4 ± 7.04
control *tara*^{GFP}	56	92.9	3.5	3.6	23.62 ± 0.05	126.7 ± 4.82
tara^{GFP}	88	92	3.4	4.5	23.45 ± 0.16	144.8 ± 5.19

N: number of flies; **R:** rhythmic; **WR:** weakly rhythmic; **AR:** arrhythmic
tau: free-running period; **Power:** measure rhythm strength;

B

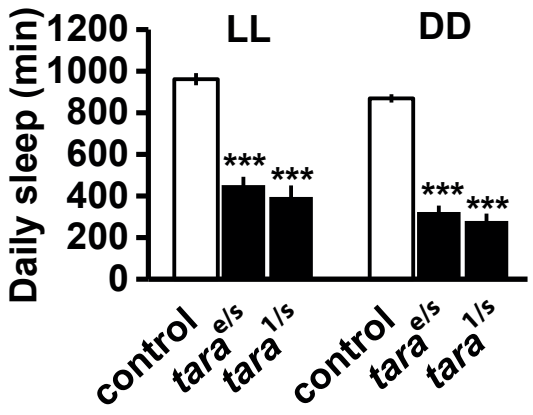

Figure S2. Circadian Phenotypes and Clock-Independent Sleep Loss of *tara* Mutant Males. (A) Free-running circadian phenotypes of male flies of the indicated genotypes in DD. χ^2 periodogram analysis was performed for each fly using the FaasX software to determine the free-running period, tau. Power, a measure of rhythmicity, corresponds to peak – significance value at p = 0.05. **(B)** Total daily sleep amount in LL and DD for control, *tara*$^{e01264/s132}$, and *tara*$^{1/s132}$ males (n=29-77 for LL; n=55-94 for DD). Sleep levels on the 3rd day in constant conditions are shown. Mean ± SEM is shown. ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test relative to control flies (B). Related to Figure 2.

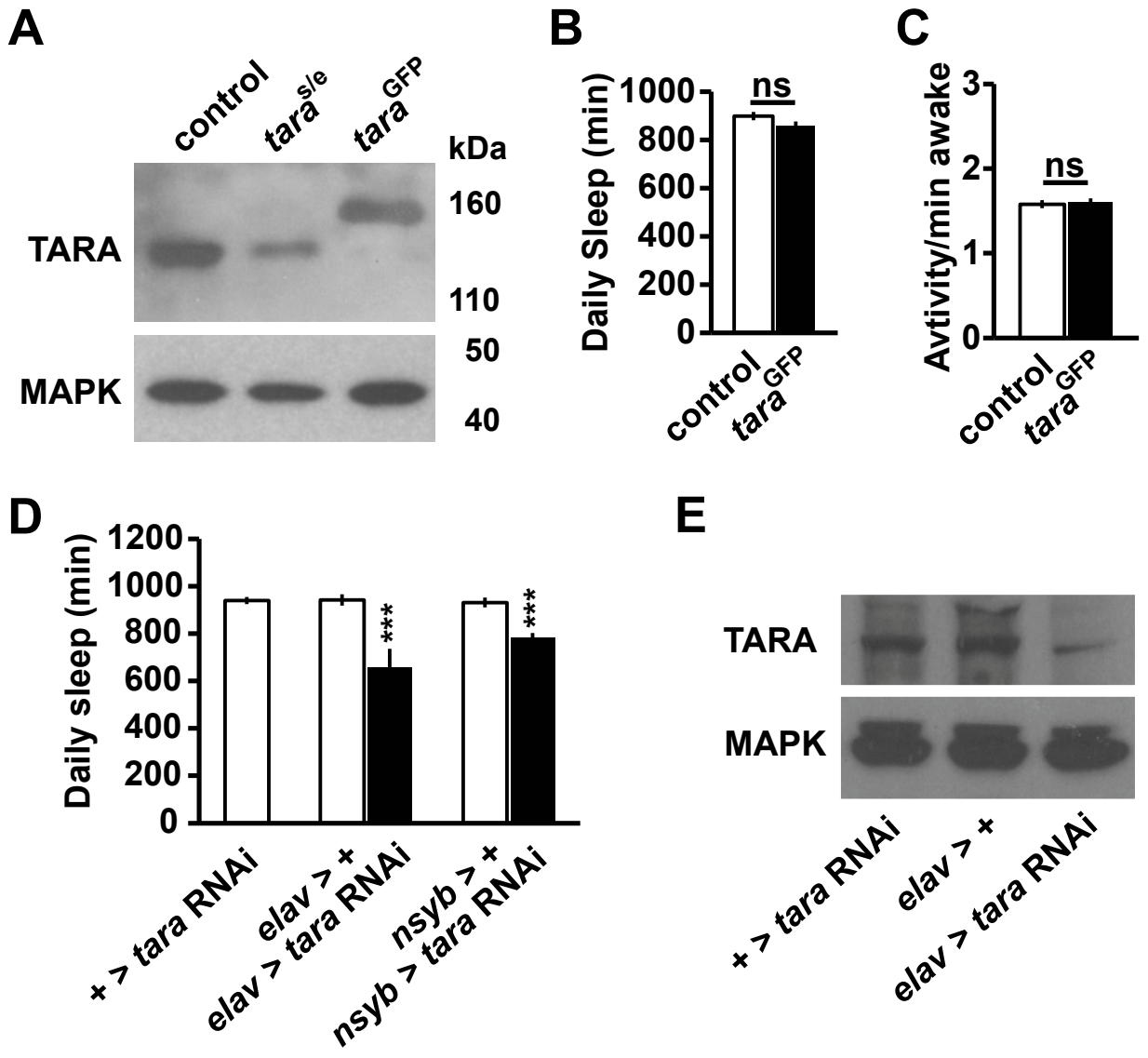

Figure S3. Characterization of *tara*^{GFP} Flies. (A) Western blot showing a band shift in homozygous *tara*^{GFP} lysates. **(B)** Total daily sleep and **(C)** waking activity of *tara*^{GFP} and control females (n=47-64). *tara*^{GFP} flies did not exhibit sleep abnormalities, suggesting TARA::GFP is functional. **(D)** Pan-neuronal *tara* knockdown using two independent Gal4 lines (*elav* and *nsyb*) resulted in a significant reduction of sleep in males (n=33-63 except for *elav* > *tara* RNAi, for which n=11). **(E)** Western blot shows a marked reduction of TARA levels in females in which *tara* was knocked down pan-neuronally (*elav* > *tara* RNAi). The experiment was performed three times with similar results. MAPK was used as loading control (A,E). Mean ± SEM is shown. ***p < 0.001, ns: not significant, Student's t-test (B,C); one-way ANOVA followed by Dunnett post hoc test relative to controls (D). Related to Figure 3.

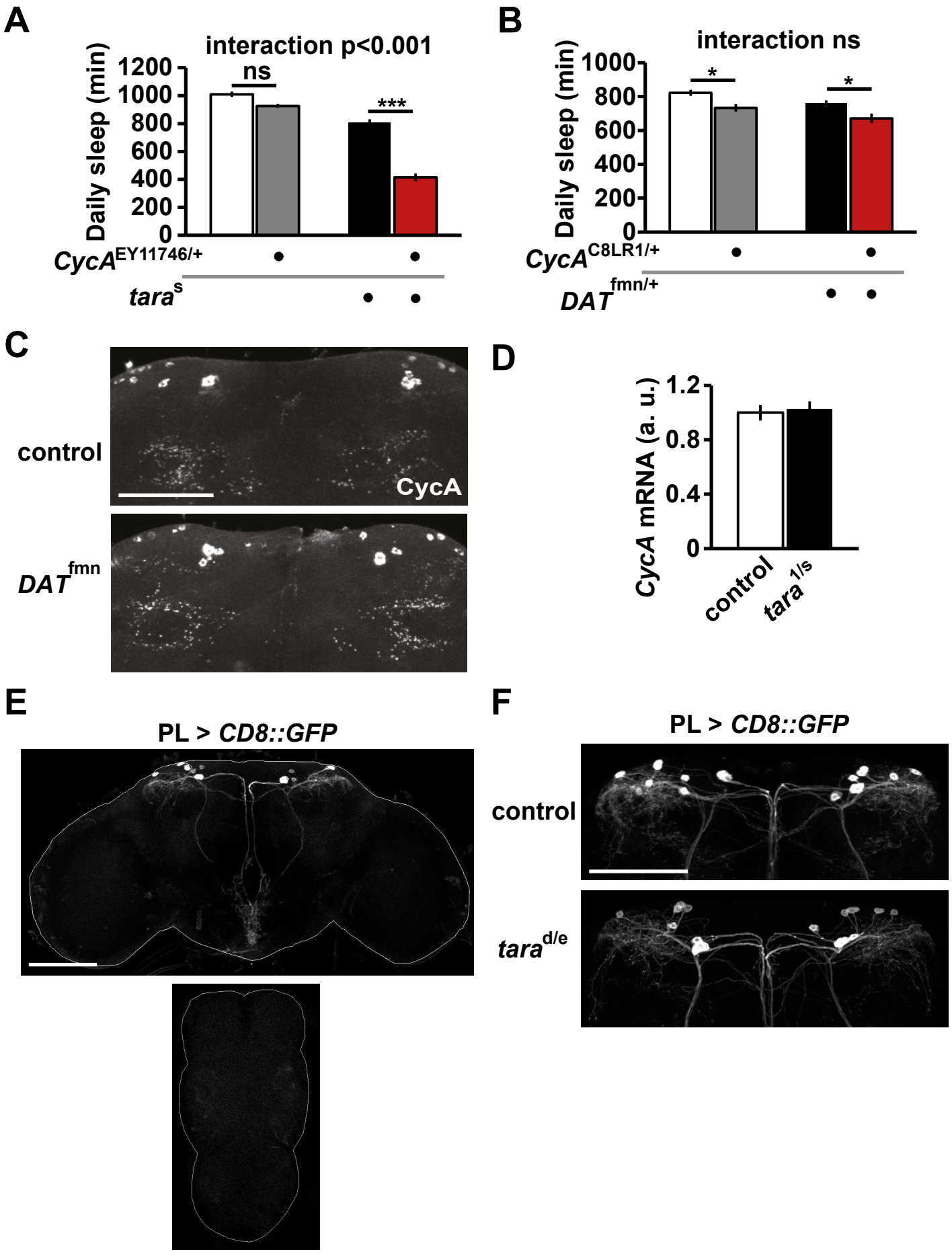
A
interaction p<0.001
ns
***
Daily sleep (min)
1200
1000
800
600
400
200
0
CycA EY11746/+
tara s
B
interaction ns
*
*
Daily sleep (min)
1000
800
600
400
200
0
CycA C8LR1/+
DAT fmn/+
C
control
CycA
DAT fmn
D
CycA mRNA (a. u.)
1.2
0.8
0.4
0
control
tara 1/s
E
PL > CD8::GFP
F
PL > CD8::GFP
control
tara d/e

Figure S4. Genetic Interaction between *tara* and *CycA*. (A) Daily sleep for male flies of the indicated genotypes (n=54-64). **(B)** Daily sleep for female flies of the indicated genotypes (n=40-56). **(C)** Maximal-intensity confocal projections of the dorsal half of the central brain of representative control and DAT^{fmn} adult flies immunostained with an antibody to CycA. In contrast to *tara* mutants, CycA levels were not altered in DAT^{fmn} mutants. Scale bar: 100 μm. **(D)** *CycA* mRNA levels relative to *actin* mRNA levels were not significantly different between $tara^{1/s132}$ and control flies (n=3). **(E)** Confocal projection of a female brain (top) and ventral nerve cord (bottom) expressing CD8::GFP under the control of PL-Gal4. GFP expression was observed in a small number of neurons in the dorsal brain. Scale bar: 100 μm. **(F)** Confocal projection of a representative control or $tara^{d40/e01264}$ central brain expressing CD8::GFP under the control of PL-Gal4. The CycA-expressing PL neurons were present and grossly normal in morphology in *tara* mutants. d40 (d, for short) is an imprecise excision allele with a sleep phenotype similar to s132 (see Experimental Procedures). Scale bar: 100 μm. Mean ± SEM is shown. *p < 0.05, ***p < 0.001, ns: not significant, two-way ANOVA followed by Tukey post hoc test (A,B); Student's t-test (D). Related to Figure 4.

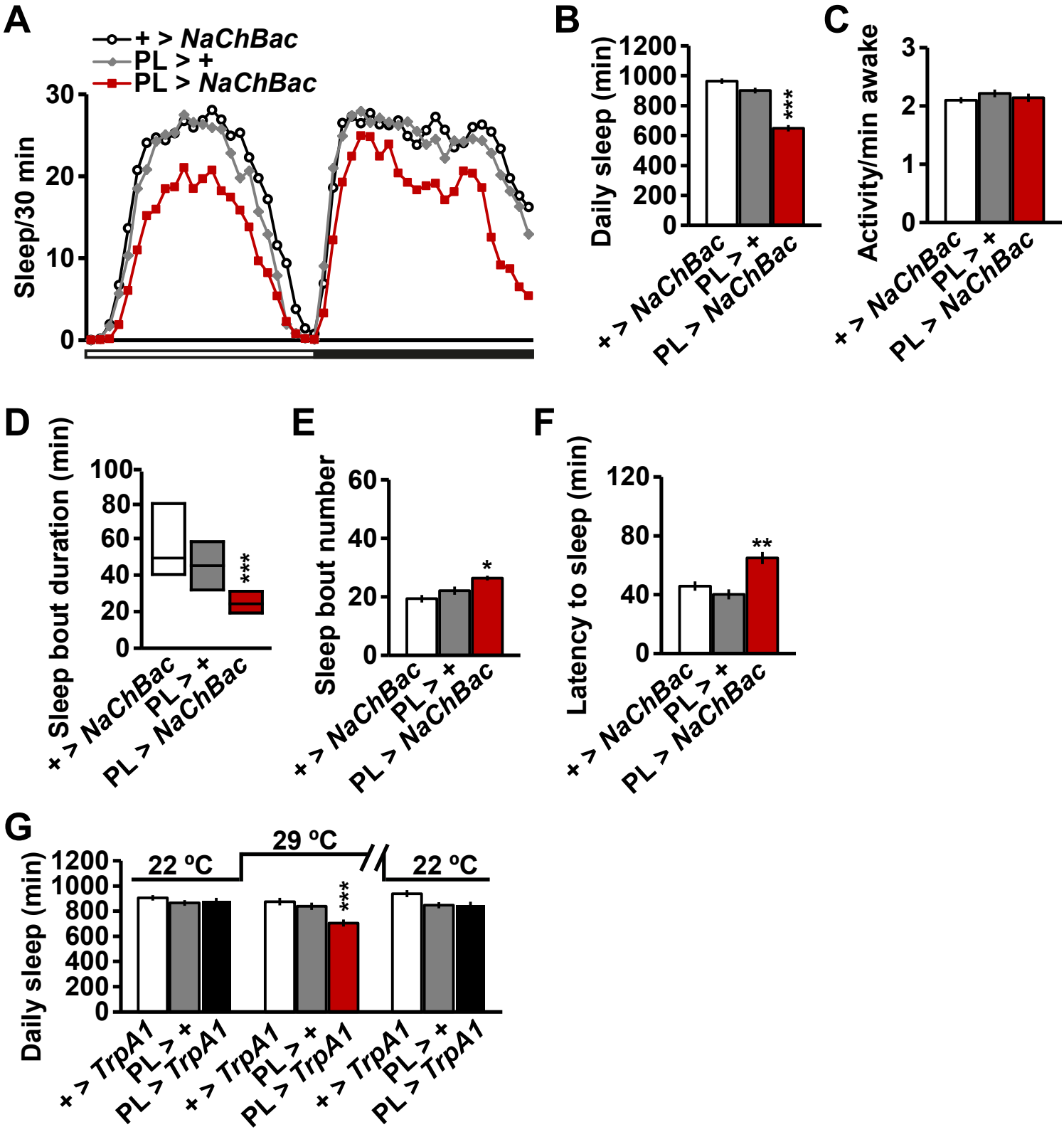

Figure S5. Activation of CycA-Expressing PL Neurons Suppresses Sleep. (A) Sleep profile for male flies expressing the NaChBac sodium channel under the control of PL-Gal4 (PL > *NaChBac*) and parental controls (n=48-64). **(B)** Daily sleep, **(C)** waking activity, **(D)** sleep bout duration, **(E)** sleep bout number, and **(F)** sleep latency at ZT12 for the flies shown in (A). **(G)** Total daily sleep for male flies carrying both UAS-*TrpA1* and PL-Gal4 (PL > *TrpA1*, n=32) relative to parental controls (n=16-32). Flies were monitored at 29°C, which activates the TrpA1 channel, and at 22°C, which inactivates the TrpA1 channel. Mean ± SEM is shown. *p < 0.05, **p < 0.01, and ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test relative to controls (B-C, E-G); Kruskal-Wallis test (D). Related to Figure 5.

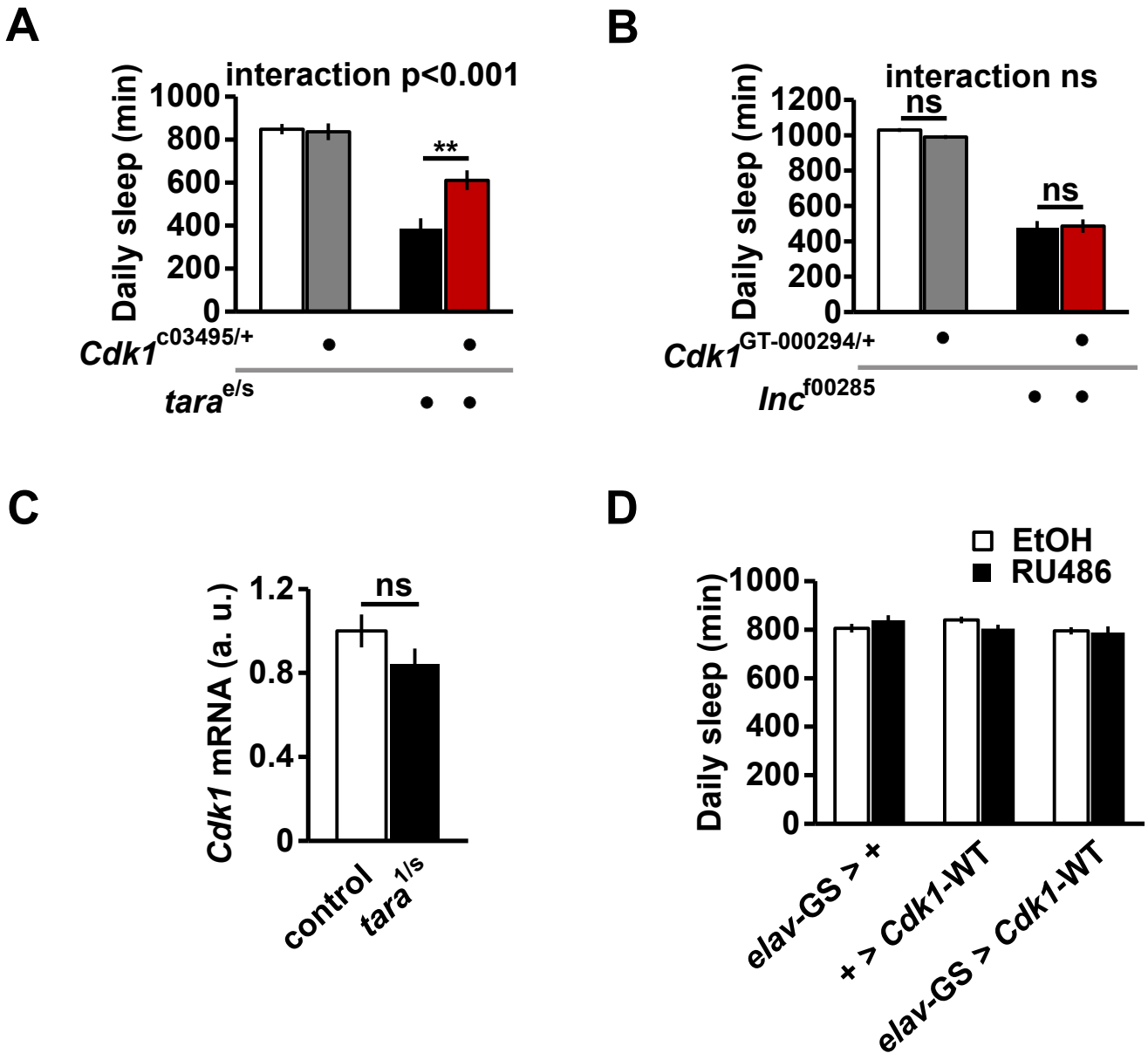

Figure S6. *tara* and *Cdk1* interact antagonistically to control sleep. (A) Daily sleep for control, *Cdk1*$^{c03495/+}$, *tara*$^{e01264/s132}$, and *Cdk1*$^{c03495/+}$; *tara*$^{e02164/s132}$ females (n=23-32). **(B)** Daily sleep for male flies of the indicated genotypes (n=26-43). **(C)** *Cdk1* mRNA levels of *tara*$^{1/s132}$ mutants were comparable to those of control flies. **(D)** Adult-stage pan-neuronal overexpression of wild-type *Cdk1* has little effect on sleep. Flies were fed either RU486 or vehicle (EtOH) (n=31-32). Mean ± SEM is shown. **p < 0.01, ns: not significant, one-way ANOVA followed by Tukey post hoc test (A, B, D); Student's t-test (C). Related to Figure 6.

Supplemental Experimental Procedures

Fly stocks

Flies were maintained at room temperature (RT) on standard food containing molasses, cornmeal, and yeast. UAS-*tara*-RNAi (JF01421), *tara*^{1}, *tara*$^{Df(3R)Exel7329}$, *CycA*^{EY11746}, *CycA*^{C8LR1}, *inc*^{f00285}, *Cdk1*$^{GT-000294}$, *Cdk1*^{c03495}, UAS-*Cdk1-myc*, UAS–*mCD8-GFP*, UAS-*NaChBac*, UAS-*TNT-G*, UAS-*TNT-IMP*, UAS-*dicer2* (*dcr2*), *elav*-Gal4, *nsyb*-Gal4, *Cha*-Gal4, *VGlut*-Gal4, *Tdc2*-Gal4, c309-Gal4, OK107-Gal4, and UAS-*TrpA1* were obtained from the Bloomington Stock Center. 104y-Gal4, *Dilp2*-Gal4, *tim*-Gal4, *Pdf*-Gal4, and *elav*-GS lines were obtained from Dr. Amita Sehgal's lab; *Dh44*-Gal4 from the Vienna *Drosophila* Resource Center; UAS-*tara*-RNAi (6889R-1) from the National Institute of Genetics, Japan; *tara*^{e01264} from the Harvard Exelixis collection; *tara::GFP* (*tara*^{yb0035}) from Dr. Lynn Cooley; Ub-*tara* and *tara*^{EP3463} from Dr. Henri-Marc Bourbon; PL-Gal4 from Dr. Jae Park; UAS-*HA::syt* from Dr. Thomas Schwarz; *DAT*^{fmn} from Dr. Kazuhiko Kume; and UAS-*Cdk1*-WT-VFP and UAS-*Cdk1*-AF-VFP from Dr. Shelagh Campbell. PL-Gal4 contains a *Corazonin* promoter fragment (504$^{\Delta 311-249}$) [36]. For improved efficiency of *tara* knockdown, two RNAi lines (JF01421 and 6889R-1) were combined with UAS-*dcr2*. All fly lines were outcrossed to an isogenic background control line (iso31) for at least four generations, except for the UAS-*tara*-RNAi lines.

Generation of excision lines

The s132 insertion maps to 573 bp upstream of the *tara-B* transcription start site. Mobilization of the P-element in the s132 line using Δ2-3 recombinase produced precise excision lines with normal sleep patterns but failed to produce imprecise excision lines.

To generate an imprecise excision line, we employed a neighboring P-element EP3463, which maps to ~50bp upstream of the *tara*-B transcriptional start site (www.flybase.org). By mobilizing the EP3463 insertion using Δ2-3 recombinase, we obtained an imprecise excision line d40, in which a portion of the P element was removed. The d40 line was outcrossed 6 times into the control iso31 background. Homozygous $tara^{d40}$ as well as transheterozygous $tara^{d40/e01264}$ flies exhibit reduced sleep.

Sleep and circadian assays

Four to seven day old flies entrained to a 12h:12h LD cycle for at least 3 days were individually placed in small glass tubes containing 5% sucrose and 2% agar at 25°C except where noted. For the GS experiment, 500 μM RU486 or vehicle (ethanol) was added to the sucrose-agar food. For experiments involving the warmth-induced TrpA1 channel, flies were raised at RT (~21°C) and entrained in LD at 22°C for at least 3 days before being monitored for 1 day at 22°C to establish a baseline, 2 days at 29°C to activate the TrpA1 channel, and 1 day at 22°C to examine recovery. Data from the first day at 29°C as well as the baseline and recovery day are presented. Activity counts were collected in 1-min bins using *Drosophila* Activity Monitoring (DAM) System (Trikinetics), and sleep was defined as a period of inactivity lasting at least 5 min [S1]. Sleep parameters were analyzed using the PySolo software [S2], except for sleep latency which was analyzed using Sleeplab (William Joiner).

Circadian assays were performed essentially as described [31]. Briefly, male flies were monitored in DD using the DAM system (Trikinetics) for six days after being entrained to an LD cycle for at least 3 days. Activity counts collected in 30-min bins were

analyzed using the FaasX software (M. Boudinot and F. Rouyer). The software uses χ^2 analysis to calculate period length and rhythm power. Rhythm power was determined for all flies including arrhythmic ones, whereas circadian period was determined only for rhythmic flies. Actograms were generated using ClockLab (Actimetrics).

Quantitative real-time reverse-transcriptase PCR (qPCR)

Total RNA was extracted from 20-30 female fly heads using TRIzol (Life Technologies), and cDNA was generated using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). qPCR was performed using SYBR green (Applied Biosystems). The following primers were used: 5'-GAA AAA GGC GCC AAA CTT AAA TTA-3' and 5'-TCG CGG AAT TCA CAT TGG AT-3' for *tara-A*; 5'-GAA AAT GTG CAC TGA GGT GAA T-3' and 5'-GTG TTG GCA TCC TTG CTG T-3' for *tara-B*; 5'-GCG GAT GAC ATA AGT GAT GG-3' and 5'-CAT GAC GCT GTA TAT TTC CGA-3' for *CycA*; and 5'-ATG GCG TGG TGT ATA AGG GT-3' and 5'-AAA TTT CTC TGA TCG CGG TT-3' for *Cdk1*.

Antibody production and Western analysis

For antigen production, a PCR product coding for 167 amino acids of TARA common to both isoforms was subcloned into the pET-28a protein expression vector using the following primers: 5'-ATG AAT TCT CGC CAT CGG AGC C-3' and 5'-ATC TCG AGA TGC GGT ACA AAG GGA TG. The His-tagged protein was purified at the Wistar Institute Protein Expression Facility, and injected into rats to generate polyclonal antibody TJR51 (Cocalico Biologicals). TJR51 recognized a ~130kD that was markedly

reduced in *tara* mutants. Since the TARA protein is predicted to be smaller at ~100kD, we examined the possibility that the annotated *tara* transcripts are incomplete. To this end, we generated a full-length UAS-*tara*-B construct, which contains the entire coding region of the *tara*-B isoform as annotated in FlyBase (www.flybase.org) but does not contain any intronic or untranslated regions. We transfected this UAS-*tara*-B construct along with an *actin*-Gal4 construct in *Drosophila* S2 cells. Overexpression of the *tara*-B transcripts in S2 cells resulted in upregulation of the ~130kD band, demonstrating that the band corresponds to the predicted TARA-B protein. As the two TARA isoforms (A and B) differ in size by only 4 amino acids, the single band likely represents both isoforms.

Western blot analysis of head extracts and quantification of immunoreactive bands were performed essentially as described [51]. Anti-TARA antibody (TJR51) was used at 1:750, anti-HA (Covance) at 1:1000, and anti-MAPK (Sigma) at 1:10000.

Transient transfection and co-immunoprecipitation (co-IP)

For Western analysis, *Drosophila* S2 cells were transfected with indicated DNA constructs in 24-well plates (150 ng of total DNA) using Effectene (Qiagen). For co-IP experiments, S2 cells were transfected with various combinations of UAS-*tara* (200 ng) and UAS-*HA-CycA* constructs [S3] (150 ng) along with *actin*-Gal4 (100 ng) in 6-well plates using Effectene (Qiagen). UAS vector DNA was included in some conditions to make the total amount of DNA equal in all conditions. Transfected cells were kept at 25°C for 2 days before being harvested. Co-IP was performed essentially as described [51] except that an antibody to HA (Covance) was used for IP and cells were lysed in

extraction buffer containing 150 mM KCl, 50 mM Tris-Cl at pH 7.0, 10 mM EDTA, 0.2% Triton X-100, 10 mM DTT, and protease inhibitor cocktail (Roche).

Immunohistochemistry

Dissected brains were fixed in 4% paraformaldehyde for 30 min at RT. Samples were blocked in 5% normal chicken serum for experiments involving CycA staining, 1% BSA for PER staining, and 5% normal goat serum for other antibody staining. The following primary antibodies were used: rabbit anti-PER [S4] at 1:8000, mouse anti-PDF (DSHB) at 1:2000, rabbit anti-GFP (Invitrogen) at 1:500, goat anti-CycA (Santa Cruz Biotechnology, #15869) at 1:50, anti-ELAV (DSHB) at 1:200, anti-REPO (DSHB) at 1:100, anti-HA (Covance) at 1:1000, and anti-MYC (Invitrogen) at 1:1000. The secondary antibodies, Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, and Alexa Fluor 647 chicken anti-goat (Invitrogen) were used at 1:400. Primary and secondary antibodies were incubated at 4°C overnight. Images were obtained on an Olympus Fluoview confocal microscope.

Statistical Analysis

Data sets with two groups were compared using *t* tests. For multiple pairwise comparisons in a data set, *t* tests with Bonferroni correction were used. For comparisons of multiple groups, one-way ANOVA tests were performed, followed by Dunnett or Tukey post-hoc tests. For genetic interaction experiments, two-way ANOVA tests were performed to test for the interaction. For comparisons of non-normally distributed data,

Kruskal-Wallis tests were performed, followed by Dunn's post hoc tests with Bonferroni correction.

Supplemental References

S1. Huber, R., Hill, S.L., Holladay, C., Biesiadecki, M., Tononi, G., and Cirelli, C. (2004). Sleep homeostasis in Drosophila melanogaster. Sleep *27*, 628-639.

S2. Gilestro, G.F., and Cirelli, C. (2009). pySolo: a complete suite for sleep analysis in Drosophila. Bioinformatics *25*, 1466-1467.

S3. Dienemann, A., and Sprenger, F. (2004). Requirements of cyclin a for mitosis are independent of its subcellular localization. Curr Biol *14*, 1117-1123.

S4. Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the Drosophila clock gene period and a lacZ reporter: mapping elements of the PER protein involved in circadian cycling. J Neurosci *17*, 676-696.