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TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in *Drosophila*

Highlights

- *tara* is a novel sleep-regulatory gene in *Drosophila*
- TARA regulates CycA levels and interacts with CycA to control sleep
- TARA promotes sleep in CycA-expressing PL neurons, a novel arousal center
- Cdk1 interacts with *tara* and CycA and acts in PL neurons to suppress sleep

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In Brief

The molecular and neural mechanisms of sleep regulation are not well understood. Afonso et al. show that TARANIS promotes sleep by regulating CycA protein levels and inhibiting Cdk1 activity in a novel arousal center.

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TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in *Drosophila*

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SUMMARY

Sleep is an essential and conserved behavior whose regulation at the molecular and anatomical level remains to be elucidated. Here, we identify TARANIS (TARA), a *Drosophila* homolog of the Trip-Br (SERTAD) family of transcriptional coregulators, as a molecule that is required for normal sleep patterns. Through a forward-genetic screen, we isolated *tara* as a novel sleep gene associated with a marked reduction in sleep amount. Targeted knockdown of *tara* suggests that it functions in cholinergic neurons to promote sleep. *tara* encodes a conserved cell-cycle protein that contains a Cyclin A (CycA)-binding homology domain. TARA regulates CycA protein levels and genetically and physically interacts with CycA to promote sleep. Furthermore, decreased levels of Cyclin-dependent kinase 1 (Cdk1), a kinase partner of CycA, rescue the short-sleeping phenotype of *tara* and *CycA* mutants, while increased Cdk1 activity mimics the *tara* and *CycA* phenotypes, suggesting that Cdk1 mediates the role of TARA and CycA in sleep regulation. Finally, we describe a novel wake-promoting role for a cluster of ~14 CycA-expressing neurons in the *pars lateralis* (PL), previously proposed to be analogous to the mammalian hypothalamus. We propose that TARANIS controls sleep amount by regulating CycA protein levels and inhibiting Cdk1 activity in a novel arousal center.

INTRODUCTION

Most animals sleep, and evidence for the essential nature of this behavior is accumulating [1–3]. However, we are far from understanding how sleep is controlled at a molecular and neural level. The fruit fly, *Drosophila melanogaster*, has emerged as a powerful model system for understanding complex behaviors such as sleep [4, 5]. Mutations in several *Drosophila* genes have been identified that cause significant alterations in sleep [5–13]. Some of these genes were selected as candidates because they were implicated in mammalian sleep [10, 11]. However, others (such as *Shaker* and *CREB*) whose role in sleep was first discovered in *Drosophila* [6, 12] have later been shown to be involved in mammalian sleep [14, 15], validating the use of *Drosophila* as a model system for sleep research. Since the strength of the *Drosophila* model system is the relative efficiency of large-scale screens, we and other investigators have conducted unbiased forward-genetic screens to identify novel genes involved in sleep regulation [6–9, 16]. Previous genetic screens for short-sleeping fly mutants have identified genes that affect neuronal excitability [6, 7], protein degradation [9, 16], and cell-cycle progression [8]. However, major gaps remain in our understanding of the molecular and anatomical basis of sleep regulation by these and other genes.

Identifying the underlying neural circuits would facilitate the investigation of sleep regulation. The relative simplicity of the *Drosophila* brain provides an opportunity to dissect these sleep circuits at a level of resolution that would be difficult to achieve in the more complex mammalian brain. Several brain regions, including the mushroom bodies, *pars intercerebralis*, dorsal fan-shaped body, clock neurons, and subsets of octopaminergic and dopaminergic neurons, have been shown to regulate sleep [17–23]. However, the recent discovery that Cyclin A (CycA) has a sleep-promoting role and is expressed in a small number of neurons distinct from brain regions detailed above [8] suggests the existence of additional neural clusters involved in sleep regulation.

From an unbiased forward-genetic screen, we discovered *taranis* (*tara*), a mutant that exhibits markedly reduced sleep amount. *tara* encodes a *Drosophila* homolog of the Trip-Br (SERTAD) family of mammalian transcriptional coregulators that are known primarily for their role in cell-cycle progression [24–27]. TARA and Trip-Br proteins contain a conserved domain found in several CycA-binding proteins [26]. Our research shows that *tara* regulates CycA levels and genetically interacts with *CycA* and its kinase partner *Cyclin-dependent kinase 1* (*Cdk1*) [28] to regulate sleep. Furthermore, we show that a cluster of CycA-expressing neurons in the dorsal brain lies in the *pars lateralis* (PL), a neurosecretory cluster previously proposed to

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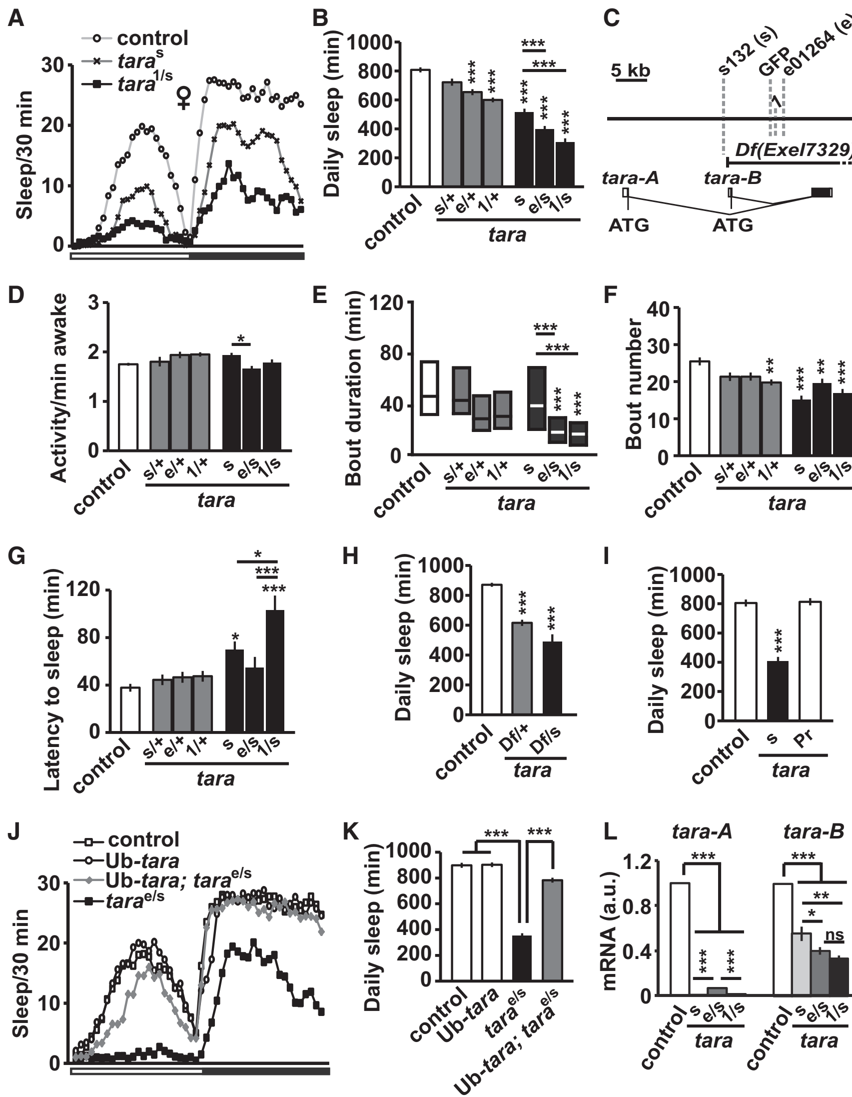

Figure 1. Sleep Phenotypes of *tara* Mutants

(A) Sleep profile of background control (white circles), *tara*^{s132} (*tara*^{s}, black X's), and *tara*$^{1/s132}$ (*tara*$^{1/s}$, black squares) female flies (n = 50–64) in 30-min bins. The white and black bars below the x axis represent 12-hr-light and 12-hr-dark periods, respectively.

(B) Total daily sleep amount for control and *tara* female flies of the indicated genotypes (n = 44–72). In this and subsequent figures, s132 and e01264 alleles are referred to as s and e, respectively.

(C) Schematic of the genomic region of the *tara* locus. Gray dashed lines indicate transposon insertion sites. The Exel7329 deficiency removes most of *tara*-A and all of *tara*-B coding regions as indicated.

(D–G) Waking activity (activity counts per waking minute) (D), sleep-bout duration (E), sleep-bout number (F), and sleep latency (time from lights off to the first sleep bout) (G) for the same female flies shown in (B). Sleep-bout duration is not normally distributed and is shown in simplified box plots, where the median and interquartile range are represented.

(H) Total daily sleep amount of control and Df(3R)Exel7329 female heterozygotes in *trans* to either a wild-type (Df/+) or *tara*^{s132} (Df/s) allele (n = 35–102).

(I) Total daily sleep of control, *tara*^{s132}, and precise excision (*tara*^{Pr}) female flies (n = 16–36).

(J) Sleep profile of female flies of the indicated genotypes (n = 53–58). The white and black bars below the x axis represent 12-hr-light and 12-hr-dark periods, respectively.

(K) Total daily sleep amount for the same flies showed in (J).

(L) *tara*-A and *tara*-B mRNA levels relative to *actin* mRNA levels in head extracts of indicated genotypes (n = 3–6). For each experiment, relative *tara* mRNA levels of the control flies were set to 1.

Mean ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant, one-way ANOVA followed by Tukey post hoc test (B, D, F, G, K, and L) or Dunnett post hoc test relative to control flies (H and I); Kruskal-Wallis test (E). For simplicity, only significant differences between the control and each mutant genotype (above the bar for the mutant) and those between *tara*^{s132}, *tara*$^{e01264/s132}$, and *tara*$^{1/s132}$ mutants (above the line for the mutant pair) are indicated. See also Figure S1.

be analogous to the mammalian hypothalamus, a major sleep center [29, 30]. Knockdown of *tara* and increased Cdk1 activity in CycA-expressing PL neurons, as well as activation of these cells, reduces sleep. Collectively, our data suggest that TARA promotes sleep through its interaction with CycA and Cdk1 in a novel arousal center.

RESULTS

Identification of *tara* as a Sleep-Regulatory Gene in *Drosophila*

In an ongoing forward-genetic screen for sleep and circadian mutants in *Drosophila* [31], we identified a novel transposon insertion line (s132) that resulted in a substantial reduction in daily sleep (Figures 1A, 1B, S1A, and S1B). Sleep was reduced in both female and male mutants relative to background controls. Using inverse-PCR, we mapped the s132 P-element insertion to the *tara* locus (Figure 1C), which suggests that TARA has a previously unappreciated role in sleep regulation. The *tara* transcription unit generates two transcripts (A and B) with alternative transcriptional and translational start sites [26] (http://flybase.org; Figure 1C). The two protein isoforms are identical except for a small number of N-terminal amino acids and appear to be functionally interchangeable [26].

For detailed characterization of the sleep phenotypes of *tara* mutants, we obtained two additional transposon insertions in the *tara* locus (*tara*^{1} and *tara*^{e01264}) from *Drosophila* stock centers (Figure 1C). s132 homozygotes are viable, but *tara*^{1} and *tara*^{e01264} homozygotes are lethal, suggesting that 1 and e01264 are stronger alleles than s132. Consistent with this view, when combined in *trans* with s132, the lethal alleles exhibited a greater reduction in sleep than s132 (Figures 1A, 1B, S1A, and S1B). The strong *tara* alleles resulted in a significant reduction in sleep even as heterozygotes (Figures 1B and

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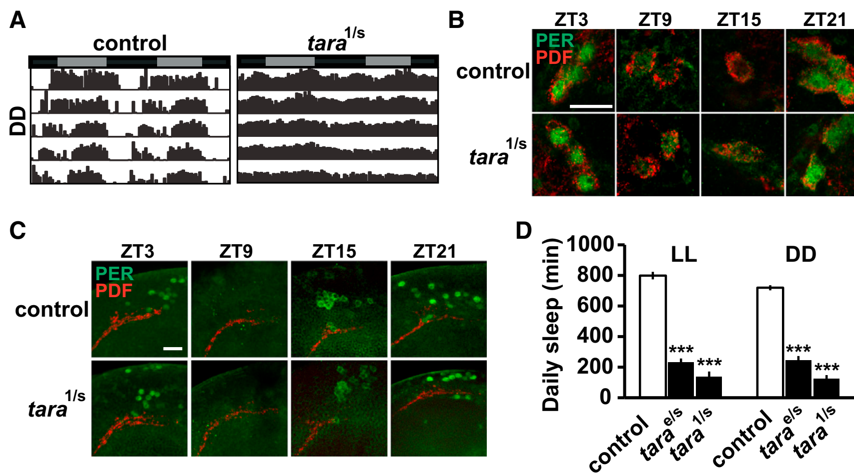

Figure 2. Circadian Phenotypes and Clock-Independent Sleep Loss of *tara* Mutants

(A) Representative circadian actogram of individual control and *tara*$^{1/s132}$ male flies in DD. Gray and black bars above the actogram indicate subjective day and night, respectively.

(B) Cycling of PER protein (green) in small ventral lateral neurons (s-LNvs) is normal in *tara*$^{1/s132}$ brains. Samples were dissected at indicated Zeitgeber times (ZT) and stained for PER and PDF (red), which was used to identify s-LNvs. Scale bar, 10 μm.

(C) Cycling of PER is also normal in a cluster of dorsal neurons (DN1s). Scale bar, 20 μm.

(D) Total daily sleep amount in LL and DD for females of indicated genotypes (n = 32–79 for LL; n = 39–96 for DD). Sleep levels on the third day in constant conditions are shown. Mean ± SEM is shown. ***$p < 0.001$, Dunnett post hoc tests relative to control flies (D).

See also Figure S2.

S1B). Whereas waking activity (activity counts per minute awake) was slightly increased in some *tara* mutants, it was not increased in strong allelic combinations (Figures 1D and S1C). Sleep-bout duration in both females and males was reduced in strong allelic combinations (Figures 1E and S1D), which suggests that TARA plays a role in sleep maintenance. The number of sleep bouts was markedly reduced in females with strong *tara* mutations (Figure 1F) and was unchanged in *tara* males (Figure S1E). In addition, sleep latency (time from lights off to the first sleep bout) was significantly increased in strong *tara* mutants (Figures 1G and S1F), revealing a role for TARA in sleep initiation. Taken together, our data demonstrate that *tara* is a novel sleep gene essential for sleep initiation and maintenance.

We undertook additional experiments to rule out the possibility that secondary, background mutations are responsible for the sleep phenotype in *tara* mutants. First, a deficiency line deleting the *tara* locus did not complement the s132 allele (Figures 1H and S1G). Second, precise excision of the s132 insertion by transposase-mediated mobilization restored normal sleep (Figures 1I and S1H). Third, ubiquitous expression of *tara*-B [26] restored sleep to nearly normal levels (Figures 1J, 1K, and S1I). These data confirm that disruption of *tara* is indeed the underlying cause of the severe sleep reduction in *tara* mutants.

As shown in Figures 1B and S1B, three *tara* allelic combinations (s132, e01264/s132, and 1/s132) yielded varying degrees of sleep reduction, suggesting that *tara*^{1} is the strongest allele and *tara*^{s132} is the weakest. To determine whether differences in *tara* mRNA levels mediate varying phenotypic strengths, we performed qRT-PCR using primers designed to distinguish between the two *tara* isoforms. *tara*^{s132} homozygous mutants had almost no detectable *tara*-A mRNA and an ~50% reduction in *tara*-B mRNA levels relative to control flies (Figure 1L). Like *tara*^{s132} mutants, *tara*$^{1/s132}$ flies had almost no detectable *tara*-A mRNA, but *tara*-B transcripts were further reduced, indicating that *tara*^{1} is a null or strongly hypomorphic allele. In *tara*$^{e01264/s132}$ flies, *tara*-A mRNA levels were slightly higher than in *tara*$^{1/s132}$ flies while *tara*-B mRNA levels were lower than in *tara*^{s132} homozygous flies. These results demonstrate that the amount of daily sleep correlates with *tara* levels. Collectively, the above data establish *tara* as a novel sleep regulatory gene.

Sleep Loss in *tara* Mutants Is Independent of the Circadian Clock and Light

To examine whether *tara* mutants exhibit circadian phenotypes, we monitored their locomotor activity in constant darkness (DD). Most *tara*$^{1/s132}$ mutants were arrhythmic or weakly rhythmic and the amplitude of their circadian rhythmicity was reduced, but the period length of all *tara* mutants was indistinguishable from that of control flies (Figures 2A and S2A). Moreover, daily cycling of the core clock protein PERIOD (PER) in *tara*$^{1/s132}$ mutants was similar to that in wild-type controls in two sets of clock neurons (Figures 2B and 2C), which suggests that dampened rhythmicity in these mutants is not due to a defect in the core molecular clock. Since arrhythmicity does not necessarily lead to short sleep (e.g., *per* and *timeless* mutants do not have reduced sleep [32]), the rhythm phenotype of *tara* mutants may not be the cause of the sleep phenotype. Our data showing that *tara*$^{e01264/s132}$ mutants displayed almost as severe a sleep reduction as *tara*$^{1/s132}$ but were largely rhythmic (Figures 1B, S1B, and S2A) support the view that the sleep and circadian phenotypes in *tara* mutants may not be linked. To test whether the sleep phenotype in *tara* mutants was due to arrhythmicity, we assayed sleep in constant light (LL), in which both control and mutant flies are arrhythmic. Indeed, *tara* mutants had greatly reduced sleep compared with controls in LL, demonstrating that the short-sleeping phenotype is not caused by arrhythmicity (Figures 2D and S2B). The short-sleeping phenotype was also observed in DD (Figures 2D and S2B), suggesting that TARA's role in sleep is independent of light. Of note, in both LL and DD, *tara*$^{1/s132}$ mutants lost over 80% of sleep relative to control flies, which is one of the most severe phenotypes documented among sleep mutants. These data show that *tara* mutants exhibit a striking reduction in sleep amount, independent of the circadian clock and light conditions.

TARA Is Required in Neurons to Control Sleep Levels

To examine the spatial requirements for TARA in regulating sleep, we generated a polyclonal antibody against the TARA protein (see Experimental Procedures). In western blots, the antibody recognized a band that is upregulated when TARA is overexpressed in *Drosophila* S2 cells. As expected, this band was

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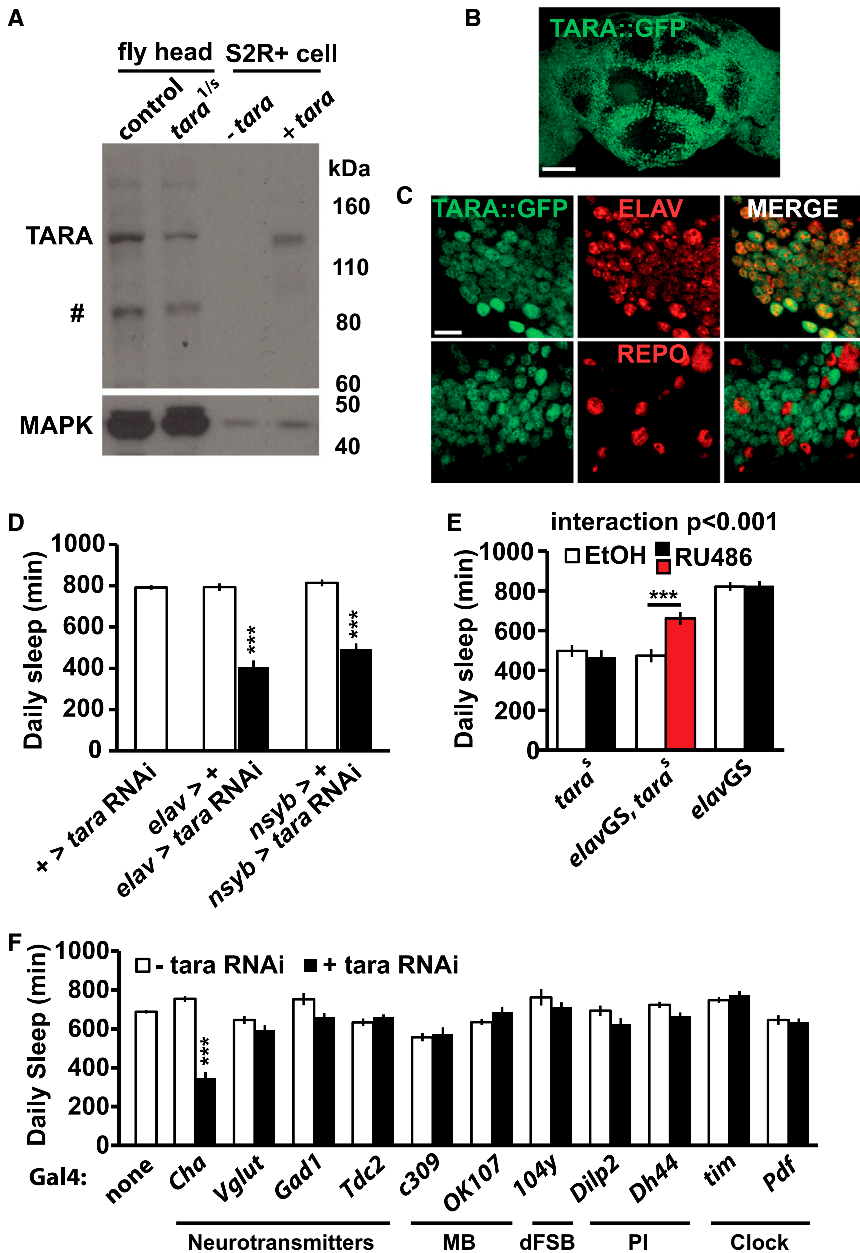

Figure 3. TARA Regulates Sleep in Neurons

(A) Western blot showing a marked reduction of TARA in *tara*$^{1/s132}$ mutants compared with control flies. Head extracts of control flies and *tara* mutants (lanes 1 and 2) as well as S2 cell extracts transfected with an empty UAS vector or a UAS-*tara* construct under the control of *actin*-Gal4 (lanes 3 and 4) were probed with a polyclonal antibody to TARA. The band that corresponds to TARA can be readily recognized by the upregulation in S2 cells transfected with *tara* cDNA. # denotes non-specific labeling or a degradation product of TARA. MAPK was used to control for loading.

(B) Maximal-intensity projection of confocal slices of the adult brain showing widespread expression of TARA::GFP. Scale bar, 50 μm.

(C) Representative confocal sections of *tara::GFP* adult brains costained with antibodies to GFP and ELAV or REPO (neuronal or glial marker, respectively). Each panel shows a single confocal slice of a region ventral to the mushroom bodies. Scale bar, 10 μm.

(D) Pan-neuronal knockdown of *tara* markedly reduces sleep. Pan-neuronal *elav*-Gal4 or *nsyb*-Gal4 was used to drive a combination of two UAS-*tara* RNAi constructs and UAS-*dcr2* (*elav*>*tara* RNAi and *nsyb*>*tara* RNAi, respectively). Flies harboring the two UAS-*tara* RNAi constructs and UAS-*dcr2* without a driver (+ > *tara* RNAi) and those harboring a driver and UAS-*dcr2* (*elav* > + or *nsyb* > +) served as controls (n = 31–58).

(E) Adult-stage expression of *tara* partially rescues the *tara* short-sleeping phenotype. Daily sleep is presented for females of the indicated genotypes in the absence (white bar) or presence (black or red bar) of RU486 (n = 24–32). Data from parental control flies show that RU486 by itself did not affect sleep.

(F) Knockdown of *tara* in cholinergic neurons reduces sleep. For each Gal4, total daily sleep of females expressing *tara* RNAi under the control of the driver (black bar) was compared to parental controls (white bar) (n = 30–173). The sleep phenotype of flies in which *tara* was knocked down in dopaminergic neurons was not determined due to lethality. Mean ± SEM is shown.

***p < 0.001, Dunnett post hoc test relative to both parental controls (D), t test with Bonferroni correction (E), Tukey post hoc test relative to both parental controls (F). See also Figure S3.

markedly downregulated in head extracts of *tara*$^{1/s132}$ mutants compared with those of control flies (Figure 3A). The identity of the band was further examined by western analysis of a previously generated GFP fusion trap in the *tara* locus (YB0035) [33], which we termed *tara::GFP*. The *GFP* exon is located upstream of the common second coding exon of both *tara*-A and *tara*-B isoforms (Figure 1C) and is expected to be incorporated into both isoforms close to the N terminus. The presumed TARA band in western blots was shifted by the addition of GFP in head extracts of *tara::GFP* flies (Figure S3A), which confirms that the band indeed represents the TARA protein. Because the polyclonal antibody did not yield a specific signal when used for immunohistochemistry, we employed the TARA::GFP fusion protein to determine the expression pattern of TARA. Homozygotes for the *tara::GFP* allele did not exhibit altered sleep levels or circadian phenotypes (Figures S2A, S3B, and S3C), indicating that the TARA::GFP fusion protein is functional. Since the *GFP* coding region is inserted into the *tara* locus in the genome, the TARA::GFP expression pattern is likely to reflect endogenous TARA expression accurately. We thus examined the localization of TARA::GFP in the adult nervous system using an anti-GFP antibody. TARA::GFP was widely expressed throughout the adult brain (Figure 3B). Costaining with neuronal and glial markers (ELAV and REPO, respectively) demonstrated that TARA is expressed in most, perhaps all, neurons but excluded from glial cells (Figure 3C).

Given the expression pattern, we sought to demonstrate a role for neuronal TARA in regulating sleep. We used RNAi to

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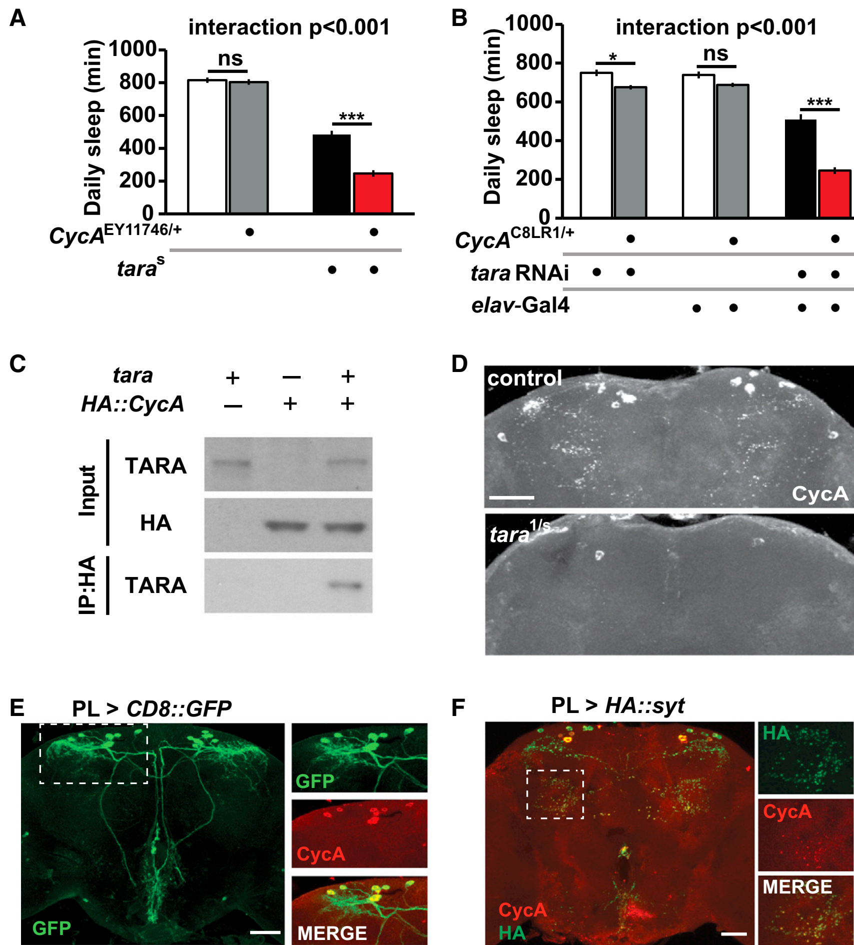

Figure 4. *tara* Interacts with *CycA* and Regulates CycA Levels

(A) Daily sleep for female flies of indicate genotypes demonstrating a synergistic interaction between $cycA^{EY11746/+}$ and $tara^{s132}$ (n = 43–60).

(B) Daily sleep for female flies of the indicated genotypes. Pan-neuronal knockdown of *tara* by RNAi (*elav>tara* RNAi) was more effective at suppressing sleep in a $CycA^{C8LR1/+}$ background than in a control background (n = 46–52).

(C) TARA and CycA form a complex in S2 cells. HA-tagged CycA was immunoprecipitated with an anti-HA antibody, and anti-TARA and anti-HA antibodies were used for western blotting. The experiment was repeated three times with similar results.

(D) Maximal-intensity confocal projections of the dorsal half of the central brain of representative control and $tara^{1/s132}$ adult females immunostained with an antibody to CycA. Scale bar, 50 μm.

(E) The central brain of an adult fly in which PL-Gal4 was used to express membrane-targeted CD8::GFP. Scale bar, 50 μm. Images on the right show colocalization of CycA and GFP driven by PL-Gal4 in the brain region indicated by the rectangle.

(F) The central brain of a fly in which the synaptic marker HA::SYT was expressed using PL-Gal4. The brain was costained with anti-HA and anti-CycA. The rectangle indicates the region magnified in the images on the right. Scale bar, 50 μm.

Mean ± SEM is shown. *p < 0.05; ***p < 0.001; ns, not significant, Tukey post hoc test. See also Figure S4.

reduce TARA expression specifically in neurons. As expected, driving *tara* RNAi with pan-neuronal drivers *elav*-Gal4 and *nsyb*-Gal4 resulted in a substantial reduction in daily sleep levels (Figures 3D and S3D). Reduced TARA expression by RNAi-mediated knockdown was confirmed by western analysis (Figure S3E). We next examined whether *tara* functions in the adult fly to regulate sleep by utilizing a UAS site in the s132 insertion to drive *tara* expression. We used GeneSwitch (GS), an RU486-dependent GAL4 protein that allows temporal control of transgenic expression [34]. Adult specific pan-neuronal expression of *tara* using the *elav*-GS driver partially rescued the short-sleeping phenotype of *tara* mutants (Figure 3E), demonstrating an adult function of *tara,* although the incomplete adult-stage rescue suggests a potential developmental role as well.

To identify neuronal groups where TARA acts to control sleep, we utilized the Gal4/UAS system to target *tara* RNAi expression to subsets of neurons. We examined several neurotransmitter systems as well as brain regions involved in sleep regulation such as the mushroom bodies (MB), dorsal fan-shaped body (dFSB), *pars intercerebralis* (PI), and clock cells. Only *tara* knockdown by *Cha*-Gal4 produced a significant reduction in sleep (Figure 3F). These data suggest that cholinergic neurons likely mediate the effects of TARA on sleep.

tara Interacts with *CycA* to Control Sleep and Regulates CycA Levels

Since CycA has been shown to promote sleep in *Drosophila* [8], and since TARA contains a conserved CycA binding homology motif, we tested whether *tara* and *CycA* act in a common genetic pathway to regulate sleep. To do so, we generated double mutants and compared their sleep behavior with those of wild-type control and single mutant flies. The $CycA^{EY11746/+}$ heterozygous mutation did not cause reduced sleep on its own, but it led to a significant reduction in sleep when combined with the $tara^{s132}$ hypomorphic mutation that has a moderate sleep phenotype (Figures 4A and S4A). This interaction was confirmed using a second allele of *CycA* ($CycA^{C8LR1/+}$) and *tara* RNAi (Figure 4B). Further, *CycA* did not exhibit a genetic interaction with the DAT^{fmn} short-sleeping mutant [13] (Figure S4B), demonstrating the specificity of the interaction between *tara* and *CycA*. These data reveal a synergistic interaction between *tara* and *CycA* and suggest they act in the same pathway to influence sleep.

Given the genetic interaction between *tara* and *CycA,* the presence of a CycA-binding homology domain in TARA, and the fact that Trip-Br1/2, mammalian homologs of TARA, have been shown to bind CycA [24], we tested whether TARA physically binds CycA in a co-immunoprecipitation assay. Indeed, we found that TARA co-immunoprecipitated with CycA in

Drosophila S2 cells (Figure 4C), suggesting that they can form a complex.

We next asked whether CycA levels are altered in *tara* mutants. We performed whole-mount immunostaining of adult brains using a CycA antibody previously shown to detect a dorsal set of CycA-positive neurons [8] (another CycA antibody previously used to detect a few additional clusters of CycA-expressing neurons is no longer available). We found that CycA protein levels were greatly reduced in the adult brain of *tara* mutants (Figure 4D). In contrast, CycA protein levels were not reduced in DAT^{fmn} mutants (Figure S4C), which demonstrates the specificity of the regulation of CycA levels by TARA. *CycA* mRNA levels were not affected in *tara* mutants (Figure S4D), indicating that TARA regulates CycA levels post-transcriptionally. Our data suggest that TARA promotes sleep in part through regulation of CycA protein levels.

We noticed that the dorsal CycA cluster might correspond to the *pars lateralis* (PL) [35], so we drove expression of CD8::GFP using PL-Gal4, a driver expressed in the PL neurons [36], while simultaneously labeling brains for CycA. Both GFP and CycA were expressed in ~14 neurons with large cell bodies in the dorsal brain (Figures 4E and S4E). The striking overlap seen between the GFP and CycA signals demonstrates that the dorsal CycA neurons indeed lie in the PL. This is significant because the PL, along with the *pars intercerebralis*, shares several features with the mammalian hypothalamus, a major sleep center [29, 30]. However, a possible contribution of the PL to sleep regulation has not been previously explored.

We employed the PL driver to determine whether the CycA-expressing cells were present in *tara* mutants. By examining flies expressing CD8::GFP under the control of PL-Gal4, we confirmed that the PL neurons were indeed present (Figure S4F). Interestingly, CycA protein was observed not only in cell bodies, but also in discrete puncta that appeared to be synapses (Figure 4D). This is noteworthy because according to the synaptic homeostasis hypothesis, waking activity leads to a net increase in synaptic strength, whereas sleep leads to overall downscaling of synapses [37]. To determine whether these puncta represent synapses, we used PL-Gal4 to express a synaptic marker (HA::SYT) [38] and demonstrated that CycA indeed localized to synaptic regions (Figure 4F). We note that CycA protein levels were downregulated in both cell bodies and synaptic regions in *tara* mutants (Figure 4D). CycA levels and function at synapses, under the control of TARA, may be important for normal sleep.

TARA Regulates Sleep in CycA-Expressing PL Neurons, which Define a New Arousal Center

To address whether TARA is required in CycA-expressing cells for sleep regulation, we examined the sleep phenotype of flies in which *tara* was knocked down using the PL driver. We found that PL-specific *tara* knockdown significantly reduced sleep (Figure 5A). We note that this manipulation produced a weaker sleep reduction than pan-neuronal knockdown of *tara* (Figure 3D), which suggests that TARA likely functions in additional groups of neurons to regulate sleep.

Our results pointed to a possible role of the PL neurons in sleep regulation. Indeed, we found that activation of the PL neurons via expression of the bacterial sodium channel NaChBac [39] led to decreased sleep (Figures 5B, 5C, S5A, and S5B). In contrast, activity levels during waking periods were not affected by PL activation (Figures 5D and S5C). Sleep-bout duration was markedly decreased while sleep-bout number showed little change, and sleep latency was significantly increased in flies with activated PL neurons (Figures 5E–5G, and S5D–S5F). These data suggest that activation of PL neurons promote wakefulness by delaying sleep onset and impairing sleep maintenance. Adult-stage specific activation of these neurons using the warmth-activated cation channel TrpA1 [40] also reduced sleep, demonstrating that this cell cluster functions in adult animals to promote wakefulness (Figures 5H, 5I, and S5G). Further, blocking the activity of PL neurons with tetanus toxin [41] significantly increased sleep (Figure 5J), which confirms the wake-promoting role of these neurons. The above data identify the PL neurons as a novel arousal center and demonstrate that TARA acts, at least in part, in CycA-positive PL neurons to promote sleep.

tara and *Cdk1* Interact Antagonistically to Regulate Sleep

CycA has been shown to bind Cdk1 and can either increase or decrease Cdk1 activity depending on the cellular context [28, 42]. We therefore asked whether *Cdk1* also interacts with *tara* for sleep regulation. We introduced a heterozygous $Cdk1^{GT\text{-}000294/+}$ mutation (the GT-000294 insertion is in the coding region and is likely to be a null allele) into a *tara* mutant background and compared their sleep with *tara* and *Cdk1* single mutants as well as with wild-type control flies. We found that the $Cdk1^{GT\text{-}000294/+}$ heterozygous mutation did not cause a sleep phenotype in a wild-type background, but it resulted in a substantial rescue of the *tara* sleep phenotype (Figures 6A and 6B). We confirmed the antagonistic interaction between *tara* and *Cdk1* using a second allele of *Cdk1* ($Cdk1^{c03495/+}$) (Figure S6A). The $Cdk1^{GT\text{-}000294/+}$ heterozygous mutation did not rescue the short-sleeping phenotype of *insomniac* (*inc*) mutants (Figure S6B) [9, 16], which demonstrates that the interaction between *tara* and *Cdk1* is not due to additive effects. In contrast, the *Cdk1* mutation did rescue the sleep phenotype of heterozygous *CycA* null mutants (Figure 6C), consistent with a model in which *tara* and *CycA* act together to antagonize *Cdk1*. Transcript levels of *Cdk1* were not significantly affected in *tara* mutants (Figure S6C), suggesting that the interaction between *tara* and *Cdk1* is not likely to be due to transcriptional regulation of *Cdk1* by TARA. The antagonistic interaction between *tara* and *Cdk1* suggests that *Cdk1* has a previously unrecognized wake-promoting role.

To investigate the potential wake-promoting role of Cdk1, we assayed sleep in flies overexpressing wild-type Cdk1 (Cdk1-WT). Since activity of Cyclin-dependent kinases is tightly controlled by a number of regulatory molecules [42–44], we also examined flies overexpressing Cdk1-AF, a mutant Cdk1 protein that has elevated kinase activity due to mutations in inhibitory phosphorylation sites [42]. Because overexpression of Cdk1-AF under the control of *elav*-Gal4 resulted in lethality, we used the RU486 inducible *elav*-GS to express Cdk1 specifically in the adult stage. Whereas RU486 had little effect on control flies, flies in which Cdk1-AF was expressed under the control of *elav*-GS exhibited significantly reduced sleep when fed RU486 (Figure 6D), which indicates that increased Cdk1 activity indeed promotes wakefulness. In contrast, overexpression of Cdk1-WT had little effect on sleep (Figure S6C), presumably because

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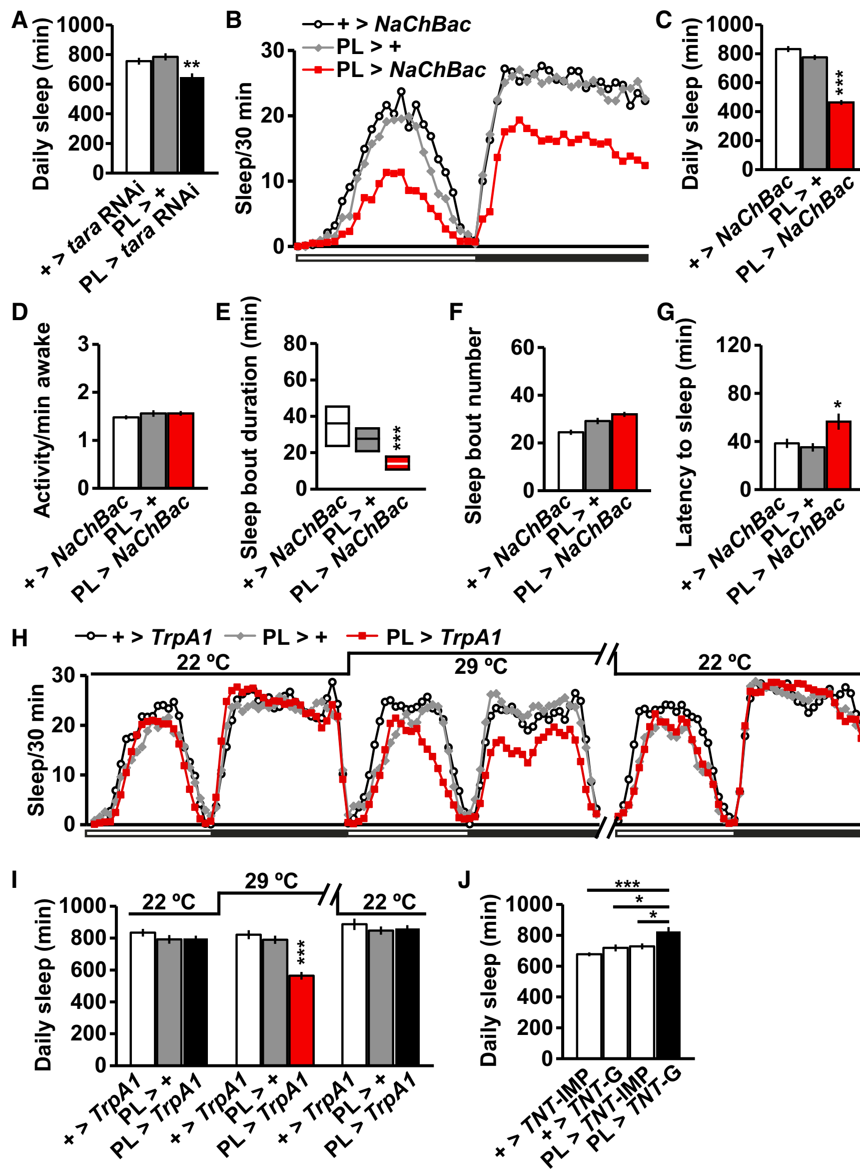

Figure 5. TARA Regulates Sleep in CycA-Expressing PL Neurons that Define a New Arousal Center

(A) Total daily sleep of female flies of the indicated genotypes (n = 32).

(B) Sleep profile for female flies expressing the NaChBac sodium channel under the control of PL-Gal4 (PL > *NaChBac*) and parental controls (n = 48–63).

(C–G) Total daily sleep (C), waking activity (D), sleep-bout duration (E), sleep-bout number (F), and latency to sleep after lights off (G) for the flies shown in (B).

(H) Sleep profile for female flies expressing *TrpA1* under the control of PL-Gal4 (PL > *TrpA1*) and 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. Sleep profile for the second day at 29°C is omitted for simplicity.

(I) Total daily sleep for flies shown in (H).

(J) Female flies expressing functional tetanus toxin under the control of PL-Gal4 (PL > *TNT-G*) exhibited a significant increase in sleep relative to flies expressing inactive tetanus toxin (PL > *TNT-IMP*) or those carrying either form of tetanus toxin transgene without the PL driver (+ > *TNT-G* and + > *TNT-IMP*) (n = 30–32).

Mean ± SEM is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, not significant; Dunnett post hoc test relative to parental controls (A, C, D, F, G, and I) or PL > *TNT-G* flies (J); Kruskal-Wallis test (E). See also Figure S5.

overexpression of wild-type Cdk1 alone was not sufficient to increase its kinase activity. To examine whether Cdk1 acts in CycA-expressing cells to regulate sleep, we assayed sleep in flies expressing the *Cdk1*-AF transgene under the control of PL-Gal4. These flies had significantly reduced sleep compared with parental control flies (Figure 6E). These data provide strong evidence for a novel role of Cdk1 in suppressing sleep.

Since CycA is expressed in synaptic regions (Figure 4F), we next asked whether Cdk1 colocalizes with CycA at synaptic regions. To address this question, we expressed MYC-tagged wild-type Cdk1 [45] in PL neurons and found that Cdk1::MYC exhibited marked overlap with CycA puncta at synaptic regions (Figure 6F). Although the synaptic localization of Cdk1::MYC could be an artifact of overexpression, the potential colocalization of Cdk1 and CycA at synaptic regions raises the interesting possibility that synaptic Cdk1 activity may be important for maintaining normal sleep amount. Together, our data demonstrate that Cdk1 interacts antagonistically with TARA and CycA and acts in PL neurons to promote wakefulness.

DISCUSSION

From an unbiased forward genetic screen, we have identified a novel sleep regulatory gene, *tara*. Our data demonstrate that TARA interacts with CycA to regulate its levels and promote sleep. We have also identified Cdk1 as a wake-promoting molecule that interacts antagonistically with TARA. Given the fact that TARA regulates CycA levels, the interaction between TARA and Cdk1 may be mediated by CycA. Our finding that *Cdk1* and *CycA* also exhibit an antagonistic interaction supports this view. The previous discovery that CycE sequesters its binding partner Cdk5 to repress its kinase activity in the adult mouse brain [46] points to a potential mechanism, namely that TARA regulates CycA levels, which in turn sequesters and inhibits Cdk1 activity. TARA and its mammalian homologs (the Trip-Br family of proteins) are known for their role in cell-cycle progression [24–27]. However, recent data have shown that Trip-Br2 is involved in lipid and oxidative metabolism in adult mice [47], demonstrating a role beyond cell-cycle control. Other cell-cycle proteins have also been implicated in processes unrelated to the cell cycle. For example, CycE functions in the adult mouse brain to regulate learning

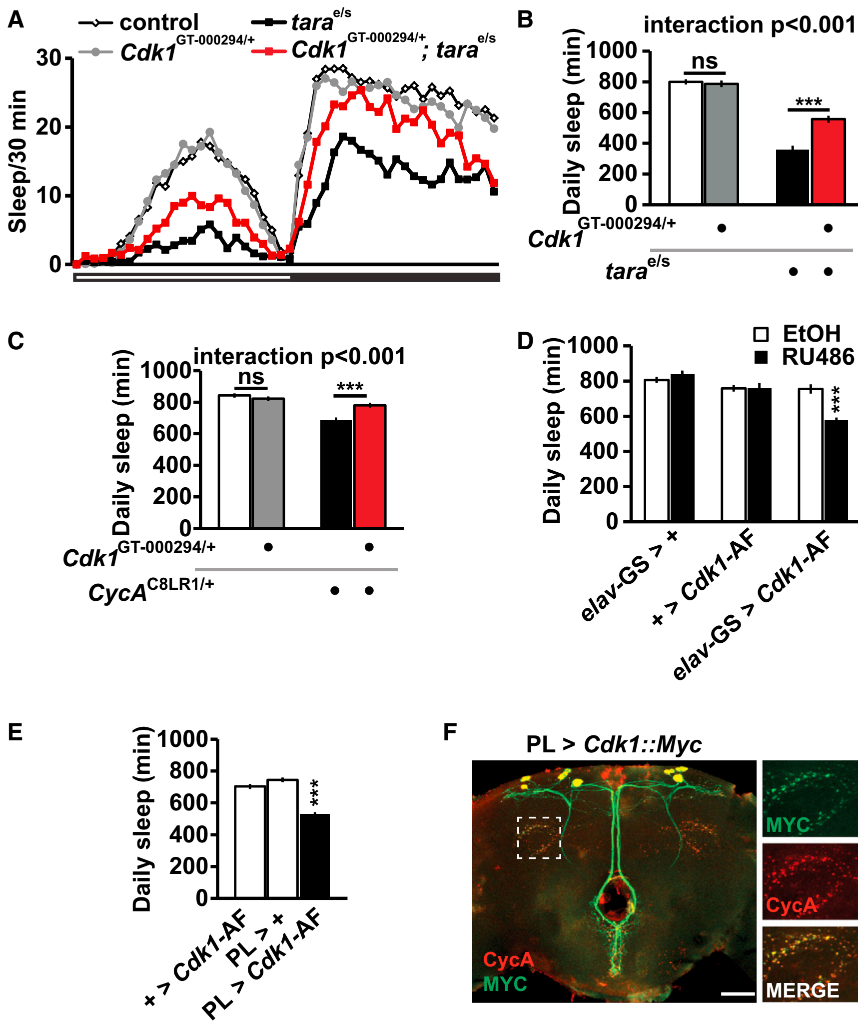

Figure 6. *Cdk1* Interacts Antagonistically with *tara* and *CycA*, and Increased Cdk1 Activity Suppresses Sleep

(A) Sleep profile for control (white diamonds), *Cdk1*$^{GT\text{-}000294/+}$ (gray circles), *tara*$^{e01264/s132}$ (black squares), and *Cdk1*$^{GT\text{-}000294/+}$; *tara*$^{e01264/s132}$ (red squares) female flies (n = 50–64).

(B) Total daily sleep for the flies shown in (A).

(C) Total daily sleep of female flies of the indicated genotypes (n = 81–88).

(D) Adult-stage specific expression of Cdk1-AF induced by feeding flies food that contain RU486 diluted in ethanol (EtOH) reduced sleep (n = 31–32).

(E) Cdk1-AF expression specifically in CycA-expressing PL neurons resulted in reduced sleep (n = 70–75).

(F) The central brain of a fly in which UAS-*Cdk1-myc* was expressed using PL-Gal4. The brain was costained with anti-MYC and anti-CycA. The rectangle indicates the region magnified in the images on the right. Scale bar, 50 μm.

Mean ± SEM is shown. ***p < 0.001; ns, not significant; Tukey post hoc test (B and C), Dunnett post hoc test relative to all other controls (D); relative to both parental controls (E). See also Figure S6.

and memory [46]. Based on the finding that CycA and its regulator Rca1 control sleep, it was hypothesized that a network of cell-cycle genes was appropriated for sleep regulation [8]. Our data showing that two additional cell-cycle proteins, TARA and Cdk1, control sleep and wakefulness provide support for that hypothesis. Moreover, the fact that TARA and CycA, factors identified in two independent unbiased genetic screens, interact with each other highlights the importance of a network of cell-cycle genes in sleep regulation.

There are two main regulatory mechanisms for sleep: the circadian mechanism that controls the timing of sleep and the homeostatic mechanism that controls the sleep amount [48]. We have shown that TARA has a profound effect on total sleep time. TARA also affects rhythmic locomotor behavior. Since TARA is expressed in clock cells (our unpublished data), whereas CycA is not [8], it is possible that TARA plays a non-CycA dependent role in clock cells to control rhythm strength. Our finding that *tara* mutants exhibit severely reduced sleep in constant light suggests that the effect of TARA on sleep amount is not linked to its effect on rhythmicity. Instead, TARA may have a role in the sleep homeostatic machinery, which will be examined in our ongoing investigation.

To fully elucidate how sleep is regulated, it is important to identify the underlying neural circuits. Here, we have shown that activation of the CycA-expressing neurons in the PL suppresses sleep while blocking their activity increases sleep, which establishes them as a novel wake-promoting center. Importantly, knockdown of *tara* and increased Cdk1 activity specifically in the PL neurons leads to decreased sleep. A simple hypothesis, consistent with our finding that both activation of PL neurons and increased Cdk1 activity in these neurons suppress sleep is that Cdk1 affects neuronal excitability and synaptic transmission. Interestingly, large-scale screens for short-sleeping mutants in fruit flies and zebrafish have identified several channel proteins such as SHAKER, REDEYE, and ETHER-A-GO-GO [6, 49, 50] and channel modulators such as SLEEPLESS and WIDE AWAKE [51, 52]. Thus, it is plausible that Cdk1 regulates sleep by phosphorylating substrates that modulate the function of synaptic ion channels or proteins involved in synaptic vesicle fusion, as has previously been demonstrated for Cdk5 at mammalian synapses [53].

Whereas our data mapped some of TARA's role in sleep regulation to a small neuronal cluster, the fact that pan-neuronal *tara* knockdown results in a stronger effect on sleep than specific knockdown in PL neurons suggests that TARA may act in multiple neuronal clusters. PL-specific restoration of TARA expression did not rescue the *tara* sleep phenotype (data not shown), further implying that the PL cluster may not be the sole anatomical locus for TARA function. Given that CycA is expressed in a few additional clusters [8], TARA may act in all

CycA-expressing neurons including those not covered by PL-Gal4. TARA may also act in non-CycA-expressing neurons. Our data demonstrate that *tara* knockdown using *Cha*-Gal4 produces as strong an effect on sleep as pan-neuronal knockdown (Figures 3D and 3F). This finding suggests that TARA acts in cholinergic neurons, although we cannot rule out the possibility that the *Cha*-Gal4 expression pattern includes some non-cholinergic cells. Taken together, our data suggest that TARA acts in PL neurons as well as unidentified clusters of cholinergic neurons to regulate sleep.

Based on genetic interaction studies, *tara* has been classified as a member of the *trithorax* group genes, which typically act as transcriptional coactivators [26, 54]. However, TARA and Trip-Br1 have been shown to up- or downregulate the activity of E2F1 transcription factor depending on the cellular context, raising the possibility that they also function as transcriptional corepressors [24, 27]. Interestingly, TARA physically interacts with CycA and affects CycA protein levels but not its mRNA expression. These findings suggest a novel non-transcriptional role for TARA, although we cannot rule out an indirect transcriptional mechanism. The hypothesis that TARA plays a non-transcriptional role in regulating CycA levels and Cdk1 activity at the synapse may provide an exciting new avenue for future research.

EXPERIMENTAL PROCEDURES

Details of experimental procedures are available in the online Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.05.037.

AUTHOR CONTRIBUTIONS

K.K. conceived the study, and D.J.S.A. and K.K. designed the experiments and analyzed the data. D.J.S.A. performed the experiments with the help of D.L., D.R.M., J.E.C.J., and H.P., and D.R. identified the dorsal CycA-expressing cells as the *pars lateralis* cluster. The manuscript was written by K.K. and D.J.S.A. with editorial input from D.R. and J.E.C.J.

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