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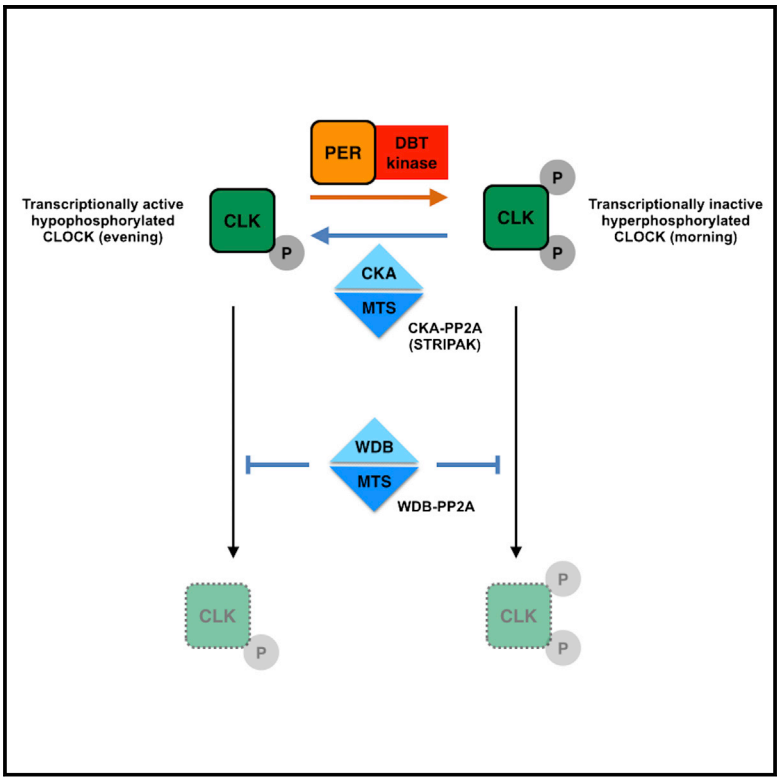
Daytime CLOCK Dephosphorylation Is Controlled by STRIPAK Complexes in *Drosophila*

Graphical Abstract



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In Brief

Andreazza et al. show that two types of PP2A phosphatase complexes promote the activity of the CLOCK transcriptional activator through different mechanisms in the *Drosophila* circadian clock. The PP2A STRIPAK complexes that include CKA and its interacting protein STRIP induce daytime CLK dephosphorylation, whereas the PP2A/WDB complexes stabilize CLOCK.

Highlights

- Different PP2A complexes control the activity of the *Drosophila* CLOCK (CLK) protein
- STRIP-containing PP2A/CKA (STRIPAK) complexes promote daytime CLK dephosphorylation
- PP2A/WDB complexes stabilize CLK

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Daytime CLOCK Dephosphorylation Is Controlled by STRIPAK Complexes in *Drosophila*

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SUMMARY

In the *Drosophila* circadian oscillator, the CLOCK/CYCLE complex activates transcription of *period (per)* and *timeless (tim)* in the evening. PER and TIM proteins then repress CLOCK (CLK) activity during the night. The pace of the oscillator depends upon post-translational regulation that affects both positive and negative components of the transcriptional loop. CLK protein is highly phosphorylated and inactive in the morning, whereas hypophosphorylated active forms are present in the evening. How this critical dephosphorylation step is mediated is unclear. We show here that two components of the STRIPAK complex, the CKA regulatory subunit of the PP2A phosphatase and its interacting protein STRIP, promote CLK dephosphorylation during the daytime. In contrast, the WDB regulatory PP2A subunit stabilizes CLK without affecting its phosphorylation state. Inhibition of the PP2A catalytic subunit and CKA downregulation affect daytime CLK similarly, suggesting that STRIPAK complexes are the main PP2A players in producing transcriptionally active hypophosphorylated CLK.

INTRODUCTION

Circadian clocks govern a wide range of physiological and behavioral rhythms to adapt organisms to the 24-hr day-night cycles generated by the rotation of the Earth on its axis. They stem upon feedback loops where transcription factors activate the expression of their own inhibitors, which repress transcription with a timely regulated delay. In the *Drosophila* circadian oscillator, the CLOCK (CLK) and CYCLE (CYC) bHLH-PAS domain transcription factors activate expression of the *period (per)* and *timeless (tim)* genes at the end of the day (Hardin, 2011; Ozkaya and Rosato, 2012). In a second loop, CLK/CYC activate the *vrille (vri)* and *pdp1ε* genes, whose protein products repress (VRI) or activate (PDP1ε) the transcription of the *Clk* gene. The delayed accumulation of PER and TIM leads to transcriptional repression of CLK/CYC during the late night, and their subsequent morning degradation allows transcription to resume in the second part of the day. PER and TIM stability is largely controlled by phosphorylation. PER is phosphorylated by the DOUBLETIME (DBT, CK1δ/ε), CK2, and NEMO kinases and polyubiquitylated by the SCF^{Slimb} ubiquitin ligase complex (Kloss et al., 1998; Price et al., 1998; Ko et al., 2002; Grima et al., 2002; Lin et al., 2002; Akten et al., 2003; Nawathean and Rosbash, 2004; Chiu et al., 2008, 2011). TIM phosphorylation involves the CK2 and SHAGGY (SGG, GSK-3) kinases, and TIM degradation also depends on SCF^{Slimb} and a Cullin-3-based ubiquitin ligase complex (Martinek et al., 2001; Grima et al., 2002, 2012; Lin et al., 2002; Meissner et al., 2008). In addition, PER and TIM are controlled by phosphatase activities: PP2A regulates PER abundance and nuclear entry (Sathyanarayanan et al., 2004), while PP1 dephosphorylates TIM (Fang et al., 2007).

CLK phosphorylation cycles with a peak in the morning and requires both PER and DBT (Lee et al., 1998; Kim and Edery, 2006; Yu et al., 2006; Hung et al., 2009). However, DBT kinase activity does not seem to be required, and it was proposed that DBT function is to recruit other kinases (Yu et al., 2009). A similar cycling was reported for CLK immunoreactivity (Hung et al., 2009; Menet et al., 2010; Lamaze et al., 2011), but relatively constant CLK levels have been observed with harsh extraction conditions (Yu et al., 2006; Sun et al., 2010; Luo et al., 2012). CLK DNA-binding and transcriptional activity are maximal in the evening when CLK phosphorylation is minimal (Yu et al., 2006; Menet et al., 2010; Abruzzi et al., 2011). The release of CLK from DNA correlates with its PER/DBT-dependent hyperphosphorylation (Yu et al., 2006; Kim and Edery, 2006; Menet et al., 2010). In addition to DBT, kinases known to be involved in CLK phosphorylation include NEMO (NMO), which destabilizes CLK (Yu et al., 2011), and CK2α that stabilizes CLK and inhibits its transcriptional activity (Szabó et al., 2013). CLK is also regulated by ubiquitylation with the HECT-domain ubiquitin ligase CTRIP destabilizing CLK (Lamaze et al., 2011), and the USP8 ubiquitin protease decreasing its transcriptional activity (Luo et al., 2012). Finally, CLK activity is controlled by repressors/activators such as CLOCKWORK ORANGE (CWO) (Matsumoto et al., 2007; Kadener et al., 2007; Lim et al., 2007; Richier et al., 2008) and the FOS ortholog KAYAK (KAY) (Ling et al., 2012).

PP2A is the main source of serine/threonine phosphatase activity in the cell (Virshup and Shenolikar, 2009). PP2A holoenzymes are predominantly heterotrimers in which the scaffold A and catalytic C subunits associate with a single variable regulatory B subunit, which defines substrate specificity and holoenzyme localization (Shi, 2009; Virshup and Shenolikar, 2009). In *Drosophila*, the scaffold and catalytic subunits are encoded by the *pp2a-29b* and *microtubule star (mts)* genes, respectively. Regulatory subunits are encoded by *twins (tws)* (B type), *widerborst (wdb)* and *PP2A-B′* (B′ type), *CG4733* (B″ type), and *Connector of kinase to AP-1 (Cka)* (B‴ type). CKA is the ortholog of the mammalian striatin proteins (Ribeiro et al., 2010). B‴-containing PP2A holoenzymes are associated with other proteins including the striatin-interacting protein (STRIP) and kinases in STRIPAK (STRiatin-Interacting Phosphatase And Kinase) complexes (Glatter et al., 2009; Goudreault et al., 2009; Herzog et al., 2012; Ribeiro et al., 2010; Kean et al., 2011; Hwang and Pallas, 2014; Ashton-Beaucage et al., 2014). In *Drosophila*, STRIPAK complexes act as negative regulators of the HIPPO pathway (Ribeiro et al., 2010) and as positive regulators of RAS/MAPK signaling (Ashton-Beaucage et al., 2014). CKA is also required for the dorsal closure during embryonic development where it complexes with KAY and JUN-RELATED ANTIGEN (JRA) transcription factors (Chen et al., 2002), but it is unknown whether STRIPAK complexes are involved. A STRIPAK-independent STRIP role in neuronal endosome organization has recently been described (Sakuma et al., 2014).

Catalytic MTS has been shown to promote high levels of nuclear PER (Sathyanarayanan et al., 2004), and downregulation of either TWS or WDB regulatory subunit induces PER degradation in *Drosophila* S2 cells, indicating that they associate with MTS to control PER oscillations (Sathyanarayanan et al., 2004). *wdb* or *tws* downregulation in S2 cells also induces CLK degradation (Kim and Edery, 2006), suggesting a CLK stabilization function for PP2A in vivo. Whether CKA-containing PP2A complexes (STRIPAK) contribute to clock mechanisms is not known. In this study, we identify both CKA and STRIP proteins as new components of the *Drosophila* molecular clock. We show that STRIPAK complexes promote the dephosphorylation of the CLK protein during daytime, whereas WDB-containing PP2A complexes control CLK protein levels.

RESULTS

A Large-Scale RNAi Screen Identifies STRIP as a New Clock Component

The NIG-Fly collection of RNAi lines was used to individually downregulate about 6,000 genes using the GAL4/UAS expression system (S.B. and F.R., unpublished data). We used the *gal1118* GAL4 driver (Blanchardon et al., 2001), which mostly targets the clock neurons expressing the pigment-dispersing factor (PDF) neuropeptide. The locomotor activity of *gal1118>RNAi* flies was monitored in constant darkness (DD), and flies with altered behavioral rhythms were selected. We identified *Strip*, which encodes the *Drosophila* ortholog of the mammalian striatin-interacting protein (STRIP) (Glatter et al., 2009; Goudreault et al., 2009; Ribeiro et al., 2010; Sakuma et al., 2014). *gal1118>Strip-RNAi1* flies showed a 2-hr lengthening of the behavioral period and a $\geq$2.5-hr period lengthening was observed when *Strip* RNAi and *dicer* were co-expressed under the control of either *gal1118* or the *tim-gal4* driver that is expressed in all clock cells (Figure 1A; Table 1). The target specificity of the *Strip* RNAi was addressed by testing two additional non-overlapping RNAi sequences (Figure S1), which also induced long-period rhythms when expressed in the clock neurons (Table S1). Head extracts of *tim>Strip-RNAi1* flies were tested for *Strip* mRNA levels and showed a 70% decrease, indicating that the RNAi indeed targeted the *Strip* transcripts (Figure S1). This experiment also showed that *Strip* mRNA levels do not cycle in head extracts. PDF-expressing small lateral neurons (s-LNvs) were not morphologically altered in the RNAi flies (Figure S1), and PER immunofluorescence analysis revealed a delayed oscillation (Figure 1B), in agreement with the long-period behavioral rhythms.

A P-element insertion that is localized in the non-coding 5′ end of *Strip* (Figure S1) was mobilized to generate deletions of the gene by imprecise excision (see Supplemental Experimental Procedures). A 4-kb deletion encompassing most of the *Strip* gene was obtained, but *Δ22130* homozygous mutants died at the pupal stage. The PDF-expressing neurons of *Δ22130* L3 larvae did not show obvious morphological defects (Figure S1) and were used for analyzing PER protein oscillations. PER levels cycled with a slow pace in the null homozygous mutants, with a 6-hr delay compared to heterozygous larvae at DD4 (Figure S1). This indicates that a functional *Strip* gene is required to set the pace of the clock to 24 hr.

Cka Downregulation Affects Behavioral Rhythms

To ask whether the circadian function of STRIP involves the STRIPAK complex, we tested the downregulation of the STRIP-associated PP2A regulatory subunit CKA. Expression of two non-overlapping *Cka* RNAi sequences in clock cells induced long-period rhythms, indicating that CKA was indeed involved in the behavioral clock (Figure 2A; Figure S2; Table 1; Table S1). Accordingly, *tim>Cka-RNAi1* flies showed a 40% decrease of *Cka* mRNA levels, whereas no cycling of the *Cka* transcripts nor of the CKA protein was observed in wild-type head extracts (Figure S2). Cka^2-null mutants are embryonic lethal, but viable adult flies could be obtained by providing daily heat-shock-controlled *Cka* expression until the emergence of the adults (Chen et al., 2002). Emerged flies were then transferred to low temperature and did not show detectable CKA immunostaining in head extracts (Figure S2). Developmentally rescued Cka^2 mutants were behaviorally tested at low temperature and became arrhythmic after a few days in DD (Table 1; Figure 2A). PDF-positive s-LNvs appeared normal in the mutants as well as in the RNAi flies (Figure S2). PER cycling was analyzed in the mutants. PER oscillations were marginally delayed during the first two DD days but were abolished with low immunostaining levels at DD6 (Figure 2B). Our data indicate that both CKA and STRIP are involved in the control of PER oscillations, strongly supporting a clock function for STRIPAK PP2A complexes.

STRIP and CKA Prevent the Accumulation of Hyperphosphorylated CLK during Daytime

A western blot analysis of head clock proteins was done to characterize the molecular defects associated with *Strip* and *Cka*

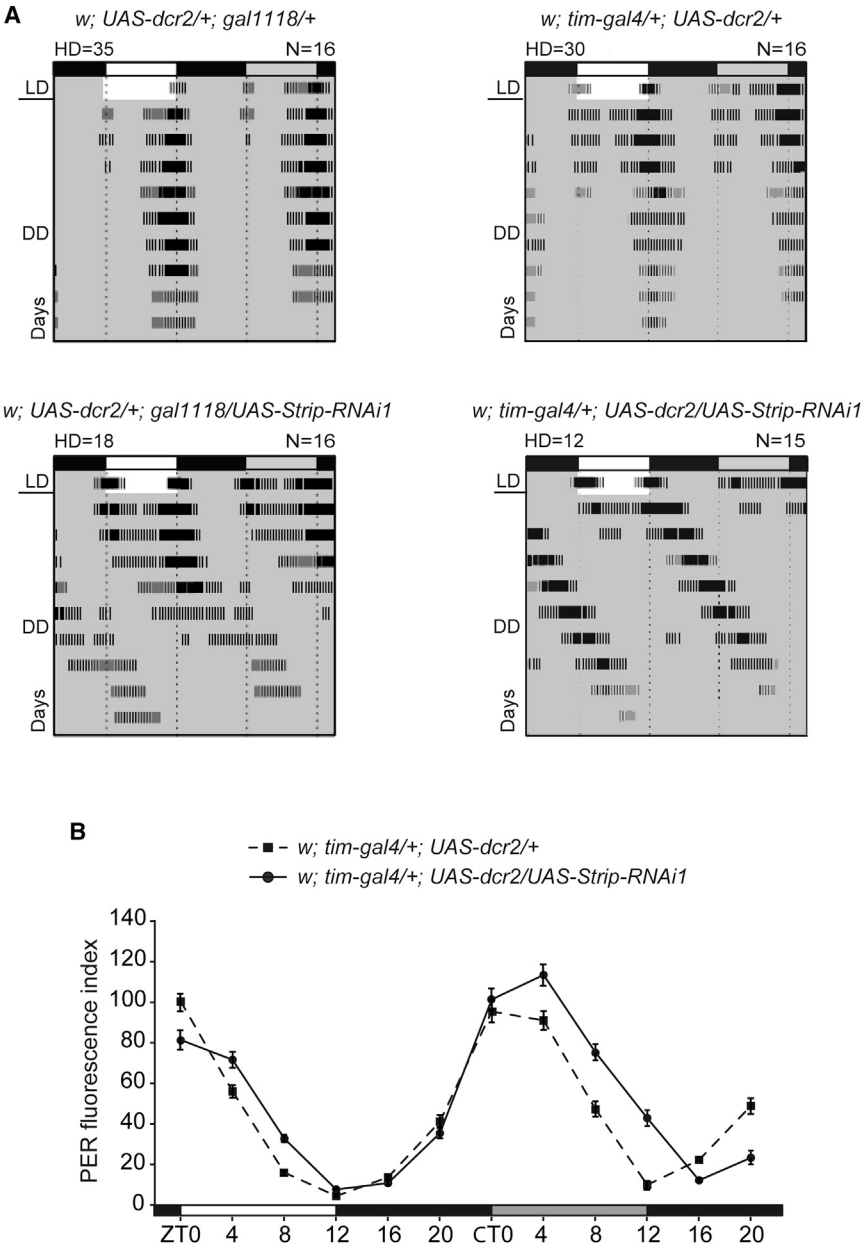

Figure 1. *Strip* Is Required for Normal Behavioral and Molecular Rhythms

(A) Averaged actograms over 1 day of light-dark (LD) and 9 days of constant darkness (DD) at 25°C. Shaded areas correspond to darkness. N, number of flies that survive until the end of the experiment. HD, hash density (see Experimental Procedures).

(B) Oscillations of the PER protein levels in the s-LNv neurons. Flies were entrained in LD for 4–5 days and then transferred to DD. Flies were dissected every 3 hr during the last LD day (ZT is Zeitgeber Time, ZT0 is lights-ON) and DD day 1 (CT, circadian time; CT0, the beginning of the first subjective day). Fluorescence index (see Experimental Procedures) is given in arbitrary units. Error bars indicate SEM. Bars indicate light (white), night (black), and subjective day (gray).

See also Figure S1.

downregulation. In DD, *tim>Strip-RNAi1* flies showed delayed PER and TIM oscillations with hyperphosphorylated forms observed at CT6-9, whereas such forms were almost absent at the same time in the controls (Figure 3A). In agreement with a slowed down molecular clock, newly synthetized low-molecular-weight PER and TIM were less abundant in the evening (CT9-12). *tim>Cka-RNAi1* flies, which displayed a milder behavioral phenotype, had virtually no PER/TIM cycling defects on the first day of DD (Figure 3C; Figure S3). However, developmentally rescued Cka^2 mutants showed a delayed PER accumulation during the night, with less phosphorylated PER at CT12-18 (Figure S3). The strong behavioral phenotype of Cka^2 mutants and *Strip*-RNAi flies suggested that another clock protein was more strongly affected in flies defective for STRIPAK complexes. *tim>Strip-RNAi1* flies showed persisting slow-migrating, hyperphosphorylated CLK forms during subjective daytime (Figure 3A), with a higher hyper/hypo-phosphorylated CLK ratio (Figure 3B). A similar phenotype was observed for *tim>Cka-RNAi1* and Cka^2 flies (Figures 3C–3E; Figure S3). The disappearance of slow migrating material after phosphatase treatment indicated that the high-molecular-weight CLK was due to hyperphosphorylation (Figure 3F). Importantly, the morning phosphorylation phenotype was also observed in light-dark conditions (LD) (Figures 3G and 3H), showing that the CLK phosphorylation defects were not secondary effects of a long period.

Flies expressing both *Strip* and *Cka* RNAi molecules did not show a stronger phenotype compared to *Strip* RNAi flies at both the behavioral and molecular levels (Table S1; Figure S3). Flies carrying a tagged version of STRIP or CKA were generated to test the effect of increased protein levels. Conversely to protein downregulation, *tim>Flag-Strip* and *tim>Cka-FlagHA* flies behaved normally (Table S1) and had no apparent perturbation of CLK protein cycling in head extracts (Figure S4; data not shown), suggesting that another STRIPAK component was rate limiting.

STRIP and CKA are part of the same STRIPAK complex in cultured *Drosophila* cells (Ribeiro et al., 2010), but whether such complexes exist in fly heads is not known. Co-immunoprecipitation experiments were repeated in S2 cells, which showed the presence of CKA-STRIP complexes (Figure S4). *tim>Flag-Strip* flies were then used to test STRIP interactions in head extracts. STRIP and CKA did strongly co-immunoprecipitate, indicating that the two proteins are part of the same

Table 1. Locomotor Activity Rhythms in DD

Genotype	n[a,b]	% R[b,c]	Period (hr) ±SEM[b]	Power ±SEM[b]
w; ; UAS-strip-RNAi1/+	16	100	23.8 ± 0.1	187 ± 19
w; UAS-Cka-RNAi1/+	32	94	23.4 ± 0.0	195 ± 10
w; ; UAS-wdb-RNAi/+	29	100	23.6 ± 0.1	193 ± 11
w; ; gal1118/+	16	100	24.1 ± 0.1	219 ± 11
w; UAS-dcr2/+; gal1118/+	16	100	24.0 ± 0.1	216 ± 16
w; tim-gal4/+	16	100	24.1 ± 0.1	205 ± 16
w; tim-gal4/+; UAS-dcr2/+	16	100	23.9 ± 0.1	178 ± 15
w; ; #22130	11	100	23.8 ± 0.1	134 ± 23
w; ; #22130/Df	16	100	24.4 ± 0.1	224 ± 15
w; ; gal1118/UAS-Strip-RNAi1	16	100	26.0 ± 0.1	129 ± 11
w; UAS-dcr2/+; gal1118/UAS-Strip-RNAi1	16	94	26.8 ± 0.1	100 ± 11
w; tim-gal4/+; UAS-dcr2/UAS-Strip-RNAi1	16	88	26.6 ± 0.1	126 ± 19
w; Cka^2, HS-Cka/CyO	18	83	24.6 ± 0.2	101 ± 20
w; Cka^2, HS-Cka	14	36	26.9 ± 2.3	50 ± 9
w; UAS-dcr2/UAS-Cka-RNAi1; gal1118/+	16	100	25.5 ± 0.1	264 ± 11
w; tim-gal4/UAS-Cka-RNAi1; UAS-dcr2/+	16	100	25.9 ± 0.1	199 ± 17
w; UAS-dcr2/+; gal1118/UAS-wdb-RNAi	16	100	25.0 ± 0.1	302 ± 8
w; tim-gal4/+; UAS-dcr2/UAS-wdb-RNAi	16	100	25.1 ± 0.1	218 ± 13
w; tim-gal4/UAS-dcr2;UAS-$dbt^{K/R}$,dbt^{ar}/+	16	6	36.0 ± 0.0[d]	75 ± 0[d]
w; tim-gal4/UAS-Cka-RNAi1;UAS-$dbt^{K/R}$,dbt^{ar}/UAS-dcr2	16	25	40.3 ± 0.4	41 ± 6
w; tim-gal4/UAS-dcr2;UAS-$dbt^{K/R}$,dbt^{ar}/UAS-Strip-RNAi1	16	25	28.0 ± 3.0	27 ± 2

All males except for the *Cka^2,HS-Cka* and *Cka^2, HS-Cka/CyO* genotypes.
[a]Number of tested flies.
[b]See Experimental Procedures.
[c]Percentage of rhythmic flies.
[d]Only one fly.

complex in the adult head (Figure S4). Conversely, neither STRIP-WDB nor STRIP-TWS complexes could be detected (Figure S4). CLK being essentially nuclear at any time (Houl et al., 2006), we asked whether STRIPAK complexes might localize in the nucleus. We could not detect significant anti-STRIP signals in wild-type brains, but STRIP-overexpressing flies showed a strong cytoplasmic signal as well as a weaker nuclear one (Figure 3I), suggesting that STRIPAK complexes could enter the nucleus.

STRIP and CKA Require PER and DBT Kinase Activity to Affect CLK Phosphorylation

PER is required for CLK hyperphosphorylation, but low phosphorylated CLK is present in per^0 mutants (Yu et al., 2006). We thus asked whether the control of CLK dephosphorylation by STRIP and/or CKA requires PER. To bypass the very low CLK levels of per^0 mutants, we used the ARK transgenic flies that express *Clk* under the control of *per* regulatory sequences and have rather high CLK levels in a per^0 background (Yu et al., 2006; Kim and Edery, 2006). Expression of either *Strip* or *Cka* RNAi did not affect CLK phosphorylation in per^0 ARK flies (Figure 4A), indicating that the CLK phosphorylation events regulated by STRIPAK require PER or the PER-dependent CLK phosphorylations. The absence of DBT impairs CLK phosphorylation (Kim and Edery, 2006), although the observation of phosphorylated CLK in flies expressing a dominant-negative form of the kinase ($DBT^{K/R}$) in a hypomorphic *dbt* background supports the idea that DBT kinase activity is not required (Yu et al., 2009). We thus used the behaviorally arrhythmic $dbt^{K/R}/dbt^{ar}$ flies to test the effects of *Strip* and *Cka* downregulation on phosphorylated CLK. *Strip* or *Cka* RNAi expression did not enhance CLK phosphorylation in this genetic background (Figure 4B), indicating that the CLK phosphorylated forms that STRIPAK act on are missing in the absence of a functional DBT kinase.

The persistence of daytime hyperphosphorylated CLK in *Cka* or *Strip* RNAi flies suggested that STRIPAK complexes might dephosphorylate CLK directly. We thus asked whether CLK associates with STRIP or CKA. Co-immunoprecipitation assays between CLK and either STRIP or CKA did not show the presence of CLK-STRIP or CLK-CKA complexes in head extracts (data not shown). Since this interaction might be very labile and/or weak, we repeated these experiments in S2 cells where induction of high protein levels might compensate for weak interactions. CLK co-immunoprecipitated with both STRIP and CKA either in the presence or in the absence of DBT (Figure 4C), strongly suggesting that CLK can associate with STRIPAK complexes.

In Contrast to CKA, the WDB PP2A Regulatory Subunit Stabilizes CLK

Downregulation of WDB and TWS PP2A regulatory subunits destabilizes PER (Sathyanarayanan et al., 2004) and CLK (Kim and

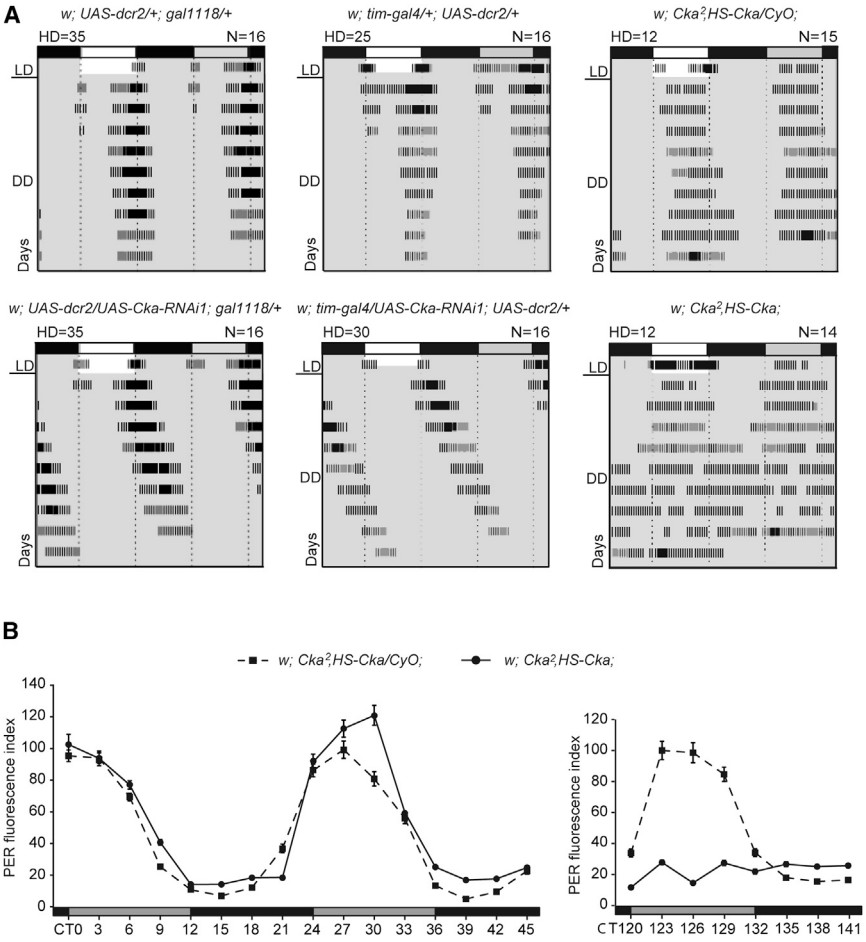

Figure 2. *Cka* Is Required for Normal Behavioral and Molecular Rhythms

(A) Averaged actograms over 1 day of light-dark (LD) and 9 days of constant darkness (DD) at 25°C (RNAi and controls) or 20°C (Cka^2 *HS-Cka*, see Experimental Procedures).

(B) Oscillations of the PER protein levels in the s-LNv neurons of Cka^2 mutants and heterozygous controls. Flies were entrained in LD for 4 days and then transferred to DD at 20°C. Flies were dissected every 3 hours during the DD days 1 and 2 (left) or during DD day 6 (right).

Error bars, SEM. See also Figure S2.

Edery, 2006) in S2 cells. However, their function in adult flies has only been tested by overexpression, leading to period lengthening *(wdb)* or arrhythmicity *(tws)* in behavioral experiments (Sathyanarayanan et al., 2004). We asked whether *wdb* or *tws* downregulation would also compromise the behavioral clock. Expressing *tws* RNAi constructs in clock neurons did not produce significant behavioral defects (data not shown). *tim>wdb-RNAi* flies had a 1-hr lengthening of the period in DD (Table 1; Figure S5) with no morphological defects of the PDF neurons (Figure S5). This mild phenotype was associated to about 50% decreased *wdb* mRNA levels in head extracts (Figure S5). In agreement with their mildly affected behavior, *tim>wdb-RNAi* flies showed robust molecular oscillations both in LD and in DD conditions, but with significantly lower levels of PER, TIM, and CLK, especially in the morning (Figures 5A–5C). No effect on CLK was observed in a per^0 genetic background (Figure 5D), indicating that PER is required for WDB activity on CLK. As for STRIP and CKA, no CLK-WDB complexes could be detected by co-immunoprecipitations in head extracts (data not shown). Combining the expression of *wdb* RNAi with either *Cka* or *Strip* RNAi produced a longer behavioral period (Table S1) and partially additive CLK molecular defects, with slightly decreased levels and higher phosphorylation during subjective daytime (Figure S3; data not shown). We finally compared the effects of downregulating WDB and CKA PP2A regulatory subunits on CLK in S2 cells. As previously reported (Kim and Edery, 2006), *wdb* double-stranded RNA (dsRNA) decreased CLK levels, although to a lesser extent in our hands (Figure S3). In the presence of added DBT, *Cka* dsRNA but not *wdb* dsRNA induced a higher shift in CLK protein mobility, likely reflecting CLK hyperphosphorylation (Figure S3). Both head extracts and S2 cells experiments thus support different roles for CKA and WDB in the regulation of the CLK protein.

mts Downregulation Affects CLK Similarly to *Strip* or *Cka* Downregulation

To evaluate the relative contributions of CKA and WDB, we asked how the inhibition of the PP2A catalytic subunit MTS would affect CLK. As previously reported (Sathyanarayanan et al., 2004), flies overexpressing a dominant-negative form of MTS (MTS^{DN}) were arrhythmic in DD (Table S1; Figure S6). However, anti-PDF staining revealed that the PDF neurons of *tim* > mts^{DN} flies were either missing or showed strong morphological defects, whereas only slight defects were seen in the larval LNs (Figure S6). Since PDF cells are required for robust DD rhythms, the results prevented a reliable molecular interpretation of the behavioral data.

Head extracts of *tim* > mts^{DN} were used to address the molecular function of MTS. As opposed to the strongly decreased protein levels observed in *tim>wdb-RNAi* flies (Figures 5A–5C), *tim* > mts^{DN} flies showed more abundant daytime phosphorylated PER and TIM proteins in both DD and LD (Figures 5E–5G). This seemed to be in contrast to the absence of PER immunoreactivity reported in larval LNs (Sathyanarayanan et al., 2004). We thus analyzed PER in the larval LNs (Figures S7 and S9), and in the adult DN1s whose number was not affected in *tim* > mts^{DN} adults (Figures S8 and S9). We observed lower apparent PER levels in the late night/early morning that were at least partly due to the more diffuse immunolabeling in the cell bodies suggesting abnormal subcellular localization, particularly in the larval LNs. The contrast with

the western blot data suggested that PER immunoreactivity did not properly reflected absolute levels of the protein in the clock neurons of MTS^{DN} flies. Conversely to PER, CLK immunoreactivity was not strongly affected in the DN1s of *tim* > mts^{DN} flies (Figures S8 and S9). However, hyperphosphorylated CLK persisted during daytime in head extracts (Figures 5E–5G), as observed with *Cka* or *Strip* downregulation but not with *wdb* downregulation. The data thus suggest that CKA-PP2A (STRIPAK) complexes are the predominant PP2A complexes for the control of the CLK protein.

PP2A Activity Enhances CLK-Dependent Transcription

Since *Strip* and *Cka* downregulation strongly affected CLK phosphorylation, we asked how it would affect CLK transcriptional activity. As readout, we measured the levels of *tim* and *vri* pre-mRNAs, which show high-amplitude oscillations in wild-type flies, on the second day of constant darkness (Figure 6). In *tim>Cka-RNAi1* and *tim>Strip-RNAi1* flies, *tim* and *vri* pre-mRNAs were decreased. In contrast, *Clk* mRNA levels were not affected, indicating that low CLK-dependent transcription was not a consequence of low *Clk* transcripts. Since daytime CLK dephosphorylation is deficient in the two genotypes (Figure 3), it seems likely that decreased phosphatase activity would prevent late day accumulation of low phosphorylated CLK, thus diminishing CLK-dependent transcription. A decrease in pre-mRNA levels was also observed with pre-*tim*, but not pre-*vri*, in *tim>wdb-RNAi* flies, which had low CLK levels around the clock, particularly in the morning (Figures 5A–5C). *tim>wdb-RNAi* flies also showed an ~30% decrease of *Clk* mRNA levels at all time points, and a slighter decrease was observed in ARK flies (Figure S10), where the contribution of the *Clk* promoter to *Clk* mRNA levels is strongly reduced in the evening (Kim et al., 2002). This suggested that transcriptional effects could contribute to the low CLK protein levels in flies with downregulated *wdb*. However, *Clk* mRNA levels were not significantly altered in *tim>wdb-RNAi* flies on the first day of DD (Figure S10), whereas CLK protein levels were strongly reduced (Figures 5A–5C), indicating that the primary effect of *wdb* downregulation is on CLK protein.

DISCUSSION

This study addresses the function of the PP2A phosphatase in the control of the CLK phosphorylation cycle. Three kinases have been identified that affect CLK apparent molecular weight and/or stability, DBT (Yu et al., 2006; Kim and Edery, 2006; Yu et al., 2009), NEMO (Yu et al., 2011), and CK2 (Szabó et al., 2013). In addition, WDB and TWS PP2A regulatory subunits have been shown to affect CLK stability in S2 cells (Kim and Edery, 2006). Our results identify two components of the STRIPAK complex, the PP2A regulatory subunit CKA and its interacting protein STRIP, as regulators of daytime CLK phosphorylation in vivo. Whereas PP2A complexes containing the WDB regulatory subunit stabilize CLK, CKA-containing STRIPAK complexes act during the day to push hyperphosphorylated morning CLK toward its low phosphorylated and transcriptionally active evening form.

Both *Strip* and *Cka* downregulation allow the stabilization of hyperphosphorylated CLK in the morning. This very similar phenotype suggests that the two proteins work together to control CLK cycling, and their presence in the same complex in adult heads strongly supports this idea. Since both proteins are known components of the STRIPAK complex (Ribeiro et al., 2010), it is very likely that STRIPAK promotes CLK dephosphorylation. However, a stronger PER/TIM phenotype was observed in *Strip* RNAi flies, compared to *Cka*-defective flies. In contrast to CLK, PER and TIM were not hyperphosphorylated but rather showed a slowed down oscillation in *Strip* RNAi flies, with more protein in the morning and a delayed evening accumulation. A STRIP-specific function on PER and TIM cannot be excluded that could be either unrelated to PP2A or a consequence of other STRIP-containing PP2A complexes. The MTS^{DN} phenotypes on PER and TIM are actually similar and could suggest the existence of CKA-independent STRIP-associated PP2A complexes. Such complexes have not been reported in flies or mammals so far, but the STRIP-like protein FAR11 was found in PP2A complexes containing a B′-type (WDB-type) PP2A regulatory subunit in yeast (Pracheil et al., 2012). MTS^{DN} has been shown to decrease PER labeling in the larval PDF neurons, whereas MTS overexpression increases nuclear PER (Sathyanarayanan et al., 2004). Our experiments indicate that this low PER labeling is reflected in low PER levels in head extracts in the early night but not in the morning when higher PER levels are observed. Weak morning PER detection in the clock neurons might thus be the consequence of both a more diffuse subcellular localization and a decrease of immunoreactivity of the protein in MTS^{DN} flies. In addition, the stronger phenotype in the larval LNs likely includes a component of developmental origin since these cells show some anatomical defects and will become more strongly altered in the adults. In contrast to MTS, WDB stabilizes PER around the clock. PP2A complexes with different regulatory subunits thus play different roles on both CLK and PER.

Our finding of CKA/CLK and STRIP/CLK complexes in S2 cells predicts a co-localization of CLK and STRIPAK in clock cells. The function of STRIPAK complexes is still poorly understood, but they are involved in various cytoplasmic processes, which support a role for STRIPAK in the cytoskeleton organization (Hyodo et al., 2012; Kean et al., 2011; Hwang and Pallas, 2014; Ashton-Beaucage et al., 2014). Anti-STRIP staining was mostly cytoplasmic in clock neurons, but the detection of a weak nuclear signal in STRIP overexpressing flies suggests that STRIPAK complexes can localize in the nucleus where they might interact with CLK. In *Neurospora*, highly phosphorylated WCC (the WCC functional homolog of CLK) is shuttled to the cytoplasm and dephosphorylated by PP2A before re-entering the nucleus to resume transcription as an active and unstable form (Schafmeier et al., 2008; Yang et al., 2004). In mammals, CLK-BMAL1 nucleocytoplasmic shuttling appears to promote both its transcriptional activity and instability (Kondratov et al., 2003; Kwon et al., 2006). CLK is constitutively nuclear in *Drosophila* clock neurons, but cytoplasmic transfer of presumably highly phosphorylated CLK has been reported in S2 cells (Kim and Edery, 2006; Maurer et al., 2009; Hung et al., 2009). It cannot be excluded that CLK is very transiently present in the cytoplasm, but we could not detect any CLK cytoplasmic localization in either wild-type or

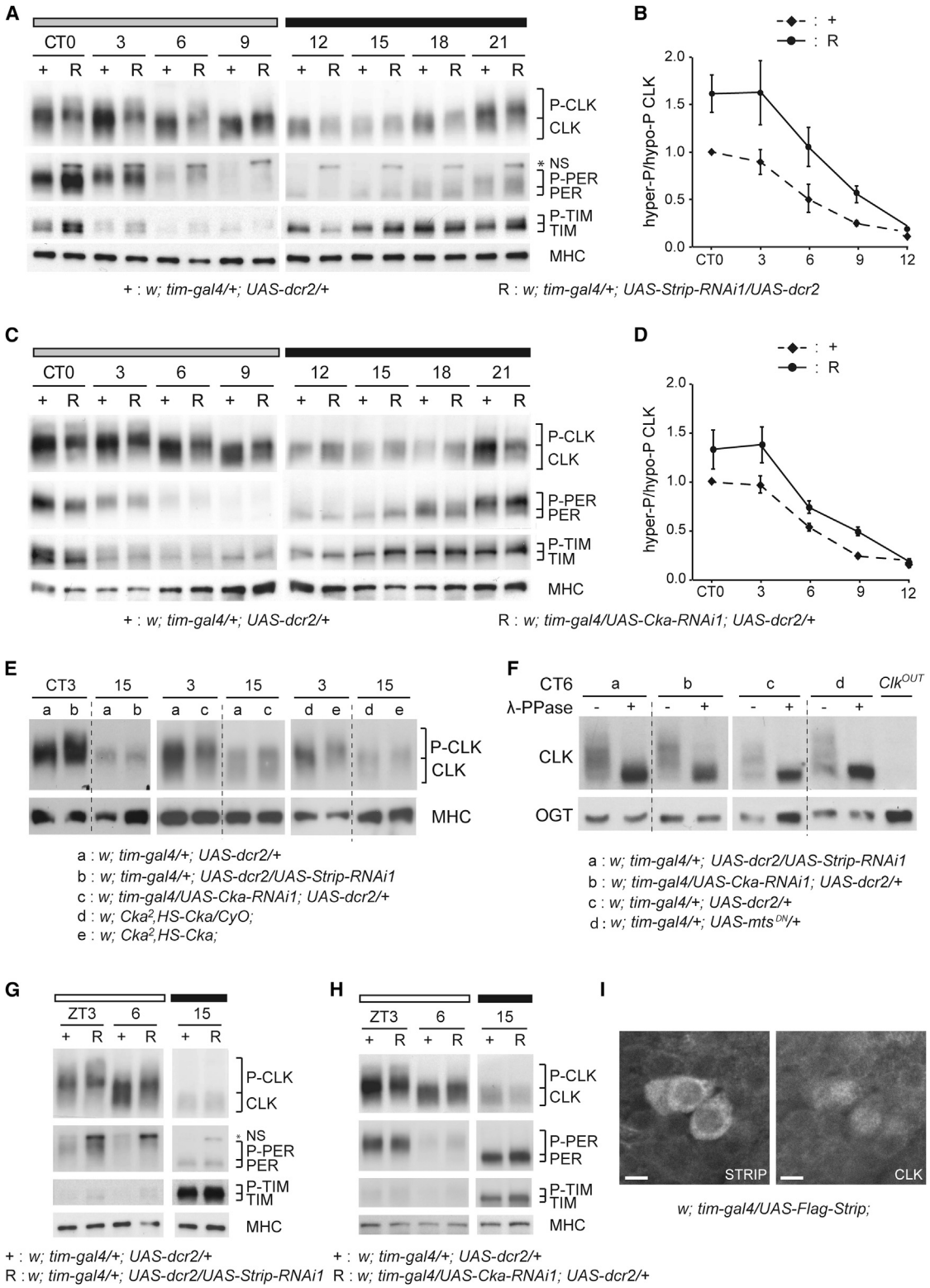

Figure 3. CKA and STRIP Promote CLK Dephosphorylation

(A, C, G, and H) Western blot of head extracts (4% Tris-glycine gels) using anti-CLK, anti-PER, and anti-TIM antibodies. Flies were entrained in LD for 4–5 days (25°C) and collected on the last LD day (G and H) or transferred to DD and collected during the first DD day (A and C). For each time point, RNAi flies (R) are loaded

(legend continued on next page)

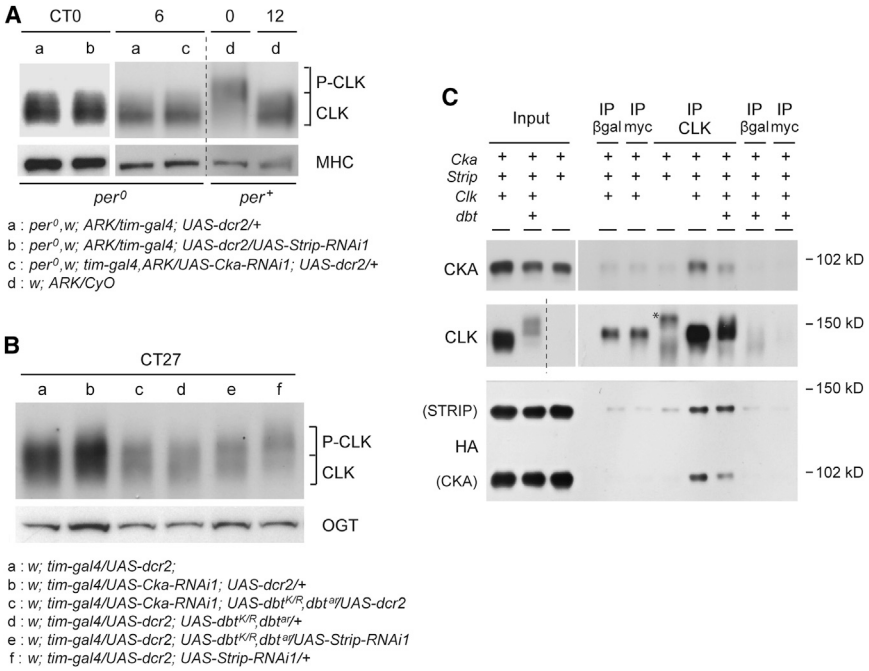

Figure 4. Effects of PER and DBT on STRIPAK Function and Analysis of STRIPAK Interactions

(A and B) Western blot analysis of the CLK protein at DD1-2. Head extracts were run on 4% (A) or 6% (B) Tris-glycine gels.

(C) Co-immunoprecipitation experiments in S2 cells. Cells were transfected with *pMt-Strip-Flag-HA* (*Strip*, 400 ng) and *pMt-Cka-Flag-HA* (*Cka*, 400 ng) plasmids and *pAc-Clk-V5* (*Clk*, 400 ng), *pAc-dbt-V5* (*Dbt*, 100 ng), or equal amounts of an empty vector, as indicated. Cell lysates were subjected to anti-CLK, anti-βGAL, and anti-MYC immunoprecipitations (IPs). Inputs and immunoprecipitates were analyzed by immunoblotting on a 6% Tris-glycine gel. For CKA inputs, a shorter film exposure was used as compared to IP extracts. Used antibodies are shown on the left side; the position of the molecular weight markers is indicated on the right side. A dotted line indicates that the two surrounding slots were not contiguous on the gel. All experiments were performed at least twice. *Non-specific band.

See also Figure S4.

Strip/*Cka* downregulated flies (data not shown). Our co-immunoprecipitation and immunocytochemistry experiments rather suggest that STRIPAK complexes can enter the nucleus. CKA activates KAY/JRA-dependent transcription in *Drosophila* embryos and cytoplasmic CKA partially relocalizes to the nucleus when co-transfected with KAY and JRA in S2 cells (Chen et al., 2002). It is not known whether this CKA function relies on STRIPAK complexes. Interestingly, KAY regulates *Clk* transcription (Ling et al., 2012), but STRIPAK complexes are unlikely to be involved since we have seen no modifications of *Clk* mRNA levels in *Cka* or *Strip* RNAi flies.

How do STRIPAK complexes control CLK phosphorylation? The simplest interpretation is that CLK is directly dephosphorylated by the MTS PP2A catalytic subunit of the STRIPAK complex. Alternatively, since STRIPAK complexes contain autoactivated kinases, PP2A-induced inhibition of some of these kinases could reduce CLK phosphorylation. In addition to HPO, other members of the Ste-20 kinase family, such as *misshapen* (*msn*) and *germinal center kinase III* (*gck-III*), co-purified with CKA in S2 cells (Ribeiro et al., 2010). It will be interesting to see whether such STRIPAK kinases also affect CLK phosphorylation. We did not observed *Cka* or *Strip* downregulation effects on the hypophosphorylated CLK present in per^0 mutants. Interestingly, the phosphorylated CLK that is present in $dbt^{K/R},dbt^{ar}$ flies was not affected by *Cka* or *Strip* downregulation. In such flies, CLK phosphorylation is thus likely impaired on sites that are critical for STRIPAK function. Alternatively, DBT might be involved in the regulation of the STRIPAK phosphatase activity. In any case, our data indicate that PER and DBT likely act upstream of STRIPAK to control CLK phosphorylation. Since PER levels are increased in *Strip* RNAi flies, could CLK hyperphosphorylation be a consequence of PER changes ? CLK is hyperphosphorylated in *Cka* RNAi and mutant flies, whereas PER is not significantly affected. Moreover, CLK is not hyperphosphorylated in flies deficient for a component of the ubiquitin/proteasome system, although they show high PER levels without phosphorylation changes (S.B. and F.R., unpublished). Altogether, these data strongly suggest that CLK hyperphosphorylation in STRIPAK-deficient flies is not a consequence of increased PER.

next to controls (+). P-CLK, P-PER, and P-TIM are hyperphosphorylated forms of CLK, PER, and TIM, respectively. NS, non-specific band (inferred by presence in per^0 extracts, data not shown). Extracts were run on 4% Tris-glycine gels.

(B and D) Quantification of the hyper-/hypo-phosphorylated CLK ratios determined by measuring band intensities. The top of the CLK band shown at CT12 is used as a reference to separate the hyper- from the hypo-phosphorylated CLK. The value of the control at CT0 was set to 1. The average values of four independent experiments are shown. Error bars, SEM.

(E) Anti-CLK western blot showing CLK hyper-phosphorylation at CT6 in *Strip* and *Cka* RNAi and *Cka* mutants compared to controls, while no effects is seen at CT15. A dotted line indicates that the two surrounding slots were not contiguous on the gel. Extracts were run on a 6% Tris-glycine gel to increase size resolution.

(F) Lambda-Phosphatase treatment of head extracts from flies collected at CT6 and run on a 6% Tris-glycine gel. Clk^{OUT} extracts were added as a CLK staining control, while the MHC or OGT staining was used as gel-loading controls.

(I) Anti-STRIP and anti-CLK staining of representative adult clock neurons (LN_ds) of flies overexpressing *Strip* in *tim*-positive cells. Shown is a single confocal microscopy plan (0.5 μm) chosen at maximum CLK signal (400× magnification). STRIP staining intensity in the nucleus is 2-fold the background value and half of the cytoplasm intensity (average values from 20 independent neurons). Scale bar, 5 μm.

See also Figure S3.

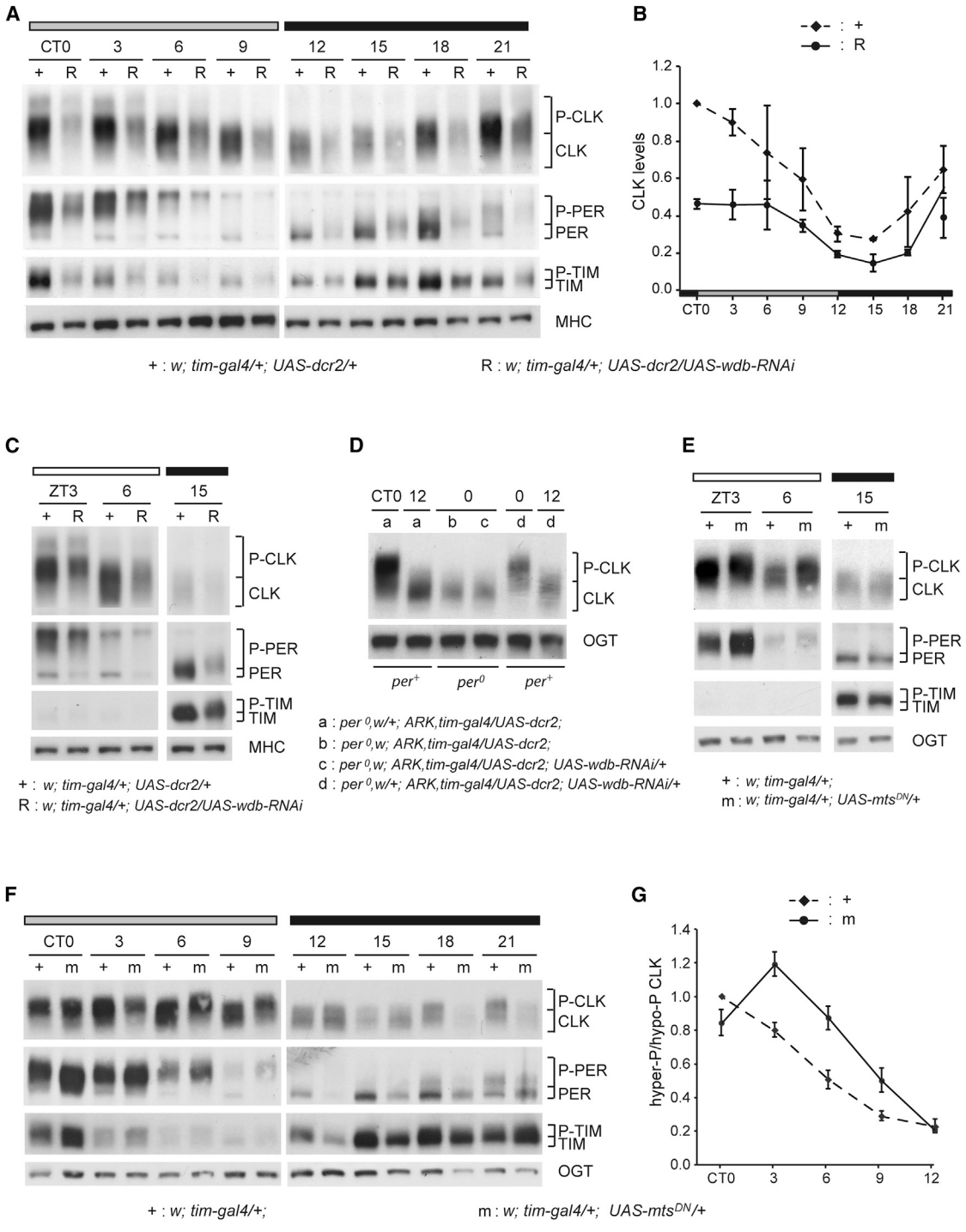

Figure 5. Effects of WDB and MTS Alterations on PER, TIM, and CLK

(A and C–F) Western blot analysis of CLK, PER, and TIM proteins in genotypes altered for WDB and MTS function. Extracts were run on 6% (A, C, and D) or 4% (E and F) Tris-glycine gels.

(B) Relative CLK levels in *wdb* RNAi and control head extracts were determined by measuring band intensities. The average values of two independent experiments are shown; error bars, SD.

(G) Quantification of the hyper-/hypo-phosphorylated CLK ratios determined by measuring band intensities. The average values of four independent experiments are shown. Error bars, SEM.

See also Figures S3 and S5–S9.

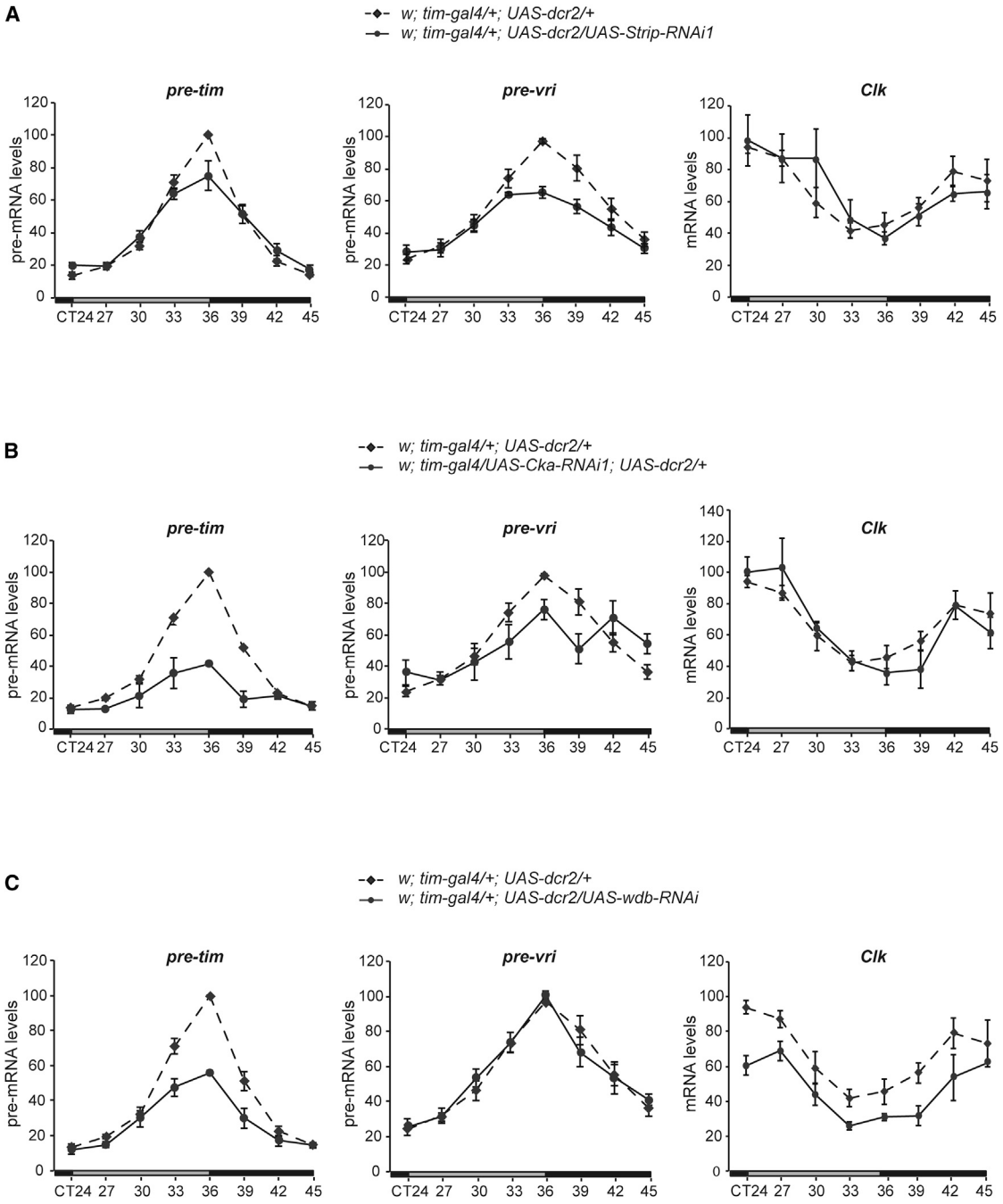

Figure 6. Transcriptional Effects of *Cka*, *Strip*, or *wdb* Downregulation

Quantitative RT-PCR measurements of *tim* and *vri* pre-mRNAs as well as *Clk* mRNA levels in head extracts of flies collected at DD day 2. Bars indicate night (black) and subjective day (gray). Average values from at least three independent experiments are normalized to the mean of peak control values set to 100. Error bar, SEM. Each panel shows a comparison between *tim* > + controls and *tim*>*Strip*-RNAi (A), *tim*>*Cka*-RNAi (B), or *tim*>*wdb*-RNAi (C). See also Figure S10.

Since downregulation of either WDB or TWS destabilizes CLK in S2 cells, it has been proposed that CLK hyperphosphorylation and degradation is controlled by a balance between PP2A and DBT (Kim and Edery, 2006). Our results show that WDB stabilizes CLK in head extracts without affecting its apparent phosphorylation level. WDB-PP2A might dephosphorylate a few sites important for CLK stability or affect components of the CLK degradation pathway. CLK stabilization occurs at all time points, although WDB function is prominent in the morning when CLK is hyperphosphorylated. As DBT (Kim and Edery, 2006; Yu et al., 2006) and CKA, WDB requires PER to affect CLK, suggesting that CLK is not a suitable PP2A-WDB substrate in the absence of PER. It is

also possible that WDB effects on CLK are due to its stabilizing effects on PER, in contrast to CKA, which does not affect PER. Inhibition of the PP2A catalytic subunit MTS affected CLK phosphorylation very similarly to CKA or STRIP downregulation, but did not show the CLK destabilization effects of WDB downregulation. Although a MTS-independent role of WDB cannot be excluded, it suggests that another type of PP2A complex is required upstream of PP2A-WDB for the control of CLK levels. Alternatively, the decreased levels of WDB-MTS complexes in *wdb* RNAi flies might affect CLK levels by favoring a competing PP2A complex that destabilizes CLK. Although TWS has a CLK-stabilizing effect in S2 cells (Kim and Edery, 2006), the antiphasic curves of *wdb* and *tws* mRNA levels (Sathyanarayanan et al., 2004) could suggest opposite roles of WDB and TWS on CLK stability in flies. In any case, the very similar effects of catalytic MTS and regulatory CKA downregulation on CLK indicate that STRIPAK complexes are the key PP2A components to control daytime CLK dephosphorylation. Although *Cka* has been identified as a CLK transcriptional target in the head (Abruzzi et al., 2011), we could not detect *Cka* mRNA cycling in head extracts. Since protein levels do not cycle, the temporal control of CKA activity on CLK might be driven by CLK phosphorylation levels. *Cka* mRNA levels cycle in intestinal cells (Karpowicz et al., 2013), and it is possible that cycling also occurs in specific cell populations in the head.

Downregulation of either WDB or CKA both decreased CLK-dependent transcription. Since *wdb* downregulation destabilizes CLK and PER, the transcriptional inhibition effects observed in *wdb* downregulated flies are likely a consequence altered activation/repression ratio with low CLK and low PER. *Cka* downregulation reduced *tim* pre-mRNA levels by about 60% and also decreased *vri* transcripts, whereas *wdb* downregulation decreased *tim* pre-mRNAs by about 50% with no detectable impact on *vri*. This suggests that *vri* transcription could be less sensitive than *tim* transcription to CLK quantities. Interestingly, the unchanged levels of *vri* pre-mRNAs are associated with lower levels of *Clk* mRNAs in *wdb* RNAi flies, suggesting that more VRI might be present to repress *Clk* transcription in comparison to *Cka* RNAi flies. The effects of *Cka* or *wdb* downregulation on *tim* transcription need to be compared to the CLK molecular defects observed in these genotypes and suggest that CLK phosphorylation is more important than CLK levels to define CLK transcriptional activity. Although higher CLK protein levels are associated with a shorter period (Kadener et al., 2008), significant differences in CLK levels have been reported to have only limited effects on the pacemaker (Kim et al., 2002), in agreement with the low-amplitude oscillation of CLK levels. Further support to this idea comes from $CK2\alpha^{Tik}$ flies, in which low levels of hypophosphorylated CLK display strong transcriptional activity (Szabó et al., 2013).

The regulation of CLK activity is a central point in the functioning of the circadian oscillator. Yet, it is a poorly understood aspect of the *Drosophila* clock. Our study reveals that CLK function is regulated by at least two types of PP2A complexes. The data support a model where WDB-MTS complexes stabilize CLK, whereas CKA-MTS (STRIPAK) complexes dephosphorylate the daytime hyperphosphorylated CLK to replenish the pool of hypophosphorylated active CLK. It remains to be determined how the ratio between different PP2A activities is controlled during a circadian cycle to determine the rate of clock-controlled transcription by defining levels and activity of both the CLK activator and the PER repressor.

EXPERIMENTAL PROCEDURES

Fly Strains and Constructs

Flies were maintained on standard cornmeal-yeast-agar medium on 12-hr:12-hr LD conditions. The transgenic fly lines used in this study as well as the generation of *Strip* mutants and constructs are described as Supplemental Experimental Procedures.

Behavioral Analysis

Locomotor activity of individual flies were measured with the *Drosophila* activity monitors (TriKinetics) at 25°C. Young adult males were first entrained to 12-hr:12-hr light-dark (LD) cycles for 2–4 days and then transferred to constant darkness (DD) for 11 days. The activity data analysis was done with the FaasX 1.16 software (Klarsfeld et al., 2003) that is derived from the Brandeis Rhythm Package (Hamblen-Coyle et al., 1989) and is freely available upon request (Apple Mac OSX only). Actograms are double-plotted graphs representing the absolute activity levels for each 0.5-hr interval, averaged over a group of flies of a given genotype. The hash density (HD = number of activity events per hash) varies according to the activity levels of the genotype. This allows the comparison of activity profiles between genotypes that display very different activity levels. Periods were calculated for each individual fly using χ^2 periodogram analysis (CycleP) and pooled to calculate a group average for each genotype. Analysis was done over 10 days from the second day of DD. The following filtering criteria were used to assess rhythmicity: power $\geq$20, width $\geq$2 hr. Power and width represent the height and width of the periodogram peak, respectively, and give the significance of the calculated period. Genotypes with a reduced number of rhythmic flies (<50%), low power (<30), and high SEM of the period (>1) are considered arrhythmic. All behavioral experiments were reproduced at least twice with very similar results.

Immunoblotting

20–40 flies (both males and females) were collected on dry ice at indicated time points and processed as previously described (Lamaze et al., 2011; Szabó et al., 2013). Isolated frozen heads were homogenized in ice-cold RBS lysis buffer (10 mM HEPES [pH 7.5], 5 mM Tris-HCl [pH 7.5], 50 mM KCl, 2 mM EDTA [pH 8.0], 10% glycerol, 1% Triton X-100, 0.4% NP-40) supplemented with anti-proteases tablets (Roche), phosphatase inhibitor cocktail 2 and 3 (Sigma), and 20 mM β-glycerophosphate. To improve extraction efficiency, extraction was performed in two steps. Tubes were homogenized for 1 min, followed by 10–15 min of ice incubation, and then homogenized for another 15–30 s. Protein concentration was determined by a Bradford (Bio-Rad) protein assay, according to the manufacturer's instructions. For SDS-PAGE, 50 μg of proteins was separated on NuPAGE Novex 4% or 6% Tris-glycine precast gels (CKA, STRIP, PER, TIM, and CLK immunoblotting) or 4%–12% Bis-Tris gels (GFP, TWS, and WDB immunoblotting) from Invitrogen and transferred to PVDF membranes. Primary and secondary antibodies used are described in Supplemental Experimental Procedures. Chemiluminescence reaction was performed using Pierce ECL-Plus Western Blotting Substrate (Thermo Scientific). All of these immunoblot analyses were repeated in two to four experiments, most of which were blotted multiple times. Quantification of band intensities was performed using the ImageJ software. For each experiment, the value of the control at CT0 was set to 1.

Immunoprecipitation

For immunoprecipitation assays frozen heads were homogenized in ice-cold HEPES/EDTA (HE) lysis buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 10 mM EDTA [pH 8.0], 0.1% NP-40, 5% glycerol) supplemented with anti-proteases tablets (Roche), phosphatase inhibitor cocktail 2 and 3 (Sigma), and 20 mM β-glycerophosphate. For FLAG immunoprecipitations, 600 μg to 1 mg of total protein extract was incubated overnight at 4°C with 25 μl EZview Red anti-FLAG M2 Affinity Gel (Sigma-Aldrich). Other immunoprecipitations were performed using 25–50 μl Protein G Sepharose 4 Fast Flow (GE Healthcare) pre-coupled with 10 μl of goat anti-CLK antibody (sc-27069, Santa Cruz

Biotechnology) or 3 μl mouse anti-HA antibody (12CA5, Roche) or 10 μl goat anti-c-MYC antibody (sc-789-G, Santa Cruz Biotechnology) or 3 μl mouse anti-ßgal antibody (Promega). Beads were washed three times for 45 min with HE lysis buffer. Purified protein and associated complexes were then eluted in 1 × NuPAGE LDS Sample Buffer (Invitrogen), 1 × Sample Reducing Agent (Invitrogen) for 10 min at 70°C. The same protocol was used for S2 cells immunoprecipitation analysis.

Cell Culture

Drosophila Schneider 2 (S2) cells were maintained in Schneider's *Drosophila* Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin solution (Sigma-Aldrich). *pAc-Clk-V5* (McDonald et al., 2001) and *pAc-dbt-V5* (Nawathean et al., 2007) were described previously. *pMt-Cka-Flag-HA* (BDGP clone FMO03251) and *pMt-Strip-Flag-HA* (BDGP clone FMO09403) were obtained from the *Drosophila* Genomics Resource Center (DGRC). Cells were transiently transfected with Effectene transfection reagent (QIAGEN) and grown in Schneider's medium for 48 hr before collection. DNA quantities were equalized for transfection by addition of empty *pAc* vector. For gene expression from metallothionein (Mt) promoter cells were exposed to 500 μM $CuSO_4$ starting from 24 hr before harvesting. For dsRNA assays, dsRNAs were produced using standard protocols (see Supplemental Experimental Procedures) and added to S2 cells 3 hr after transfection in a serum-free medium, supplied with serum and antibiotics 1 hr later. Cells were harvested 48 hr after dsRNA treatment, centrifuged for 6 min at 2,000 × *g* at room temperature, washed once with PBS, and pellet frozen at −80°C until extraction. Protein extraction was achieved by lysing cells in 60–100 μl of ice-cold HE buffer by means of pipetting and vortexing. After centrifugation for 10 min at 14,000 rpm 4°C, supernatants were quantified (Bradford assay). Extracts were then processed for western blot or immunoprecipitation assays.

Quantitative RT-PCR

Total RNA was prepared from adult fly heads (about 35 heads per sample) using the Promega SV Total RNA Isolation System. 300 ng of total RNA were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) with RNase inhibitor and random primers following the manufacturer's instructions. Quantitative PCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR System using the SYBR green detection protocol. Detailed protocols are given as Supplemental Experimental Procedures.

Brain Immunostainings

Larval and adult flies were collected at the indicated circadian times (CTs) and dissected. Samples were incubated 3–4 days with primary antibodies and overnight with secondary antibodies (see Supplemental Experimental Procedures). Whole brains were mounted in slides with ProLong (Roche) mounting medium and imaged using a AxioImager Z1 fluorescence microscope equipped with an ApoTome structured illumination module (Zeiss) or a Leica SP8 confocal system. Fluorescence intensity of individual cells was quantified from digital images of single focal planes with the NIH ImageJ software. For quantification, a fluorescence index was calculated by applying the formula: I = 100*(S–B)/B (S (Signal) is the fluorescence intensity of the positive cell; B (Background) is the average intensity of the region adjacent to the positive cell), which gives the fluorescence percentage above background. Index values were then averaged for the considered subset of cells (i.e., four PDF-positive s-LN_vs) among 12–20 brain hemispheres for each time point.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, ten figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.033.

AUTHOR CONTRIBUTIONS

S.A., S.B., and B.M. performed and analyzed behavioral experiments, S.A. and B.M. generated the *Strip* mutants and the UAS-*Strip* construct. S.A., A.L., and C.P. performed and analyzed immunoprecipitation and western blot experiments. E.C. performed and analyzed brain immunolabelings. P.P. and E.J. performed and analyzed quantitative PCR experiments. S.A. and F.R. wrote the paper.

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