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Identification of Light-Sensitive Phosphorylation Sites on PERIOD That Regulate the Pace of Circadian Rhythms in *Drosophila*

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The main components regulating the pace of circadian (≅24 h) clocks in animals are PERIOD (PER) proteins, transcriptional regulators that undergo daily changes in levels and nuclear accumulation by means of complex multisite phosphorylation programs. In the present study, we investigated the function of two phosphorylation sites, at Ser826 and Ser828, located in a putative nuclear localization signal (NLS) on the *Drosophila melanogaster* PER protein. These sites are phosphorylated by DOUBLETIME (DBT; *Drosophila* homolog of CK1δ/ε), the key circadian kinase regulating the daily changes in PER stability and phosphorylation. Mutant flies in which phosphorylation at Ser826/Ser828 is blocked manifest behavioral rhythms with periods slightly longer than 1 h and with altered temperature compensation properties. Intriguingly, although phosphorylation at these sites does not influence PER stability, timing of nuclear entry, or transcriptional autoinhibition, the phospho-occupancy at Ser826/Ser828 is rapidly stimulated by light and blocked by TIMELESS (TIM), the major photosensitive clock component in *Drosophila* and a crucial binding partner of PER. Our findings identify the first phosphorylation sites on core clock proteins that are acutely regulated by photic cues and suggest that some phosphosites on PER proteins can modulate the pace of downstream behavioral rhythms without altering central aspects of the clock mechanism.

A wide variety of life forms exhibit circadian (~24 h) rhythms in metabolism, physiology, and behavior, which are governed by cellular "clocks" based on the expression of species- or tissue-specific sets of clock genes (reviewed in reference 1). In general, clock mechanisms are biochemical oscillators built on interlocked loops of transcriptional negative feedback and protein degradation, wherein a "master" clock transcription factor drives expression of one or more key repressor proteins that, after a delay, feed back to inhibit the transcription factor until the repressor(s) declines in abundance, enabling another round of gene expression (2). This molecular logic of circadian clocks is usually referred to as transcriptional-translational feedback loops (TTFLs). Studies based on a wide range of model systems indicate that the daily changes in the levels of the key clock feedback repressor(s) are driven by complex temporal phosphorylation programs that dictate the pace of the clock (3–6). In animals, PERIOD (PER) proteins are the central components of the negative arm of the clock mechanism and behave as the primary phosphotimer regulating clock speed (3, 4). A major effect of phosphorylation on regulating the pace of the clock is via evoking temporal changes in the stability of PER proteins, which yields daily cycles in their levels that are inextricably linked to clock progression.

Studies of *Drosophila melanogaster* have been instrumental in our understanding of clock mechanisms in general and mammalian ones in particular. The *D. melanogaster* intracellular clock mechanism is comprised of interlocked transcriptional feedback loops with overlaying posttranslational regulatory circuits (reviewed in reference 7). Prominent players in the first or major TTFL are PER (referred to here as *Drosophila* PER [dPER]), TIMELESS (TIM), CLOCK (dCLK), and CYCLE (CYC; homolog of mammalian BMAL1). dCLK and CYC are transcription factors of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) superfamily that heterodimerize to stimulate the daily transcription of *dper* and *tim*, in addition to other clock and downstream genes. dPER plays a pivotal role in driving cyclical gene expression by undergoing daily translocation from the cytoplasm to the nucleus, where it functions as a critical nexus in the phase-specific inhibition of dCLK-CYC transcriptional activity. Kinases are key players in controlling when in a daily cycle dPER engages in autoinhibition by regulating its stability, timing of nuclear entry, and duration in the nucleus, and possibly its repressor potency (reviewed in reference 3).

Much progress has been made in understanding the role of phosphorylation in regulating dPER's daily life cycle. At midday, *dper* and *tim* mRNA levels begin to rise, but dPER and TIM protein levels remain low during the day. The instability of dPER is due mainly to phosphorylation by the DOUBLETIME (DBT; *Drosophila* homolog of CK1δ/ε) kinase (8, 9), whereas TIM is degraded in a light-mediated pathway that involves the circadian photoreceptor CRYPTOCHROME (CRY) (reviewed in reference 10). After nightfall, TIM levels increase, and this enhances the interaction with dPER, which protects dPER against DBT-mediated degradation. In addition, the interaction of dPER and TIM promotes the translocation of both (in addition to PER-bound DBT) from the cytoplasm to the nucleus, an event that occurs

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around midnight (11–13). In the nucleus, dPER acts as a scaffold to seed ill-defined repressor complexes that block dCLK-CYC-mediated transcription (14–16). As TIM levels begin to drop in the late night/early morning, dPER becomes hyperphosphorylated and is recognized by the F-box protein β-TrCP (termed SLIMB in *Drosophila*), which targets dPER for rapid degradation via the proteasome (17, 18). A rapid decline in the nuclear level of dPER relieves autoinhibition and enables another round of circadian gene expression. Although DBT is the main kinase underlying the progressive phosphorylation of dPER and temporal changes in its stability, other kinases, such as CK2 (19, 20), glycogen synthase kinase 3β (GSK-3β; known as SHAGGY [SGG] in *Drosophila*) (21), and NEMO (22, 23), and protein phosphatases (such as PP1 and PP2A) (24, 25) also regulate dPER metabolism and function.

Using mass spectrometry, we and others identified over 30 phosphosites on dPER (22, 26–29). Intriguingly, many of these sites are organized in phosphoclusters that appear to have different functions and are phosphorylated in an ordered or hierarchical manner. To date, all of the phosphosites that regulate the periodicity of behavioral rhythms appear to have primary effects on dPER stability and/or timing of nuclear translocation. To further explore the role of dPER phosphorylation, we investigated the function of two closely spaced phosphorylation sites (Ser826 and Ser828) that lie within a putative nuclear localization signal (NLS) in dPER (30). Our findings identify a new class of phosphosites on PER proteins that are acutely photoresponsive and appear to regulate the pace of downstream behavioral rhythms without affecting the central clockworks.

MATERIALS AND METHODS

Generation of transgenic flies. To generate transgenic flies carrying *dper* mutations, we used a previously characterized vector that contains a 13.2-kb *dper* genomic fragment tagged with the sequences for an HA epitope and multiple histidine residues (10×His) at the carboxyl terminus (13.2per^+-HAHis) (15). An XbaI-BamHI subfragment of this vector including sequences encoding amino acids (aa) 1 to 870 of dPER was subcloned into the pGEM7 vector (Promega), and the resulting plasmid was used as the template for site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent). The desired mutated *dper* regions were confirmed by DNA sequencing and used to replace the corresponding fragment in the 13.2per^+-HAHis plasmid. Transgenic flies were generated by Genetics Services Inc. (Sudbury, MA), using standard P element-mediated transformation techniques and w^{1118} per^+ (referred to as either $wper^+$ or *w*) embryos as hosts. For each construct, several independent germ line transformants in the $wper^+$ background were obtained, yielding a wild-type version ($wper^+$; p{*dper*-HAHis}), herein referred to more simply as p{*dper*}, and several mutant versions: p{*dper*(S826A)}, p{*dper*(S828A)}, p{*dper*(S826D)}, p{*dper*(S828D)}, p{*dper*(S826A/S828A)}, and p{*dper*(S826D/S828D)}. The transgenes were crossed into a $wper^{01}$ background, and thus the only functional copy of *dper* was expressed from the transgene. p{*dper*/ΔDBD} flies (which contain a deletion in the corresponding DBT binding domain [deleting the sequence for aa 755 to 809] on *dper*) were generated and described previously (31). *w*;;cry^{01} flies, which are null mutants for the circadian light receptor cryptochrome (*cry*), were generated and characterized previously (32). All flies were routinely reared at room temperature (RT; 22 to 25°C) and maintained in vials or bottles containing standard agar-cornmeal-sugar-yeast-Tegosept medium.

Behavioral assays. Locomotor activity was continuously monitored and recorded in either 15- or 30-min bins by placing individual adult male flies in glass tubes and using a *Drosophila* activity monitoring system from Trikinetics (Waltham, MA) as previously described (33). Briefly, 3- to 7-day-old male flies were kept in incubators at the indicated temperature (18, 25, or 29°C) and entrained for at least five daily light-dark (LD) cycles. For the LD cycles, flies were exposed to one of several regimens that differed in day length (photoperiod), namely, the standard condition of 12 h of light and 12 h of dark (12:12 LD) or a regimen with a shorter photoperiod (9:15 LD). In all cases, zeitgeber time zero (ZT0) was defined as the start of the light period. Cool white fluorescent light (~2,000 lx) was used during LD cycles, and the temperature did not vary by more than 0.5°C between the light and dark periods. After the LD cycles, flies were kept at the same temperature for at least 7 days under constant dark conditions (DD) to determine the free-running period. Data analysis was performed on a Macintosh computer with FaasX software (kindly provided by F. Rouyer, CNRS, Gif-sur-Yvette, France). Rhythmic flies were defined by chi-square periodogram analysis with the following settings: power of ≥10 and width of ≥2. Values for individual flies were pooled to obtain an average value for each independent line analyzed. For each construct, locomotor activity rhythms were measured for at least two independent lines in the $wper^{01}$ genetic background and are representative of behavioral results obtained with other independent transgenic lines (data not shown).

Graphical representations of daily activity patterns (actograms; see Fig. 3) were generated using the eduction option of FaasX software. Briefly, activity counts were collected in 15-min bins, and the data for individual flies were pooled to generate group averages for the last day of LD entrainment followed by the next 2 days in constant darkness. The timing of the peaks and onsets for the morning and evening bouts of activity was calculated as previously described, using the unix command line version of the Brandeis Rhythm Package (BRP) phase module (34). The morning and evening onsets of activity were defined as the times in the day when 50% of the peak activity was attained prior to the respective peak, which we previously showed was a reliable indicator of the phase in the morning and evening activity bouts (34). The values shown in Table 2 were based on group averages of data collected in 30-min bins over at least 2 days of LD cycles for multiple flies of the same genotype.

Plasmids for S2 cell expression. Most of the plasmids used in this study were described previously, including pAct-*per*-V5/His (18), pAct-*per*(513-1224)-V5/His (30), pAct-3xFlag-6×His-*per*-6xmyc (26), pMT-*dbt*-V5/His (18), pMT-*sgg*-V5/His (29), pMT-*CK2*α and pMT-*CK2*β-V5/His (35), pMT-*nemo*-HA (22), pMT-*Clk*-V5/His (35), and pAct-E-box-luc (14), and pAct-ren.luc was a gift from R. Padgett (Rutgers University). To generate *dper* constructs containing point mutations (e.g., encoding S826A, S828A, and S826/828A mutations), we used the previously described pAct-*per*-V5/His vector (18) and pAct-*per*(514-1224)-V5/His (30) in combination with a QuikChange site-directed mutagenesis kit (Agilent). Final constructs were confirmed by sequencing prior to use.

S2 cell culture-based assays. S2 cells and *Drosophila* Expression System (DES) expression medium were obtained from Life Technologies, and transient transfections were performed using Effectene (Qiagen) according to the manufacturer's instructions. For each transient-transfection assay, 0.8 μg of a *dper*- or *tim*-containing plasmid and 0.2 μg of pMT vector expressing a kinase or empty control pMT-V5/His plasmid were used. Induction of pMT-driven kinases was achieved by adding 500 μM $CuSO_4$ to the culture medium 24 h after transfection, as previously described (18, 36). Cells were collected at the indicated times after induction. Where indicated, the proteasome inhibitor MG132 (50 μM; Sigma) and cycloheximide (10 μg/ml; Sigma) were added to the medium 18 h after *dbt* induction, and cells were collected 4 h later. For stability assay in the absence of *de novo* protein synthesis, cycloheximide (10 μg/ml; Sigma) was added to the medium 18 h after *dbt* induction, and cells were collected at the indicated times.

To measure CLK-mediated transactivation in tissue culture, we used the standard approach of E-box-mediated transcription of a *luciferase* (*luc*) reporter in combination with the Dual-Glo luciferase assay system (Promega) as described previously (37). Briefly, 1×10^6 S2 cells in 1 ml of S2 cell medium supplemented with 10% bovine serum were seeded into

12-well plates. One day after seeding, cells were transfected with 2 ng of a pMT-*Clk* vector, either alone or mixed with 1.5 or 10 ng of a *dper*-carrying pAct-based plasmid, in addition to 10 ng pAc-E-box-luc (14) and 25 ng pAct-ren.luc. An empty pAct-V5/His vector was used to balance the total amount of plasmids transfected into each well. One day after transfection, *dClk* expression was induced by adding 500 μM $CuSO_4$ (final concentration) to the medium, and after another day, luminescence activities from the firefly and renilla luciferases were measured in 75 μl of cell suspension, using protocols supplied by the manufacturer (Promega).

Generation of anti-dPER pS826/pS828 phospho-specific antibody. Affinity-purified anti-dPER pS826/pS828 antibodies were generated by Proteintech Group, Inc. Briefly, two rabbits were immunized with a 16-aa peptide (pS826/pS828; amino acids 820 to 835 [GIKRGGpSHpSWEGEA NK]; "p" indicates phosphate, and numbering is based on the full-length dPER sequence) conjugated to the carrier keyhole limpet hemocyanin. Antisera were affinity purified on a resin containing the pS826/pS828 peptide, yielding anti-pS826/pS828 Rb1 and Rb2 antibodies. In this study, only the Rb2 antibody was used, as it has a higher affinity. Enzyme-linked immunosorbent assays (ELISAs) using peptides that were nonphosphorylated (S826/S828), singly phosphorylated (pS826/S828 and S826/pS828), or doubly phosphorylated (pS826/pS828) showed that the anti-pS26/pS828 antibody recognized only the doubly phosphorylated version (Fig. 1B).

Immunoblotting. To prepare cell extracts for immunoblotting of proteins in cultured S2 cells, the cells were harvested, washed in phosphate-buffered saline (PBS), and homogenized using EB2 solution (20 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 5 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% Triton X-100, 25 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) supplemented with complete EDTA-free protease inhibitor cocktail (Roche) (26). Extracts from fly heads were prepared as previously described (38). Briefly, flies were collected by freezing at the indicated times under LD or DD conditions, and total fly head extracts were prepared by homogenization in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.5 mM PMSF), with the addition of complete EDTA-free protease inhibitor cocktail and PhosStop (Roche) (38). Extracts were resolved using either 6% or 4 to 15% SDS-polyacrylamide gels to detect dPER and other proteins, as indicated in the figure legends. Primary antibodies were used at the following dilutions: rat antihemagglutinin (anti-HA) (3F10; Roche), 1:1,000; rat anti-TIM R3 (39), 1:2,000; mouse antitubulin (Sigma), 1:7,000; mouse anti-V5 (Invitrogen), 1:5,000; and mouse anti-c-MYC (9E10; Sigma), 1:5,000. Appropriate horseradish peroxidase (HRP)-conjugated IgG secondary antibodies were used at a 1:2,000 dilution (GE Healthcare).

IP and phosphatase treatment. To examine the phosphorylation of dPER on S826/S828 by use of the anti-pS826/pS828 antibody, we first purified dPER by subjecting extracts to immunoprecipitation (IP) as described previously (26). Briefly, extracts from S2 cells or ~800 fly heads were prepared using EB2 buffer supplemented with complete EDTA-free protease inhibitor cocktail (Roche) or modified RIPA buffer supplemented with a protease inhibitor cocktail and PhosStop (Roche), respectively. About 20 μl of each extract was kept for input analysis, and to the rest of the extracts, 30 μl of anti-V5–agarose (Sigma) or anti-HA–agarose (Sigma) resin was added, depending on the epitope tag on *dper*, followed by incubation with gentle rotation at 4°C for 4 h. For experiments involving *w*;;*cry*^{01} flies, extracts were incubated with 3 μl of anti-dPER antibody (GP-73) (39), followed by 1 h of incubation with GammaBind Plus Sepharose beads (GE Healthcare). Beads were collected, washed for 10 min with the lysis buffer, and then resuspended in 30 μl of 1× SDS-PAGE sample buffer and incubated for 5 min at 100°C. The resulting supernatants were resolved by immunoblotting, using 6% and 4 to 15% SDS-polyacrylamide gels for full-length dPER and truncated dPER(513-1224), respectively. To detect phosphorylated S826/S828, immunoblots were incubated with rabbit anti-pS826/pS828 phospho-specific antibody at a

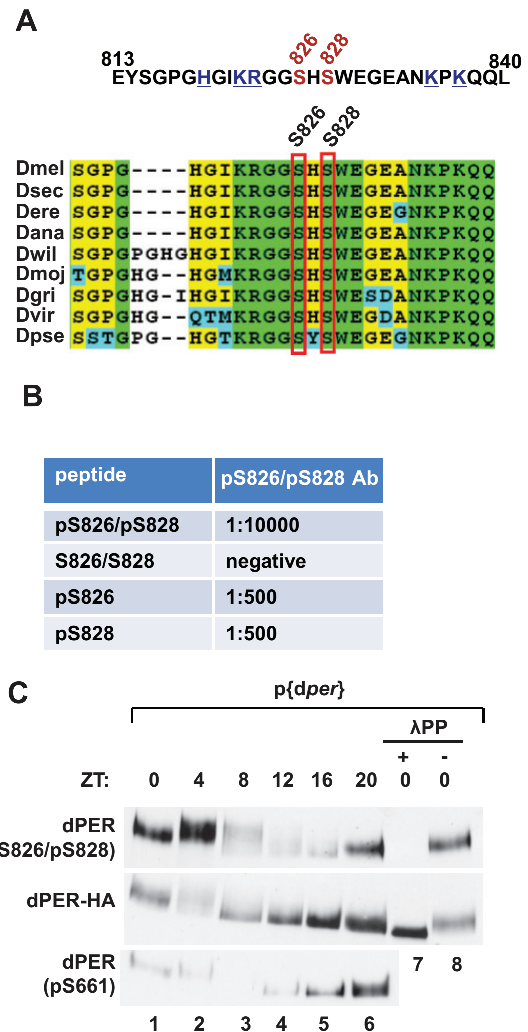

FIG 1 Late night/early morning phosphorylation of Ser826 and Ser828 in the NLS-2 region of dPER. (A) (Top) Sequence of dPER from residues 813 to 840, with S826/S828 displayed in red and basic residues of a previously characterized bipartite nuclear localization signal (NLS) underlined. (Bottom) The NLS-2 region, including Ser826 and Ser828, is conserved in different species of *Drosophila*. Dmel, *D. melanogaster*; Dsec, *D. sechellia*; Dere, *D. erecta*; Dana, *D. ananassae*; Dwil, *D. willistoni*; Dmoj, *D. mojavensis*; Dgri, *D. grimshawi*; Dvir, *D. virilis*; Dpse, *D. pseudoobscura*. (B) Results of ELISA testing with the anti-pS826/pS828 antibody (Rb2) in the presence of peptides that differ in phosphorylation at Ser826 and Ser828 of dPER. (C) Adult flies were collected at the indicated times (ZT [hours]), and head extracts were prepared. dPER-HA was immunoprecipitated (IP) with anti-HA beads; for ZT0, the sample was divided in two and the aliquots treated in the presence (+) or absence (−) of lambda phosphatase (λPP). Recovered immune complexes were resolved by 6% SDS-PAGE and blotted in the presence of anti-HA to measure total dPER levels or with phospho-specific antibodies (pS826/pS828 or p661) to measure different dPER phosphosites. Similar results were obtained in at least two independent experiments, and a representative example is shown.

1:1,000 to 1:2,000 dilution. For input analysis, extracts were resolved using 4 to 15% SDS-polyacrylamide gels unless otherwise stated.

For phosphatase treatment, immune complexes bound to anti-HA– or anti-V5–agarose beads (see above) were washed twice with modified RIPA buffer followed by another wash in λ-protein phosphatase (λ-PP) buffer (NEB). Immune complexes were then resuspended in 40 μl λ-PP buffer, and one aliquot was treated with λ-PP (NEB) for 30 min at 30°C, whereas another aliquot was mock treated in the absence of λ-PP. λ-PP

buffer was removed, and immune complexes were resuspended in 1× SDS sample buffer and directly analyzed by immunoblotting as described above.

Confocal imaging of adult brains. Whole mounts of adult brains were prepared and imaged as described previously (40), with the following modifications. Briefly, adult flies were collected at the indicated times during a daily LD cycle and fixed for 3 h in 4% paraformaldehyde with 0.1% Triton X-100 at RT in darkness. After fixation, brains were dissected in cold PBS and washed twice with PBT solution (PBS containing 0.5% Triton X-100). Brains were incubated in blocking solution (PBT with 10% goat serum) for 1 h at RT and then overnight at 4°C, with the addition of primary antibodies at the following final dilutions: anti-dPER (41), 1:200; and anti-PDF C7 (42), 1:200. Subsequently, brains were washed three times with PBT and then incubated overnight in blocking solution with the secondary antibody Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen) or Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen), both at a final dilution of 1:200. After several washes with PBT, brains were transferred to slides and mounted with Vectashield (Vector Laboratories) on a coverslip. Confocal images were obtained with a Leica SP2 confocal microscope and processed with LCS Lite software.

Measuring *dper* mRNA levels. The relative levels of *dper* mRNA in fly head extracts were measured by semiquantitative reverse transcription-PCR as described previously (43). Briefly, ~100 adult flies were entrained in the respective LD regimens (i.e., 12:12 LD or 9:15 LD) for 4 days and then collected during the last day of LD or the first day of DD, at the indicated times, by freezing on dry ice. For each time point, heads were isolated and total RNA extracted using Tri reagent (Sigma) in accordance with the manufacturer's instructions. Approximately 1 μg of total RNA was reverse transcribed using a ThermoScript RT-PCR kit (Life Technologies) in the presence of oligo$(dT)_{20}$ as a primer. To amplify *dper* and the noncycling control mRNA encoding the cap binding protein 20 (*cbp20*), a 2-μl aliquot of the reaction mixture was further processed by PCR, using the *dper*-specific primers P7197 and P6869 and the *cbp20*-specific primers CBP236 and CBP594, respectively, as previously described (43).

RESULTS

Phosphorylation of two Ser residues in a putative NLS on dPER occurs in the late night/early morning and is mediated by DBT. Among the phosphosites we identified on dPER expressed in cultured *Drosophila* S2 cells by using mass spectrometry were Ser826 and Ser828 (26), whereas a more recent study using similar methodology further supports phosphorylation at Ser826 (27). We were initially prompted to investigate the role of dPER phosphorylation at Ser826/Ser828 because these sites are embedded in a sequence that has signature motifs of a bipartite NLS (Fig. 1A, top panel) and, more importantly, was shown to function in this capacity when evaluated in *Drosophila* S2 cells (30). Moreover, Ser826/Ser828 and the surrounding putative NLS sequences are highly conserved in *Drosophila* (Fig. 1A, bottom panel). In keeping with the nomenclature of a recent study, we refer to this putative dPER NLS as NLS-2 (27).

To better study the phosphorylation of Ser826/Ser828, we sought to generate phospho-specific antibodies, a strategy we previously used successfully to examine the *in vivo* phosphorylation of dPER at other phosphosites (22, 26, 29). Due to the closeness of Ser826 and Ser828, we were concerned that antibodies recognizing only a single phosphosite might not detect the doubly phosphorylated version *in vivo*, and thus we used a doubly phosphorylated peptide (pS826/pS828) as the immunogen (see Materials and Methods). ELISA showed that the anti-dPER pS826/pS828 antibody used here mainly recognizes the doubly phosphorylated peptide (Fig. 1B).

To determine if our phospho-specific antibody recognizes dPER *in vivo*, we used transgenic flies in which the only functional copy of *dper* is a transgene that is a wild-type copy of *dper* modified with an HA epitope tag to facilitate purification of dPER (termed p{*dper*}) (29, 31). Flies were entrained (synchronized) under standard conditions of 12 h of light and 12 h of dark (12:12 LD) at 25°C and were collected at different times in the day, and head extracts were prepared. Subsequently, dPER was immunoprecipitated and then immunoblotted in the presence of anti-dPER pS826/pS828 phospho-specific antibody. Strong staining was observed at several times throughout the daily cycle but not when extracts were first treated with phosphatase, verifying the phospho-specificity of our anti-dPER pS826/pS828 antibody (Fig. 1C, top panel, compare lanes 7 and 8). When we probed for total dPER levels, we observed the expected wild-type pattern whereby dPER was first detected as a newly synthesized hypophosphorylated species (fastest-migrating species) at ~ZT8, underwent progressive increases in phosphorylation, peaked in abundance at ~ZT20, and attained the most highly phosphorylated isoforms around ZT4, concomitant with rapid decreases in levels (Fig. 1C, middle panel) (44). Intriguingly, significant phosphorylation of Ser826/Ser828 was first observed at ZT20, with peak values at ZT4, despite the fact that total levels of dPER began to decline rapidly during the early day (Fig. 1C, middle panel). This staining pattern is very different from that obtained for a dPER phosphorylation site (S661) we previously showed to affect the timing of dPER nuclear entry, which is readily observed at ZT16 and peaks at ZT20 (Fig. 1C, bottom panel) (29).

Several lines of evidence using cultured S2 cells and flies indicate that Ser826 and Ser828 are phosphorylated by DBT, consistent with our earlier results obtained using mass spectrometry (26) (Fig. 2). In prior work, we showed that although some endogenous kinases can phosphorylate dPER at several sites in S2 cells, the coexpression of *dbt* with recombinant *dper* evokes the slow conversion of hypophosphorylated dPER to hyperphosphorylated variants that are recognized by SLIMB for rapid degradation by the proteasome, recapitulating the main features of the dPER phosphorylation program observed in flies (18, 36). Phosphorylation of Ser826/Ser828 was detected when *dbt* was coexpressed with *dper* in S2 cells but not in the absence of recombinant *dbt* (Fig. 2A, top panel, compare lanes 1 and 3). We evaluated several clock-relevant kinases, but only *dbt* resulted in significant phosphorylation of Ser826/Ser828 (Fig. 2B). In flies, efficient phosphorylation of dPER by DBT requires the DBT binding domain (DBD) on dPER, and removal of this region (dPER/ΔDBD) results in flies that produce very stable dPER, with few changes in abundance or phosphorylation throughout the daily cycle (31, 37). As was the case when we used other dPER phospho-specific antibodies specific to sites that are phosphorylated by DBT (22, 26), when we probed samples with the anti-dPER pS826/pS828 antibody, there was little signal with dPER(ΔDBD) compared to dPER, even though the levels of dPER(ΔDBD) were much higher than those of dPER (Fig. 2C, lanes 1 and 3). Together, our results strongly suggest that DBT directly phosphorylates Ser826 and Ser828 on dPER.

As expected, DBT-induced phosphorylation was also abolished when we probed S2 cell extracts containing a dPER mutant version in which S826 and S828 were replaced by Ala residues [dPER(S826A/S828A)] (Fig. 2D, lanes 1 and 2). Likewise (as described in more detail below), dPER was not recognized by the anti-pS826/pS828 antibody in transgenic flies bearing a *dper* mu-

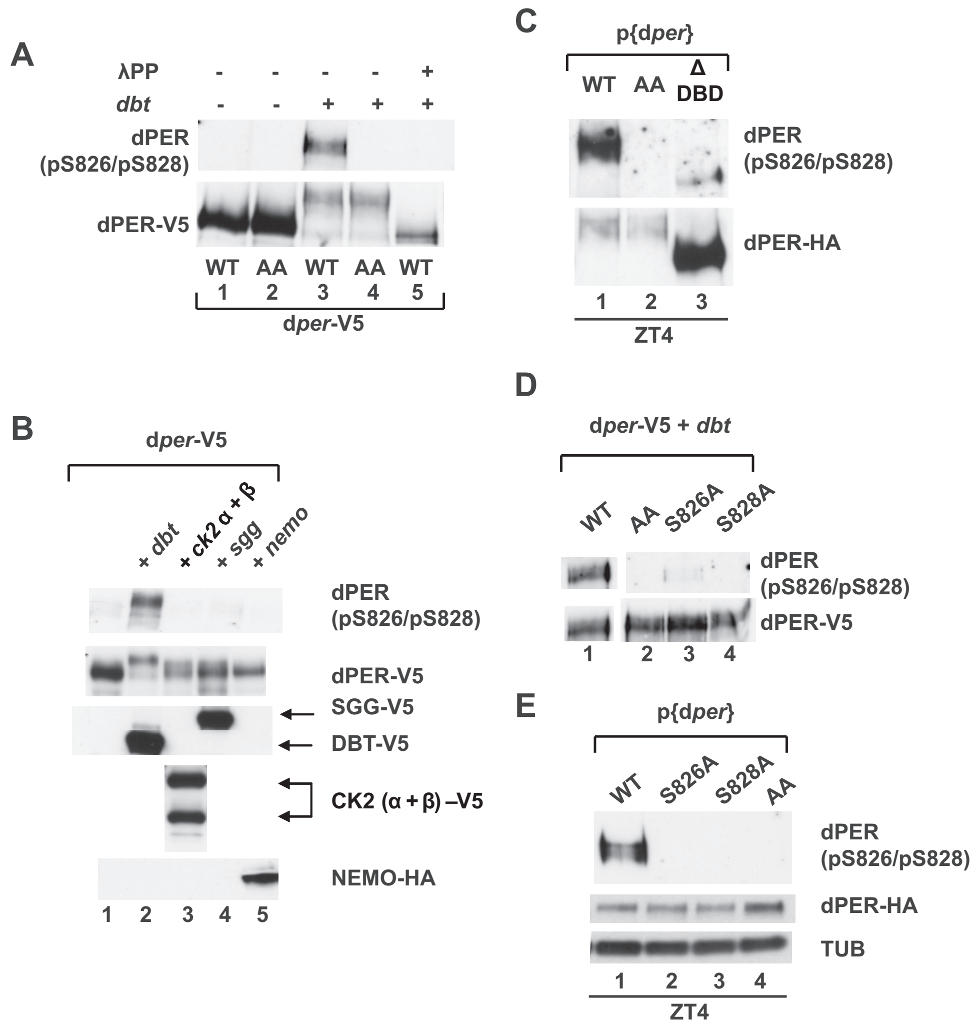

FIG 2 Ser826 and Ser828 of dPER are phosphorylated by the DOUBLETIME kinase. (A) S2 cells were transiently transfected with pAct-*dper*-V5/His (WT) or pAct-*dper*-(S826A/S828A)-V5/His (AA), as indicated at the bottom of panel, in the presence (+) or absence (−) of pMT-*dbt*-V5/His (*dbt*). Extracts were subjected to IP using anti-V5 beads, and the sample in lane 5 was further treated with lambda phosphatase (λPP). Immune complexes were resolved by 6% SDS-PAGE, and immunoblots were incubated with anti-V5 or anti-pS826/pS828 antibody. (B) S2 cells were transfected with *dper*-V5 alone (lane 1) or cotransfected with different pMT versions of plasmids encoding clock-relevant kinases (lanes 2 to 5) as indicated at the top. At 24 h postinduction, a small portion of the extract was directly analyzed by immunoblotting (4 to 15% SDS gradient gel) in the presence of anti-V5 or anti-HA antibody to detect total dPER levels (dPER-V5) or the relevant recombinant kinases (as indicated on the right). The rest of the extract was immunoprecipitated to recover dPER and immunoblotted in the presence of anti-pS826/pS828 antibody (top panel). (C) Flies with the indicated genotypes [WT, p{*dper*}; AA, p{*dper*(S826A/S828A)}; and ΔDBD, p{*dper*(ΔDBD)}] were collected at ZT4, and head extracts were prepared, followed by IP with anti-HA beads and immunoblotting with anti-pS826/pS828 or anti-HA antibody (as indicated on the right). (D) S2 cells were cotransfected with pMT-*dbt*- and pAct-driven versions of *dper*-V5. WT, wild type; AA, *dper*(S826A/S826A); S826A, *dper*(S826A); S828A, *dper*(S828A). After induction of *dbt* for 24 h, cell extracts were either directly probed by immunoblotting for total dPER levels by use of anti-V5 antibody or subjected to IP with anti-V5 antibody resins followed by immunoblotting in the presence of phospho-specific anti-pS826/pS828 antibody. (E) Transgenic adult flies of the indicated genotypes [WT, p{*dper*}; S826A, p{*dper*(S826A)}; S828A, p{*dper*(S828A)}; and AA, p{*dper*(S826A/S828A)}] were collected at ZT4, head extracts were prepared, and a small portion was immunoblotted in the presence of anti-HA antibody to detect total dPER levels (dPER-HA) or antitubulin antibody (TUB) as a loading control. The majority of the head extract was subjected to IP using anti-HA antibody resins and immunoblotted in the presence of phospho-specific anti-pS826/pS828 antibody. Results similar to those shown in each panel were obtained in at least three independent experiments.

tant with the S826A/S828A mutations (Fig. 2E, lanes 1 and 4). We also tested singly mutated versions of *dper* (S826A or S828A) produced in S2 cells or flies and did not detect staining with the anti-pS826/pS828 antibody (Fig. 2D and E), consistent with the specificity of the phospho-specific antibody (Fig. 1B). Attempts to raise singly phosphorylated phospho-specific antibodies (i.e., specific to pS826 or pS828) were not successful, so we do not know if blocking phosphorylation at one site affects phosphorylation at the neighboring site.

Blocking phosphorylation at Ser826/Ser828 shortens behavioral rhythms and affects temperature compensation of period length. To study the physiological role of phosphorylation at Ser826/Ser828 in circadian timing, we generated a series of transgenic flies that produce HA epitope-tagged versions of dPER with Ser-to-Ala replacements to block phosphorylation at S826, S828, or both, i.e., dPER(S828A), dPER(S828A), and dPER(S826A/S828A). In addition, we also generated versions in which each or both of the phosphorylated residues were replaced with Asp as a

TABLE 1 Locomotor activity rhythms for mutant and control *dper* transgenics

Genotype[a]	Photoperiod (LD)[b]	Period (h) (mean ± SEM)[c]	Power[d]	Rhythmicity (%)[e]	Total no. of flies[f]
wper^0; *per*$^+$-HA(S826A/S828A) (1M)	12:12	22.3 ± 0.06**	145.2	96.8	32
wper^0; *per*$^+$-HA(S826A/S828A) (2M)	12:12	22.4 ± 0.11**	92.2	100	12
wper^0; *per*$^+$-HA(S826D/S828D) (2M)	12:12	23.9 ± 0.13**	90.8	95.8	25
wper^0; *per*$^+$-HA(S826D/S828D) (4F)	12:12	23.8 ± 0.06**	117.9	96.9	32
wper^0; *per*$^+$-HA (1M)	12:12	23.5 ± 0.05	139.3	100	30
wper^0; *per*$^+$-HA (2M)	12:12	23.4 ± 0.07	125.2	100	32
wper^0; *per*$^+$-HA(S826A/S828A) (1M)	9:15	22.3 ± 0.07**	114.5	90	30
wper^0; *per*$^+$-HA (1M)	9:15	23.5 ± 0.06	146.7	100	26

[a] Independent transgenic lines are indicated by the designations in parentheses.
[b] Young male flies were maintained at 25°C and exposed to 5 days of the indicated LD cycle (12:12 or 9:15), followed by 7 days of constant darkness (DD), to measure locomotor activity rhythms.
[c] **, $P < 0.01$ (*t* test; pairwise comparison); indicates a significant difference in period values for transgenic flies bearing a mutant form of *dper* and wild-type transgenic controls (i.e., *wper*^{0}; *per*$^+$-HA) under the same photoperiod conditions.
[d] Power is a measure of the strength or amplitude of the rhythm and is given in arbitrary units.
[e] Percentage of flies showing locomotor rhythms with power values of ≥10 and width values of ≥2.
[f] Total number of flies that survived until the end of the experimental period.

potential phosphomimetic, i.e., dPER(S826D), dPER(S828D), and dPER(S826D/S828D). Finally, we also made new transgenic lines bearing the wild-type version of the *dper* transgene. The effects of the different transgenes were examined in the *per*-null *wper*^{0} background (45), whereby the only functional copy of *dper* is provided by the transgene. At least two independent lines of each genotype were analyzed. Flies were kept at 25°C and entrained for 4 days under 12:12 LD conditions, followed by 7 days in DD (constant dark) to determine their free-running period. As previously shown, *wper*^{0} flies expressing the wild-type *dper* transgene with an HA tag (p{*dper*}) exhibited strong rhythms with ~24-h periods (Table 1) (31, 46).

The p{*dper*(S826A/S828A)} flies [referred to as *wper*^{0}; *per*$^+$-HA(S826A/S828A) in the tables to better identify the genotypes] manifested a robust activity rhythm that was shorter by slightly more than 1 h than that of the wild-type controls ($P < 0.001$) (Table 1). With standard 12:12 LD cycles, wild-type *D. melanogaster* flies exhibit two main clock-controlled bouts of activity, with a morning peak centered on ZT0 and an evening peak centered on ZT12, that begin to rise prior to the light-dark transitions (Fig. 3) (reviewed in reference 33). In addition to the anticipatory morning and evening activity bouts, *D. melanogaster* flies routinely exhibit transient increases in activity at the light-dark transitions, termed "startle" or masking responses (47). The onset of activity is generally considered a reliable phase indicator for measuring the relative timing of the morning and evening bouts of activity (e.g., see reference 34). The upswing in morning activity began about 0.6 h earlier in p{*dper*(S826A/S828A)} flies ($P < 0.001$) (Table 2), consistent with the shorter free-running period, although the timing of the evening activity bout did not show a significant difference (Fig. 3A and Table 2).

We also examined the daily activity rhythm with a shorter photoperiod (9:15 LD) to better visualize the timing of the morning activity peak separate from the lights-on startle response (Fig. 3B). With the shorter photoperiod, the timing of evening activity in p{*dper*(S826A/S828A)} flies was around 30 min earlier, but the morning activity occurred substantially earlier (~1.4 h; $P < 0.001$) (Table 2). This preferential effect on the timing of the morning activity bout for a *dper* mutant is quite unusual, because to the best of our knowledge, *D. melanogaster* clock mutants or genetically altered transgenic flies with short-period behavioral rhythms that have been analyzed in daily light-dark cycles, including numerous dPER phosphosite mutants, manifest noticeably advanced evening bouts, whereas the morning activity

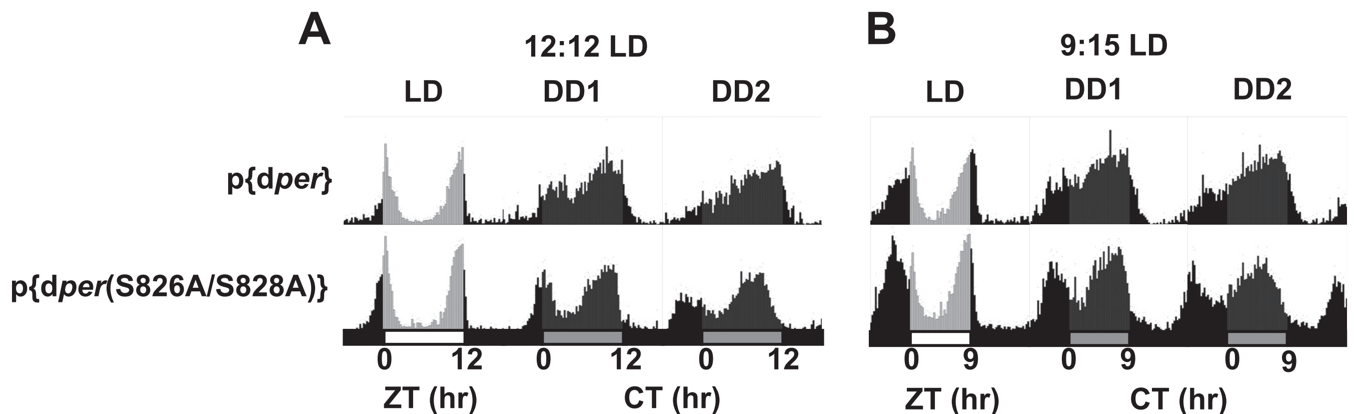

FIG 3 Blocking phosphorylation of S826/S828 alters the daily distribution of activity. (A and B) Transgenic adult flies carrying a wild-type or mutant version of *dper* (as indicated on the left) were exposed to 5 days of LD cycles with the indicated photoperiod (i.e., 12:12 or 9:15) followed by 7 days in complete darkness (DD). Activity histograms are shown for the last LD entrainment cycle and the next 2 days under free-running conditions.

TABLE 2 Timing of daily activity in mutant and control *dper* transgenic flies under different photoperiod conditions

Genotype[a]	Photoperiod (LD)	n[b]	Zeitgeber time (h) (mean ± SEM)[c]			
			Morning peak	Morning onset[d]	Evening onset[e]	Evening peak
wper[0]; *per*[+]-HA(S826A/S828A) (1M)	12:12	27	1 ± 0.08**	23.24 ± 0.11**	10 ± 0.05	11.85 ± 0.05
wper[0]; *per*[+]-HA (1M)	12:12	30	1.4 ± 0.13	23.83 ± 0.08	9.9 ± 0.05	11.75 ± 0.05
wper[0]; *per*[+]-HA(S826A/S828A) (1M)	9:15	28	22.49 ± 0.12**	20.29 ± 0.12**	6.65 ± 0.13*	8.78 ± 0.07*
wper[0]; *per*[+]-HA (1M)	9:15	30	23.83 ± 0.16	21.65 ± 0.19	7.1 ± 0.14	9.25 ± 0.11

[a] Young male flies were maintained under the indicated photoperiod conditions at 25°C for 5 days. The last 2 days' worth of activity data were averaged for each individual fly, and then the group average was determined.
[b] Number of flies that survived the entire testing period.
[c] ZT0 was defined as the lights-on time. *, $P < 0.05$ compared to the value for the wild-type control under the same photoperiod conditions; **, $P < 0.001$ compared to the value for the wild-type control under the same photoperiod conditions.
[d] Morning onset was defined as the time in the day when 50% of the value for the morning peak of activity was attained prior to the morning peak of activity.
[e] Evening onset was defined as the time in the day when 50% of the value for the evening peak of activity was attained prior to the evening peak activity.

peak shows smaller or no changes (e.g., see references 22, 40, 46, and 48 to 52).

The flies with single Ser-to-Ala replacements [p{*dper*(S826A)} and p{*dper*(S828A)}] also showed shorter rhythms, especially the S828A variant, but with smaller changes, in general, than that with the doubly mutated version (Table 3), indicating that phosphorylation at both sites has a more potent effect on behavioral rhythms. A recent study found no effect on behavioral periods from mutating Ser826 but did not evaluate mutations in both Ser826 and Ser828 or Ser828 by itself (27). Mutant versions of dPER in which both Ser826 and Ser828 were replaced by Asp residues resulted in a small but significant increase in period length of about 30 min ($P < 0.01$) (Table 1), suggesting that phosphorylation at these sites generally functions to slow down behavioral rhythms.

A hallmark feature of circadian rhythms is that the period length is relatively stable over a wide range of physiologically relevant temperatures; this is known as temperature compensation (53). Whereas the control transgenic flies harboring a wild-type copy of *dper* exhibited excellent temperature compensation properties, the period for p{*dper*(S826A/S828A)} flies shortened as the temperature increased (Fig. 4). Intriguingly, for the Asp double mutant [p{*dper*(S826A/S828A)}], the period increased at higher temperatures. Thus, at 29°C, there was an approximately 2-h difference in the free-running behavioral periods of flies in which phosphorylation at Ser826/Ser828 was abrogated and those that potentially mimic constitutive phosphorylation at these sites (Fig. 4). The fact that the double Ala (S826A/S828A) and Asp (S826D/S828D) mutants influenced period length in opposite directions further suggests a modulatory role for the phosphorylation status

TABLE 3 Locomotor activity rhythms for mutant *dper* transgenics and controls[a]

Genotype[b]	Period (h) (mean ± SEM)[c]	Power[d]	Rhythmicity (%)[e]	Total no. of flies[f]
wper[0]; *per*[+]-HA(S826A) (1M)	23.6 ± 0.09	119.3	96.8	32
wper[0]; *per*[+]-HA(S826A) (2M)	22.9 ± 0.09	98.4	78.1	32
wper[0]; *per*[+]-HA(S826A) (4M)	22.7 ± 0.1	105.2	95.8	32
wper[0]; *per*[+]-HA(S826A) (6M)	22.9 ± 0.05	139.7	87.5	32
Avg for *wper*[0]; *per*[+]-HA(S826A) lines	23.0 ± 0.08*#			
wper[0]; *per*[+]-HA(S826D) (1M)	23.3 ± 0.15	104	87.1	32
wper[0]; *per*[+]-HA(S826D) (2M)	23.9 ± 0.22	140.3	100	13
wper[0]; *per*[+]-HA(S826D) (5M)	23.3 ± 0.07	111.9	93.5	32
Avg for *wper*[0]; *per*[+]-HA(S826D) lines	23.5 ± 0.16			
wper[0]; *per*[+]-HA(S828A) (1M)	22.6 ± 0.1	87.3	87.5	32
wper[0]; *per*[+]-HA(S828A) (4M)	22.9 ± 0.31	92.2	83.3	32
wper[0]; *per*[+]-HA(S828A) (5M)	22.7 ± 0.14	116.5	89.7	32
wper[0]; *per*[+]-HA(S828A) (7M)	22.4 ± 0.05	142.7	93.5	32
Avg for *wper*[0]; *per*[+]-HA(S828A) lines	22.6 ± 0.18*			
wper[0]; *per*[+]-HA(S828D) (1M)	23.2 ± 0.08	147.8	100	32
wper[0]; *per*[+]-HA(S828D) (3M)	23.2 ± 0.09	145.8	96.9	32
wper[0]; *per*[+]-HA(S828D) (4M)	23.8 ± 0.2	55.8	57.1	32
wper[0]; *per*[+]-HA(S828D) (5F)	24.8 ± 0.1	116.8	100	32
Avg for *wper*[0]; *per*[+]-HA(S828D) lines	23.7 ± 0.13			

[a] Young male flies were maintained at 25°C and exposed to 5 days of 12:12 LD cycles followed by 7 days of DD.
[b] Independent transgenic lines are indicated by the designations in parentheses.
[c] *, $P < 0.05$ compared to the value for wild-type controls (1M and 2M); #, $P < 0.001$ compared to the value for the double alanine mutant lines, i.e., *wper*[0]; *per*[+]-HA(S826A/S828A) lines (1M and 2M).
[d] Power is a measure of the strength or amplitude of the rhythm and is given in arbitrary units.
[e] Percentage of flies showing locomotor rhythms with power values of ≥10 and width values of ≥2.
[f] Total number of flies that survived until the end of the experimental period.

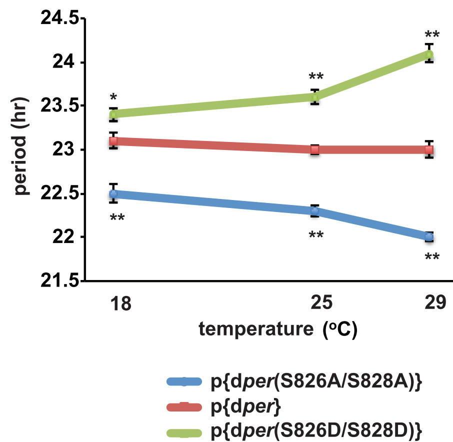

FIG 4 Replacing the dPER Ser residues at positions 826 and 828 with Ala or Asp impairs temperature compensation of period length. Adult transgenic flies of the indicated genotypes were entrained under 12:12 LD conditions for 4 days at the indicated temperature (18, 25, and 29°C), followed by DD for 7 days, and the free-running locomotor activity periods were plotted. *, $P < 0.05$; **, $P < 0.01$ (*t* test; pairwise comparison to the period for the wild-type p{*dper*} control). In addition, analysis of variance (ANOVA) showed a significant effect of temperature on period length in p{*dper*(S826A/S828A)} and p{*dper*(S826D/S828D)} transgenics ($P < 0.0001$; one-way ANOVA) but not in wild-type p{*dper*} flies ($P = 0.5847$; one-way ANOVA).

of Ser826/Ser828 in governing the pace of behavioral rhythms. Although the mechanism underlying temperature compensation of circadian rhythms in *D. melanogaster* is not clear, several *dper* mutants with shortened behavioral periods exhibit similar mild defects in temperature compensation whereby period length is shorter at warmer temperatures (50, 53, 54). However, not all *dper* mutants that have short periods show deficiencies in temperature compensation capabilities (48).

There are no discernible effects on the clockworks by blocking phosphorylation at Ser826/Ser828. Because p{*dper*(S826A/S828A)} flies yielded the most significant changes in behavioral periods, we focused on these flies to examine the molecular status of the clock by tracking the daily rhythms in *dper* protein and mRNA levels. Although behavioral periodicity was only modestly affected in p{*dper*(S826A/S828A)} flies, prior work with transgenic fly models showed that alterations in clock genes that shorten behavioral rhythms by 1 to 2 h are nonetheless accompanied by clear changes in some aspect of the clockworks, such as changes in *dper* protein and mRNA cycles, the dPER temporal phosphorylation program, or the timing of dPER nuclear entry (e.g., see references 26, 29, 51, and 52). In contrast, we did not observe any differences in the *dper* mRNA or protein cycle in p{*dper*(S826A/S828A)} flies (Fig. 5).

For example, the dPER(S826A/S828A) protein exhibited daily rhythms in abundance and phosphorylation that were indistinguishable from those observed for control wild-type transgenics (Fig. 5A and B), although a 1-h difference that affects only the timing of daily changes in dPER levels and phosphorylation would be difficult to detect using immunoblotting. Similar results were also obtained with S2 cells, in which the kinetics of DBT-mediated progressive phosphorylation and degradation of hyperphosphorylated isoforms of dPER were similar for dPER(S826A/S828A) and wild-type dPER (Fig. 5C). In addition, dPER and dPER(S826A/S828A) had virtually identical stabilities in S2 cells in the presence of cycloheximide to block *de novo* protein synthesis (Fig. 5D). Together, our results indicate that phosphorylation at Ser826/Ser828 has little to no effect on dPER stability/levels or its overall global phosphorylation program. These findings are starkly different from those for other characterized phosphosites on dPER that are phosphorylated by DBT, which have significant effects on dPER stability, temporal phosphorylation, or both (22, 26–28) (see Discussion).

As mentioned above, Garbe et al. (27) also identified Ser826, and when they tested a dPER(S826A) version in S2 cells, they found no effect on stability or progressive phosphorylation, similar to our findings for the dPER(S826/828A) double mutant (Fig. 5C and D). However, they did note that dPER(S826A) had significantly less repressor function when evaluated by the standard S2 cell assay of inhibiting dCLK-mediated transcription of a *per-luciferase* (*per-luc*) reporter. This was interpreted as suggesting that phosphorylation at Ser826 stimulates dPER nuclear entry/accumulation (27). Likewise, we obtained a similar result when we evaluated the Ser826/Ser828 double mutant in a smaller version of dPER [dPER(513-1224)] missing a cytoplasmic localization determinant, enhancing the ability to measure dPER repressor function (12, 30) (Fig. 5E). dPER(513-1224/S826A/S828A) was about 20 to 30% less efficient at repressing dCLK-mediated transcription than dPER(513-1224). When we assayed the same mutant, but this time containing a heterologous NLS [dPER(513-1224/S826A/S828A)-NLS], essentially wild-type repressor activity was observed (Fig. 5E), consistent with the idea that phosphorylation at Ser826 and/or Ser828 promotes dPER nuclear localization.

However, analysis of the *dper* mRNA daily rhythm by using high-resolution 1.5-h time points showed virtually identical curves for p{*dper*} and p{*dper*(S826A/S828A)} flies, even coinciding at the small dip around ZT12 (Fig. 5F), suggesting that *in vivo* phosphorylation of Ser826/Ser828 has minimal effects, at most, on the timing of dPER nuclear entry (or its repressor potential). Furthermore, even with short photoperiods (9:15 LD), where the distribution of daily activity in p{*dper*(S826A/S828A)} flies was clearly altered compared to that of p{*dper*} flies (Fig. 3), both genotypes had indistinguishable *dper* mRNA cycling profiles (Fig. 5G). As mentioned above, this inability to detect a molecular difference was unlikely to be due to the modest effects on behavioral periods of blocking phosphorylation at Ser826/Ser828, as prior work showed that several clock gene mutants/manipulations that either lengthen or shorten behavioral periods by about 1 to 2 h nonetheless are accompanied by altered *dper* mRNA cycling profiles, even when analyzed using a lower-resolution 4-h sampling frequency (e.g., see references 22, 26, 29, 51, and 52).

We also measured daily cycles in *dper* mRNA and protein levels during the first day in constant darkness (Fig. 6), conditions that are expected to enhance the ability to detect any differences in circadian molecular cycles that differ due to variations in speed and/or phase. However, the *dper* mRNA cycles peaked at the same time and were very similar for p{*dper*(S826A/S828A)} and p{*dper*} flies during the first day in constant darkness (Fig. 6D). In addition, we did not observe any significant reproducible differences in the daily changes in dPER levels and phosphorylation (Fig. 6A to C). Thus, the results obtained for conditions of constant darkness further indicate that blocking phosphorylation at Ser826/Ser828 has little to no effect on the central transcriptional-translational feedback loop (TTFL) underlying the clockworks in

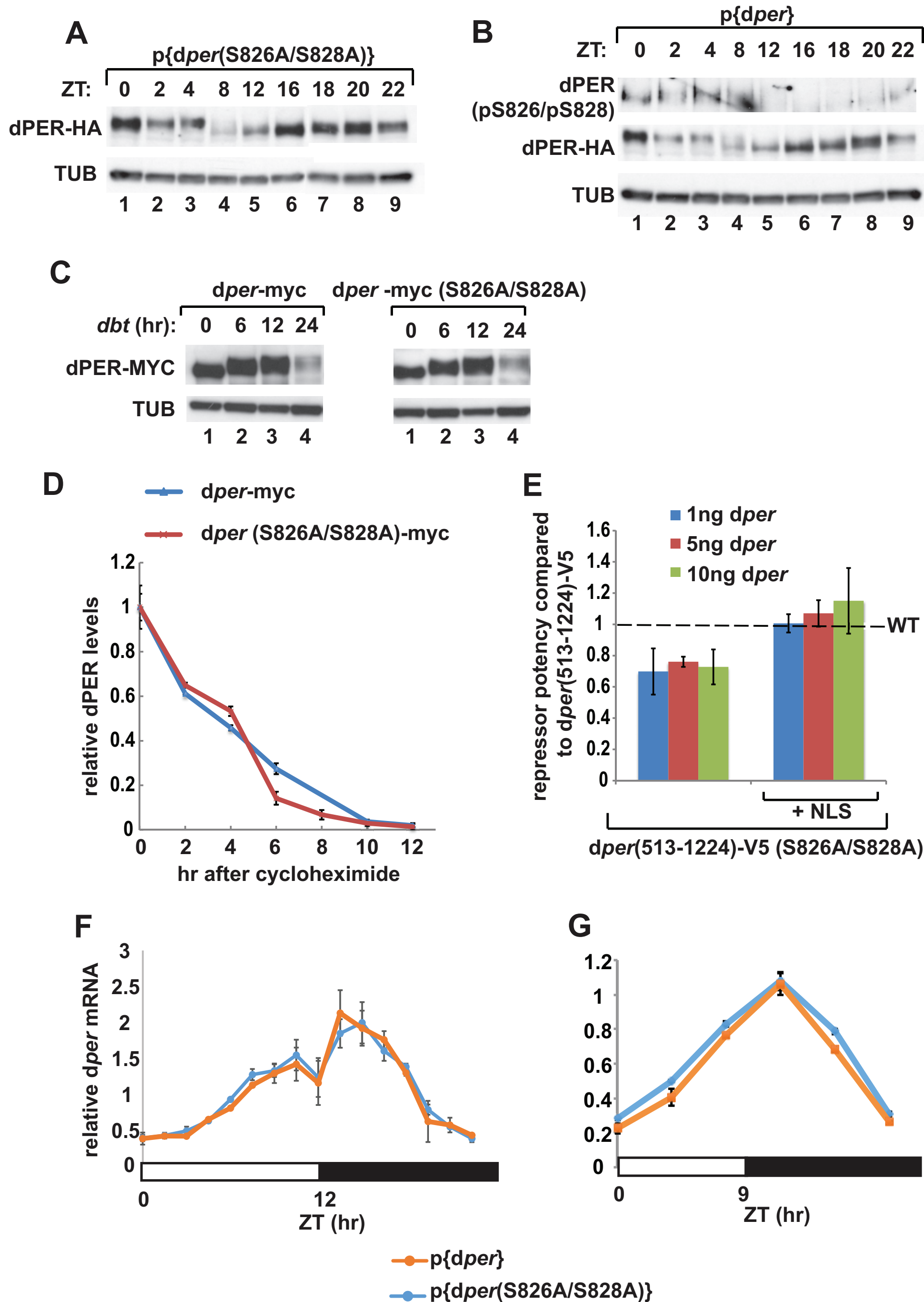

FIG 5 No significant effects of blocking phosphorylation at Ser826/Ser828 on the daily *dper* mRNA or protein cycles in flies. (A and B) Adult p{*dper*(S826A/S828A)} (A) and p{*dper*} (B) flies were collected at the indicated times during the LD cycle. Head extracts were either directly resolved in 4 to 15% SDS-polyacrylamide gels and blotted in the presence of anti-HA or antitubulin antibody (A and B) or subjected to IP with anti-HA beads followed by the analysis of immune complexes by 6% SDS-PAGE and immunoblotting with anti-pS826/pS828 antibody (B). (C) Different versions of recombinant *dper-myc* plasmids were used to transfect S2 cells either with or without pMT-*dbt*. Cells were harvested at the indicated times post-*dbt* induction, and cell extracts were probed by immunoblotting in the presence of anti-MYC antibody (dPER-MYC) or antitubulin antibody. The immunoblots shown in panels A to C are representative of at least three independent experiments. (D) S2 cells were treated with cycloheximide and collected at the indicated times, and extracts were subjected to immunoblotting in the presence of either anti-MYC (dPER-MYC) or antitubulin antibody as a loading control. The data shown are the results of quantification of dPER levels from several independent cycloheximide experiments. (E) Different amounts of plasmids encoding a wild-type fragment of dPER [*dper*(513-1224)-V5] and two mutant versions [*dper*(513-1224/S826A/S828A)-V5 and *dper*(513-1224/S826A/S828A/NLS)-V5 (denoted +NLS)] were used to transfect S2 cells and measure the repression of *dClk*-dependent expression of a luciferase reporter (see Materials and Methods). For each amount of plasmid, the repression value for the wild-type version was set to 1 (dotted line), and all other values were normalized. Values shown were obtained by pooling results from several independent experiments. (F and G) Daily *dper* mRNA cycling from head extracts of p{*dper*} and p{*dper*(S826A/S828A)} flies under different light regimens, i.e., 12:12 (F) and 9:15 (G). Data from at least two independent experiments were pooled to obtain the average profiles shown (data are means ± standard errors of the means [SEM]).

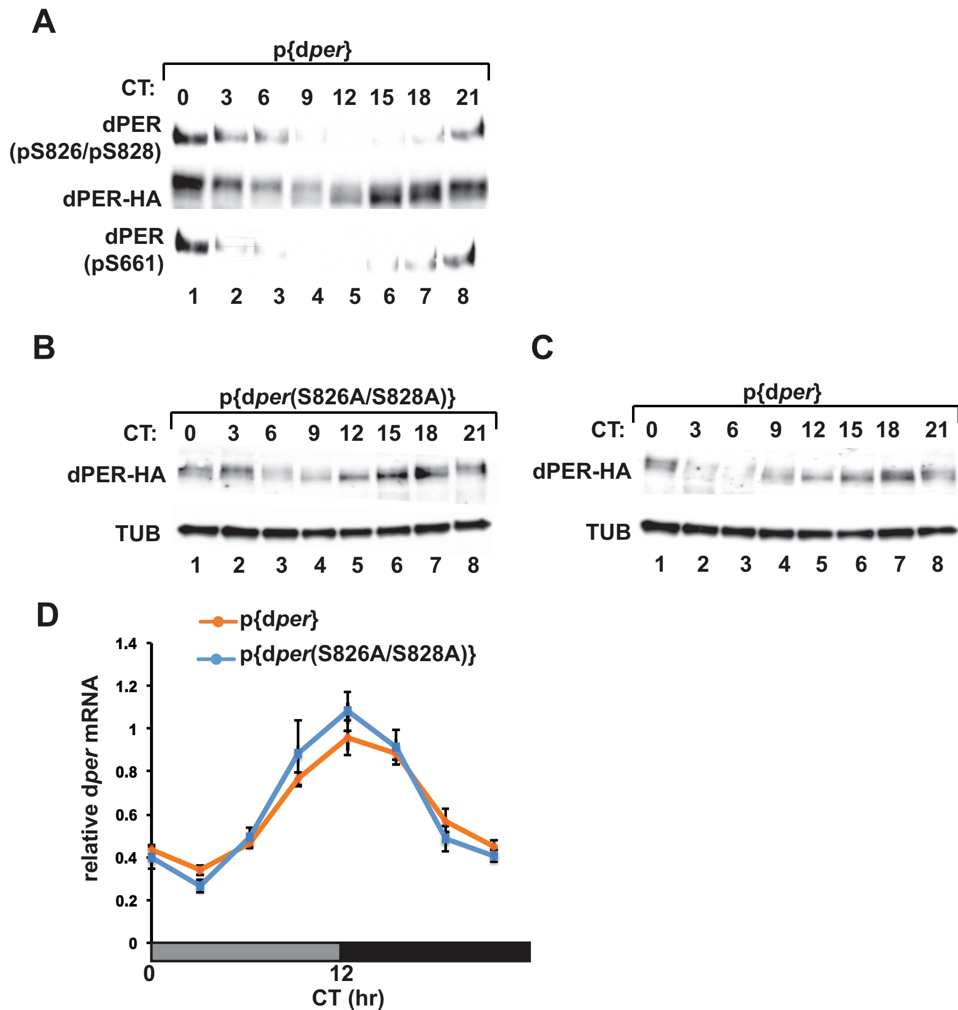

FIG 6 The S826A/S828A mutations have little to no effect on the *dper* protein and mRNA cycles under constant dark conditions. Adult p{*dper*(S826A/S828A)} and p{*dper*} flies were entrained for 4 days under LD conditions, followed by constant darkness (DD). Flies were collected every 3 h during the first day of DD, at the indicated times (CT [hours]). (A) Head extracts were subjected to IP with anti-HA beads followed by the analysis of immune complexes by 6% SDS-PAGE and immunoblotting with anti-pS826/pS828 antibody (top panel), anti-HA antibody (middle panel), or anti-pS661 antibody (bottom panel). (B and C) Head extracts were directly resolved in 4 to 15% SDS-polyacrylamide gels and blotted in the presence of anti-HA or antitubulin antibody. (D) Daily *dper* mRNA cycling in head extracts of p{*dper*} and p{*dper*(S826A/S828A)} flies. Data from at least two independent experiments were pooled to obtain the average profiles shown (data are means ± SEM).

Drosophila. Analysis of dPER in DD also revealed that the daily timing of S826/S828 phosphorylation occurred during the late night/early day (Fig. 6A, top panel), similar to that observed under LD conditions (Fig. 1C), indicating that its timing is at least partly an intrinsic property of the dPER phosphorylation program that continues under free-running conditions (see below).

Mutations that block phosphorylation at Ser826/Ser828 do not affect the timing of dPER nuclear entry. To directly test if phosphorylation of Ser826/Ser828 modulates dPER nuclear localization *in vivo*, we focused on the small ventrolateral neurons (s-LNvs), which are the key pacemaker neurons in the adult brain that control the period length of circadian rhythms (reviewed in reference 55). The majority of s-LNvs express the circadian-relevant neuropeptide pigment dispersing factor (PDF), which can be used as a convenient cytoplasmic marker for these cells. In agreement with other studies, wild-type dPER transitioned from mostly cytoplasmic at ZT18 (characterized by "doughnut" staining of dPER) to mixed cytoplasmic and nuclear at ZT19 and ZT20 and was predominately nuclear by ZT21 (characterized by punctate staining of dPER) (Fig. 7A and B) (11, 56). Our results clearly indicate that the nuclear entry time of dPER in p{*dper*(S826A/S828A)} flies is indistinguishable from that in their wild-type counterparts (Fig. 7A to C). These results are consistent with the lack of an effect of the p{*dper*(S826A/S828A)} mutant on *dper* mRNA cycling. Furthermore, the daily declines in the levels of dPER in the nuclei of s-LNvs were highly similar for p{*dper*(S826A/S828A)} and control flies (Fig. 7D). This result is consistent with the lack of an effect of the S826A/S828A mutant on the daily upswing in *dper* mRNA levels (Fig. 5F), which follows the termination of dPER-mediated transcriptional repression in the nucleus.

Phosphorylation of Ser826/Ser828 is enhanced by light and attenuated by TIM. While the combined behavioral and molecular analysis appeared to imply that phosphorylation of Ser826 and

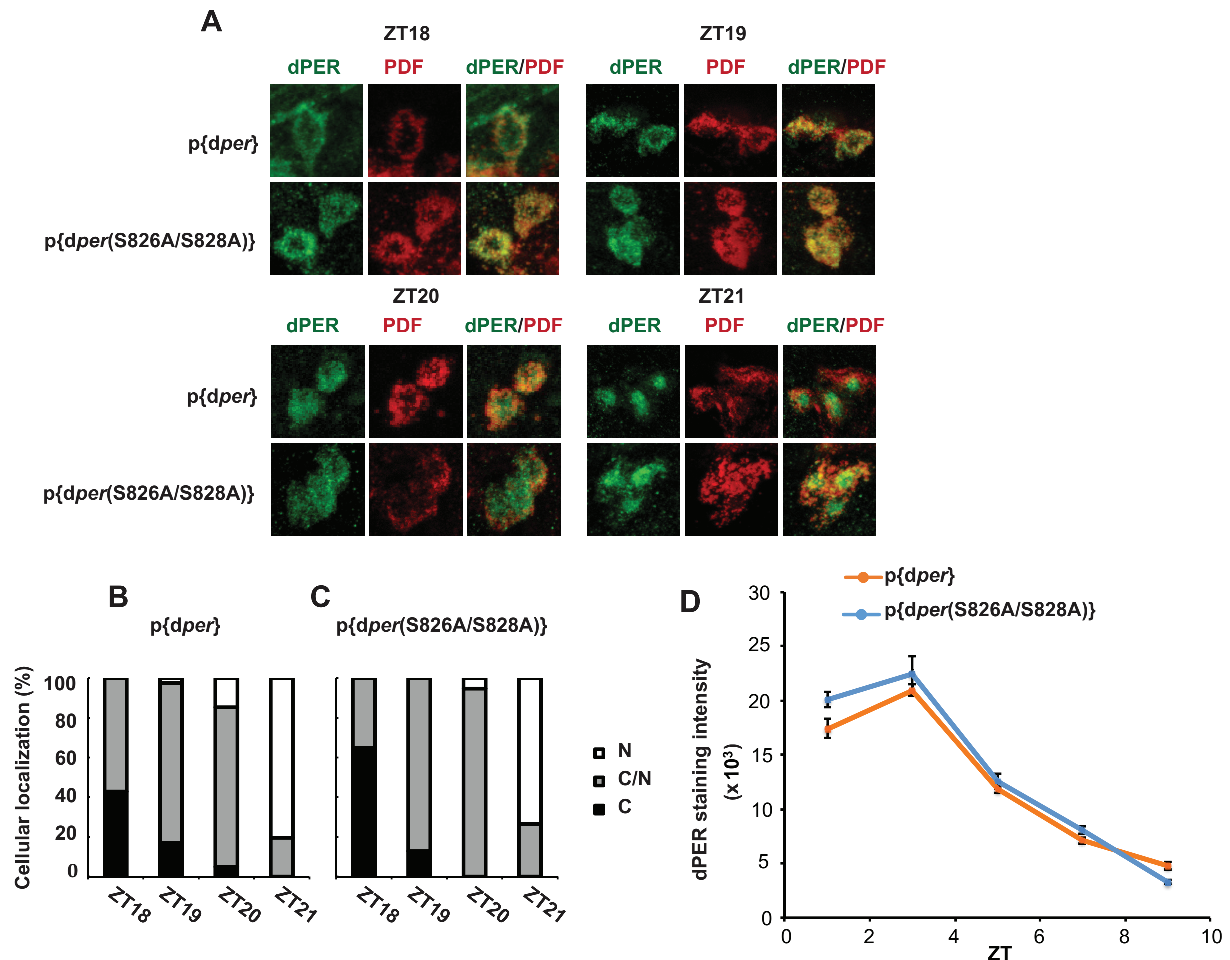

FIG 7 Blocking phosphorylation at S826/S828 does not affect the timing of dPER nuclear entry in key brain pacemaker neurons or its daily downswing. (A) Adult flies expressing a wild-type or mutant *dper* version (indicated on the left) were collected at the indicated times during the LD cycle, and isolated brains were processed for whole-mount immunohistochemistry. Representative images of s-LNvs of each genotype are shown. dPER staining is shown in green, whereas PDF, which serves as a cytoplasmic marker for s-LNvs, is shown in red. (B and C) For each genotype, the subcellular localization was determined for ~30 s-LNvs from ~10 brains in two independent experiments. N, nucleus; C/N, cytoplasm and nucleus; C, cytoplasm. (D) For each genotype, the staining intensity of dPER in the nucleus was determined for ~30 s-LNvs from ~10 brains in two independent experiments.

Ser828 on dPER has little to no effect on the core clock mechanism, we were intrigued by the kinetics of phosphorylation at these sites in flies. Because phosphorylation of Ser826/Ser828 peaked in the early morning (Fig. 1C), this raised the possibility that it is stimulated by light. To test this idea, we entrained wild-type p{*dper*} flies to several days of LD, and for one group, we advanced the lights-on time by 2 h, to ZT22. Remarkably, the staining intensity of phosphorylation at Ser826/Ser828 rapidly increased in the presence of premature exposure to nocturnal light (Fig. 8A, compare lanes 3 and 2 and lanes 5 and 4). The observation that the clock mechanism responded correctly to the premature start of the light phase was verified by measuring the abundance of TIM, which is rapidly degraded by photic stimulation (Fig. 8A, e.g., compare lanes 2 and 3).

We further analyzed the photosensitivity of Ser826/Ser828 phosphorylation by using flies in which CRY function is abolished (*cry*^{01}) (32, 57). CRY is the main circadian photoreceptor in *Drosophila*, and in *cry*^{01} mutants, TIM is not degraded in the presence of light (Fig. 8B) and circadian photosensitivity is strongly attenuated (32, 57). Intriguingly, even in the presence of light, there was little to no phosphorylation of Ser826/Ser828 in *cry*^{01} flies (Fig. 8B and C). Other dPER phosphosites that we analyzed by using phospho-specific antibodies did not show rapid light-mediated increases in staining intensity (data not shown), revealing that the phospho-occupancy at Ser826/Ser828 is specifically responsive to photic signals. Because light triggers the rapid degradation of TIM and TIM is a key binding partner of dPER (10), we used S2 cells to test if TIM might attenuate phosphorylation of dPER at Ser826/Ser828. Indeed, in the presence of TIM, there was little phosphorylation of Ser826/Ser828 by DBT (Fig. 8D). Although earlier studies showed that TIM can slow down the DBT-mediated hyperphosphorylation of dPER, it does not block it (18, 58–60), further demonstrating that Ser826 and Ser828 are particularly sensitive to regulation by TIM. The observation that phosphorylation at S826/S828 had a daily timing in LD cycles similar to that in DD (Fig. 1C and 6A) was not unexpected, because daily rhythms in TIM levels, with the downswing phase beginning in the mid- to late night, persist under constant dark conditions (61).

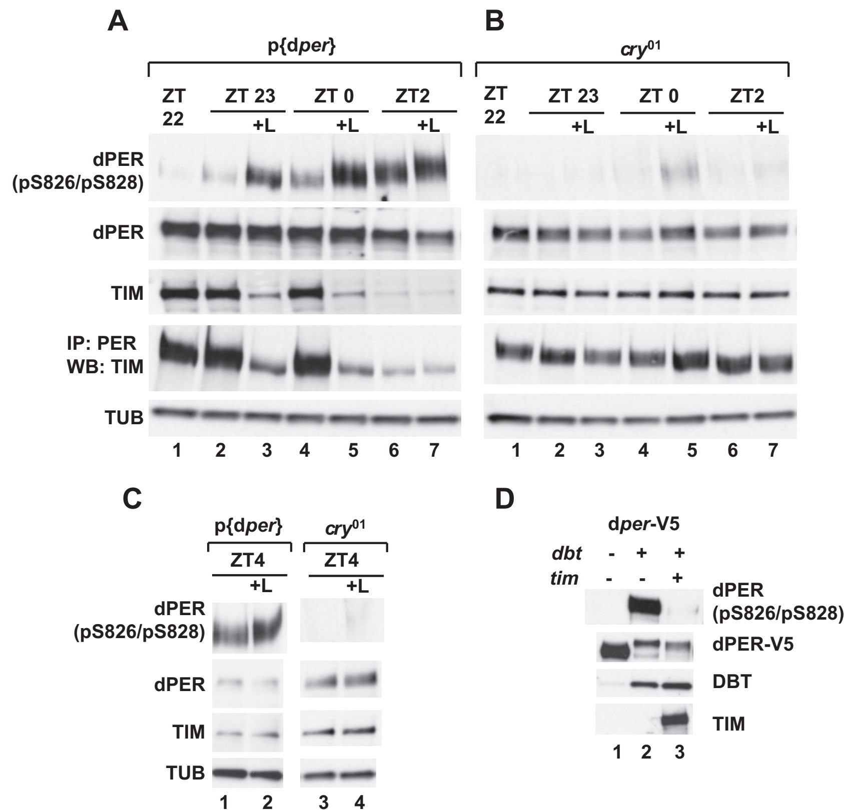

FIG 8 Phosphorylation of S826/S828 is enhanced by light, dependent on CRY, and blocked by TIM. (A to C) Wild-type control adult flies (A and C) or *cry*^{01} mutant flies (B and C) were entrained for 4 days under a 12:12 LD regimen. During the last dark phase of the LD cycle, the lights-on time was advanced by 2 h, to ZT22, for one group of flies (+L), whereas lights-on occurred at the normal time, ZT24/0, for the other group. Flies were collected at the indicated times during the LD cycle (relative to the control group with lights-on at ZT24), head extracts were prepared, and a small amount was saved for direct analysis by 4 to 15% SDS-PAGE followed by immunoblotting with anti-dPER, anti-TIM, or anti-TUB antibody. To detect phosphorylation at S826/S828 by using the anti-pS826/pS828 antibody, the rest of each extract was first subjected to IP with anti-dPER GP-73 antibody in the presence of Sepharose beads, and immune complexes were recovered. The immune complexes were then split and resolved by 6% SDS-PAGE. One part was analyzed by immunoblotting in the presence of anti-pS826/pS828 antibody, whereas the other part was analyzed in the presence of anti-TIM antibody (IP: PER, WB: TIM). (D) S2 cells expressing pAct-d*per*-V5/His, with (+) or without (−) pAct-*tim*-HA or pMT-*dbt*-V5/His, were treated for 4 h with the proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide after 18 h of *dbt* induction. Extracts were immunoprecipitated with anti-V5 beads and analyzed by immunoblotting in the presence of anti-pS826/pS828 antibody (6% gels) or anti-TIM, anti-V5, or anti-DBT antibody (4 to 15% gradient gels). Similar results were obtained in three independent experiments, and representative examples are shown.

Because phosphorylation at Ser826/Ser828 is regulated by light, we sought to determine if p{*dper*(S826A/S828A)} flies exhibited any defects in photic entrainment. We used several standard assays, such as short light pulses to evoke phase shifts, determining the rate of reentrainment to a novel photoperiod, and entrainment to daily light-dark cycles with different light intensities (e.g., see reference 62). However, we did not observe any differences between p{*dper*(S826A/S828A)} and wild-type control flies (data not shown), suggesting that phosphorylation of Ser826/Ser828 does not play a significant role in circadian behavioral photosensitivity.

DISCUSSION

In animals, a complex phosphorylation program drives daily cycles in the levels of PER proteins that are central to setting clock speed. This posttranslational regulatory mechanism intertwines with cyclical gene expression because PER proteins interact with central clock transcription factors in the nucleus to seed repressor complexes. By regulating PER stability and cytoplasmic-nuclear localization, phosphorylation plays a key role in restricting the duration and timing of PER protein engagement in transcriptional repression. Over 30 Ser/Thr phosphorylation sites have been mapped on dPER, and the majority are localized in clusters that appear to have discrete functions in regulating dPER stability and/or timing of nuclear entry in key pacemaker cells (22, 26–29, 63). In this study, we investigated the function of a possible phosphocluster that includes two closely spaced phosphosites, Ser826 and Ser828, embedded within a putative NLS.

The physiological functions of many phosphosites and phosphoclusters on dPER have been studied by generating transgenic flies that have one or more of the phosphosites replaced by Ala and, in some cases, Asp (as a possible phosphomimetic). Abolishing phosphorylation at some sites or clusters can lengthen or shorten behavioral rhythms by more than 6 h, whereas some phosphosites appear to have little to no effect on period length (22, 26–29, 63). Blocking phosphorylation at Ser826/Ser828 decreases behavioral rhythms by about 1 h (Table 1). Thus, within the realm of dPER phosphosites analyzed, Ser826 and Ser828 have modest effects on the period length of daily rhythms in

locomotor activity. However, the fact that the directions of period change are opposite between Ala and Asp replacements at Ser826/Ser828 (Table 1), a situation exacerbated at higher temperatures (Fig. 4), strongly implies that the phospho-occupancy at these sites plays a physiologically significant modulatory role in determining the period length of circadian rhythms. Why differences in the circadian periods of p{*dper*(S826A/S828A)} and p{*dper*(S826D/S828D)} flies become greater at higher temperatures is not clear, but many period-altering alleles of *dper* affect temperature compensation (50, 53, 54).

A surprising result from our studies is that mutating Ser826/Ser828 did not lead to any observable changes in the clockworks in a variety of standard assays (Fig. 5 and 6). Most notably, the *dper* mRNA daily cycling profile offers a robust quantitative measure to probe for alterations in the speed/phase of the core clock mechanism, as changes in dPER protein levels/stability, nuclear entry time, duration in the nucleus, or transcriptional repressor function or possible effects of mutations on mRNA stability/levels can alter the daily rhythms in *dper* mRNA levels due to the tight feedback circuitry between the dPER protein and its cognate mRNA. The lack of a noticeable effect on *dper* mRNA cycling is unlikely to be due simply to a limitation in detection. In prior work, we showed that phosphosite mutants that alter behavioral rhythms by as little as 1 to 2 h still have noticeable effects on one or more aspects of the TTFL, with primary influences on dPER stability and/or timing of nuclear entry in key pacemaker neurons that are also reflected in changes to daily clock mRNA cycles (22, 26, 29). For example, in a situation behaviorally comparable to that of p{*dper*(S826A/S828A)} flies, transgenic flies in which we changed the key phosphodegron on dPER at Ser47 to an Asp [dPER(S47D)] also manifested 1 to 1.5 h shorter behavioral rhythms; however, under the standard conditions of 12:12 LD, the *dper* mRNA rhythms in these flies had substantially higher amplitudes and attained peak levels earlier, consistent with the faster degradation of dPER(S47D) in the nucleus (26). A similar advanced *dper* mRNA cycle of higher amplitude was also observed in another transgenic model, with a period that was about 1.5 h shorter under 12:12 LD conditions, despite the fact that in this case the clock speed was altered by mutating phosphorylation sites on dCLK (52).

In the aforementioned cases, differences in *dper* mRNA cycling were observed even with 4-h sampling frequencies, whereas we used a much higher resolution of 1.5 h (Fig. 5F). In addition, differences in *dper* mRNA cycling were not observed during the first day of constant darkness (Fig. 6) or even with the shorter photoperiod of 9:15 LD conditions (Fig. 5G), whereby changes in the distribution of daily activity between wild-type controls and the S826A/S828A mutant were more pronounced (Fig. 3B). This is not to imply that all short-period mutants will have a similar effect on circadian transcriptional feedback but to point out that mutants with 1- to 1.5-h shorter periods still manifest noticeable differences in one or more aspects of the daily cycles in *dper*-encoded protein and/or mRNA, which is not surprising because *dper* is the central factor regulating the period of circadian rhythms.

Consistent with no effect on transcriptional feedback regulation or daily cycles in dPER levels and progressive phosphorylation, we also did not observe a role for Ser826/Ser828 in the timing of dPER translocation from the cytoplasm to the nucleus in s-LNvs (Fig. 7) or several other brain clock neurons that we measured (data not shown). Although there are approximately 150 pacemaker neurons in the adult brain that regulate different aspects of the daily wake-sleep cycle in *D. melanogaster*, under normal conditions the PDF-expressing s-LNvs are the key clock cells within the circadian neural network that govern the pace of behavioral rhythms (55; but see references 64 and 65). Thus, if a mutation in *dper* (or any other clock gene) that leads to a change in free-running behavioral periods is due to an alteration in the timing of dPER nuclear entry, this should be readily observable in s-LNvs, as the transition from the cytoplasm to the nucleus normally occurs in a small time window around ZT19 (11, 56). Indeed, prior work identified several phosphoclusters on dPER that have primary effects on the timing of dPER nuclear entry into s-LNvs (29, 63). For example, blocking phosphorylation at a hierarchical phosphocluster that is comprised of phosphosites at Ser657 and Ser661 leads to 1- to 2-h longer behavioral periods that also delay dPER nuclear entry into s-LNvs by the same amount of time and are associated with significant changes in the timing of the daily upswing in *dper* mRNA levels (29).

The kinetics of phosphorylation at Ser826/Ser828 also raises doubts about a role in governing the transition from the cytoplasm to the nucleus. Phosphorylation at Ser826/Ser828 occurs mainly in the late night/early morning, after nuclear translocation is essentially complete, whereas for Ser657/Ser661 these sites are extensively phosphorylated prior to dPER nuclear entry (29) (Fig. 1C). In work to be reported elsewhere, mutations that target key residues of NLS-2 do not alter the timing of dPER nuclear entry (E. Yildirim and I. Edery, unpublished data). Thus, despite the ability of NLS-2 to function as a *bona fide* NLS in cultured *Drosophila* S2 cells using recombinant versions of *dper* (30), it apparently does not function in this capacity in flies and/or is masked by redundant functions. The regulation of when dPER transitions from the cytoplasm to the nucleus is likely complex, as it appears to involve several regions on dPER and numerous *trans*-acting factors, such as TIM and several kinases (e.g., see references 13, 29, 63, and 66).

While the molecular underpinnings for how phosphorylation at Ser826/Ser828 contributes to the maintenance of normal, ~24-h periods are not clear, to the best of our knowledge our findings are the first to identify phosphorylation sites on any core clock protein whose phospho-occupancy responds rapidly to light (Fig. 8). Our results demonstrate that the loss of TIM binding to dPER as a result of the light-mediated degradation of TIM via the well-characterized circadian photic signaling pathway involving CRY (10) somehow enhances the ability of DBT to phosphorylate Ser826/Ser828. Prior work has shown that the interaction of TIM with dPER stabilizes dPER against DBT-mediated degradation, although it is not clear if this is directly due to attenuating phosphorylation at one or more phosphosites (13, 18, 58, 60, 67). Although we did not find any effects of mutating Ser826/Ser828 on circadian behavioral photosensitivity (data not shown), we noticed that the alignment of the morning activity peak in daily light-dark cycles was affected more than the evening bout (Fig. 3 and Table 2). This is unusual because period-altering mutants mainly affect the timing of the evening bout (e.g., see references 22, 46 to 48, 50, and 68). While this is highly speculative, the fact that phosphorylation at Ser826/Ser828 rapidly increases in the late night/early morning, as TIM levels drop, offers a possible basis for a preferential effect of mutating these sites on the morning activity bout. However, the morning bout of activity is mainly governed

by the s-LNvs (69, 70), in which we did not see an effect on dPER nuclear entry/duration by blocking phosphorylation at Ser826/Ser828 (Fig. 7).

How might blocking phosphorylation at Ser826/Ser828 lead to faster-running daily wake-sleep cycles but no apparent effect on the clockworks? One possibility is based on the multicellular organization of the circadian neural network driving behavioral rhythms. Although it appears that under normal circumstances the PDF-expressing s-LNvs are the key pacemaker neurons driving free-running behavioral rhythms, recent findings suggest that the circadian neural circuitry in the *Drosophila* brain is less hierarchical than previously thought and that neurons other than the s-LNvs can make strong contributions to behavioral periodicity (64, 65). Within this framework, the S826A/S828A mutations might speed up the clockworks in only a few non-s-LNv cells, an event that would somehow alter the circadian neural hierarchy such that behavioral rhythms would be governed by the faster-running cells, not the s-LNvs. To complete this scenario, these hypothetical faster-running cells would not affect the rest of the circadian neural network, which would lead to us observing no effects when assaying s-LNvs or head extracts. Additionally, changes in clock speed might not even be necessary, because certain clock cells already run intrinsically faster than the slower-paced s-LNvs; thus, blocking phosphorylation at Ser826/Ser828 might diminish the impact of the s-LNvs and/or increase the relative strength of faster-running pacemaker cells in the overall neural network driving behavioral rhythms (e.g., see reference 64).

Another, nonexclusive possibility is based on a recent study analyzing phosphorylation of the key clock protein FREQUENCY (FRQ) in *Neurospora* (71). It was suggested that whereas some phosphorylation events dictate the daily turnover of clock proteins (termination-signaling phosphorylation [TSP]), others operate in conveying temporal information from the clock to output pathways (clock-signaling phosphorylation [CSP]). Phosphorylation of S826/S828 on dPER might operate as a CSP that conveys temporal information from the core clock mechanism to downstream pathways regulating the daily wake-sleep cycle. In this regard, it is interesting that a mutation in *tim* (*tim*$^{\text{blind}}$) which affects TIM phosphorylation also led to changes in behavioral periods, with little to no observable changes in the daily *dper* mRNA or protein cycles, suggesting that TIM has an as yet uncharacterized function in output from the clock (72).

Clearly, future work is required to understand the molecular basis for why the dPER phosphosites at Ser826/Ser828 modulate the periodicity of behavioral rhythms. Nonetheless, despite this limitation in our study, the findings strongly suggest the existence of a novel class of phosphorylation sites on dPER that can modulate the period of behavioral rhythms in a manner independent of general effects on its daily stability/levels, timing of nuclear translocation, duration in the nucleus, or function in transcriptional autoinhibition.

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