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Phosphorylation of the Transcription Activator CLOCK Regulates Progression through a ~24-h Feedback Loop to Influence the Circadian Period in *Drosophila*[*]

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Background: CLOCK phosphorylation coincides with circadian rhythms in transcription.
Results: CLOCK phosphorylation sites are identified that regulate the timing and level of transcriptional activity and influence circadian period.
Conclusion: CLOCK phosphorylation influences the circadian period by regulating transcriptional activity and progression through the circadian cycle.
Significance: This study shows that CLOCK phosphorylation contributes to circadian period determination in *Drosophila*.

Circadian (≅24 h) clocks control daily rhythms in metabolism, physiology, and behavior in animals, plants, and microbes. In *Drosophila*, these clocks keep circadian time via transcriptional feedback loops in which CLOCK-CYCLE (CLK-CYC) initiates transcription of *period* (*per*) and *timeless* (*tim*), accumulating levels of PER and TIM proteins feed back to inhibit CLK-CYC, and degradation of PER and TIM allows CLK-CYC to initiate the next cycle of transcription. The timing of key events in this feedback loop are controlled by, or coincide with, rhythms in PER and CLK phosphorylation, where PER and CLK phosphorylation is high during transcriptional repression. PER phosphorylation at specific sites controls its subcellular localization, activity, and stability, but comparatively little is known about the identity and function of CLK phosphorylation sites. Here we identify eight CLK phosphorylation sites via mass spectrometry and determine how phosphorylation at these sites impacts behavioral and molecular rhythms by transgenic rescue of a new *Clk* null mutant. Eliminating phosphorylation at four of these sites accelerates the feedback loop to shorten the circadian period, whereas loss of CLK phosphorylation at serine 859 increases CLK activity, thereby increasing PER levels and accelerating transcriptional repression. These results demonstrate that CLK phosphorylation influences the circadian period by regulating CLK activity and progression through the feedback loop.

Daily rhythms in metabolism, physiology, and behavior are controlled by circadian clocks in a wide array of organisms. Although the molecular components of these clocks are not universally conserved, a common transcriptional feedback loop mechanism functions to keep circadian time in eukaryotes (1). In *Drosophila* this feedback loop is activated by the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) transcription factors CLOCK (CLK)[2] and CYCLE (CYC); CLK-CYC heterodimers bind E-boxes to initiate transcription of the transcriptional repressors *period* (*per*) and *timeless* (*tim*) during the early evening (2). Accumulating levels of cytoplasmic PER and TIM form a complex with the casein kinase 1δ/ϵ ortholog DOUBLE-TIME (DBT) and enter the nucleus late in the evening to inhibit CLK-CYC activity, thus repressing *per* and *tim* transcription. In the early morning PER and TIM undergo proteasomal degradation, thus releasing repression and permitting the next round of CLK-CYC transcription.

Post-translational modifications of clock proteins determine circadian oscillator pace and amplitude. As PER-TIM-DBT complexes accumulate in the cytoplasm, phosphorylation of PER and TIM by the glycogen synthase kinase 3β ortholog SHAGGY (SGG) and PER by casein kinase 2 promote their nuclear localization (3–6). Nuclear localization of PER is inhibited by *O*-GlcNAcylation (7), thereby imposing a delay in transcriptional repression. After nuclear entry, PER is progressively phosphorylated by DBT (8–10). The terminal DBT phosphorylation site on PER, serine 47 (Ser^{47}), promotes PER degradation by forming a binding site for the E3 ubiquitin ligase SLIMB (11). The timing of PER Ser^{47} phosphorylation, and thus PER degradation, is regulated by prior phosphorylation within the "per-short" (Ser^{596}, Ser^{589}, Ser^{585}, and Thr^{583}) domain (11–13). Phosphorylation in the per-short domain is initiated by the pro-

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[2] The abbreviations used are: CLK, CLOCK; CYC, CYCLE; PER, PERIOD; TIM, TIMELESS; DBT, DOUBLE TIME; SGG, SHAGGY; NMO, NEMO; ZT, Zeitgeber time; S2, Schneider 2; *per*-luc, *per* promoter-driven luciferase; FLP, Flippase; DN, dorsal neuron; ZT2, Zeitgeber time 2.

line-directed kinase NEMO (NMO) at Ser^{596}, which promotes DBT phosphorylation at Ser^{589}, Ser^{585}, and Thr^{583} to delay Ser^{47} phosphorylation and PER degradation (8). Work in cultured S2 cells identified 25–30 phosphorylation sites on PER, suggesting that phosphorylation of sites outside the per-short domain and Ser^{47} regulate PER degradation and/or repressor activity (11, 13). Indeed, two sites (Thr^{610} and Ser^{613}) in the "per-short downstream" domain function to regulate phosphorylation of Ser^{859}, and thus control PER degradation (12, 13). Phosphorylation-dependent regulation of PER subcellular localization and stability is conserved in mammals; the stabilization and nuclear retention of hPER2 in familial advanced sleep phase syndrome is due to the loss of casein kinase 1δ/ϵ-dependent phosphorylation at Ser^{662} (14), although another study attributes PER degradation to casein kinase 1δ/ϵ-dependent phosphorylation at a different site (15).

In the nucleus, PER-DBT-TIM complexes bind to CLK and inhibit CLK-CYC transcription (16–18). Coincident with PER-DBT-TIM binding, CLK becomes progressively phosphorylated and is released from E-box sequences (18). Although CLK phosphorylation is dependent on DBT (19, 20), neither CLK phosphorylation nor transcriptional repression require DBT catalytic activity (20), suggesting that DBT mediates CLK phosphorylation by other kinases. Recent work shows that casein kinase 2 stabilizes CLK and decreases its transcriptional activity in a PER-dependent manner (21). NMO has also been implicated in CLK phosphorylation by promoting CLK degradation to slow the pace of the circadian cycle, but whether NMO directly phosphorylates CLK is not known (22). When PER is degraded, hypophosphorylated CLK starts accumulating coincident with CLK-CYC-dependent transcription (18). These data suggest that CLK phosphorylation is important for CLK stability, CLK-CYC E-box binding and/or CLK-CYC transcriptional activity. As in flies, mammalian Clock is phosphorylated coincident with transcriptional repression (23, 24). Phosphorylation at Ser^{38} and Ser^{42} weakened ClocK-Bmal1 transactivation and Clock nuclear localization (24), whereas glycogen synthase kinase 3 promotes Clock degradation via phosphorylation at Ser^{427} (24, 25). However, Clock phosphorylation also plays a role in transactivation as serum-induced transcription of *mPer1* in fibroblasts is preceded by PKC-dependent Clock phosphorylation (26).

To determine the role that CLK phosphorylation plays in the *Drosophila* clock, we used mass spectrometry to identify eight phosphorylated serines in CLK, of which seven were in the C-terminal half of CLK harboring the activation domain (27, 28). The impact of phosphorylation at these Ser residues was determined by testing whether transgenes that express mutant CLK proteins with Ser to non-phosphorylatable alanine (Ala) substitutions rescue behavioral and molecular rhythms in a novel *Clk* null mutant (*Clk*^{out}). We found that five of the eight Ser to Ala mutants rescued behavioral rhythms having short periods, but did not alter CLK levels or phosphorylation state in fly heads. One of these mutants, S859A increased CLK activity, thereby accelerating transcriptional feedback, whereas the other mutants appear to accelerate or bypass a step that delays transcriptional activation or repression. These results indicate that phosphorylation decreases CLK activity or imposes a delay to lengthen circadian period, thereby serving as a determinant for setting the pace of the circadian oscillator.

EXPERIMENTAL PROCEDURES

Fly Strains—The following fly strains were obtained from the Bloomington *Drosophila* Stock Center (IN): w^{1118}, pBac{WH}*Clk*[f06808], pBac{WH}*Clk*[f03095], P{hsFLP} y^1 w^{1118}; Dr^1/TM3 *Sb*, P[lacW]*nmo*P1 (*nmo*^{P1}), Df(3L)Exel6279 (*nmo*Df), w;Cyo-P{Tub-pBac\T}2/wgSp-1, and *yw*;PBac{y$^+$-*attp*-9A}VK00018. *Clk*^{out} was recombined with *nmo*^{P1} or *nmo*Df to generate *nmo*^{P1} *Clk*^{out} and *nmo*Df *Clk*^{out} double mutants.

Generating the Clk^{out} Deletion Mutant—A deletion within *Clk* was generated by recombination between FRT sites from PBac{WH}*Clk*[f06808] (hereafter f06808), which inserted 64 bp into the first exon of *Clk*, and PBac{WH}*Clk*[f03095] (hereafter f03095), which is inserted 5334 bp upstream of exon 1 (29, 30). Flippase (FLP)-induced recombination was induced by a daily 1-h heat shock at 37 °C given to hsFLP;f06808/f03095 larvae and pupae (31). Three recombinants were recovered based on a lighter orange eye color due to loss of one pBac element, and each produced a deletion rather than a duplication of intervening *Clk* sequences. The remaining pBac insert in each recombinant strain was excised by crossing recombinants to w;Cyo-P{Tub-pBac\T}2/wgSp-1 flies that express pBac transposase (30), and white eyed progeny lacking the remaining pBac element were selected. The deleted region was amplified with a primer situated upstream of the f03095 insertion site (5′-CGGAATATTGGACAACAAACAG-3′) and downstream of the f06808 insertion site (5′-CAGCAGTGGAATCTTAATACAG-3′) and sequenced. In each case the deletion encompassed all sequences between the initial pBac insertion sites. The newly generated *Clk* deletion was named *Clk*^{out}.

Generating a Clk Rescue Transgene—A *Clk*-containing P[acman] transgene was generated using recombineering-mediated gap repair (32). To prepare the P[acman] vector, homology arms were amplified from genomic DNA with primers *Clk*LA-f (5′-ATGTGGCGCGCCGCCCCAAAAATCCATAAATGCT-3′) and *Clk*LA-r (5′-GTGTTGGATCCAGGGGTGTTATAGAGAGGGACA-3′) for the left arm and *Clk*RA-f (5′-GTGTGGATCCGCAGAGTGAAACCTGTGCAA-3′) and *Clk*RA-r (5′-ATATATGTGCGGCCGCTCCCGGTTATGAGTTTTTCG-3′) for the right arm via PCR, and cloned as AscI-BamHI and BamHI-NotI fragments into AscI and NotI digested *attB*-P[acman]-Ap^R vector (modified to remove the SphI site) to form *attB*-P[acman]*Clk*LARA. Recombination-competent SW102 cells harboring the BAC clone RP98 5K6 (BACPAC Resource Center), which contains the *Clk* genomic region, were transformed with the *attB*-P[acman]*Clk*LARA vector (linearized with BamHI). One of the resulting ampicillin-resistant recombinants, termed *attB*-P[acman]-*Clk*, was verified by PCR and sequencing, and contains 15.5 kb of the genomic sequence beginning ~8 kb upstream of the *Clk* translation start and ending ~2.5 kb downstream of the *Clk* stop codon.

To introduce a V5 epitope tag at the C terminus of the *Clk* open reading frame (ORF), a 3′ genomic fragment of *Clk* (from 351 bp upstream to 1580 bp downstream of the translation

stop) was cloned into pGEM-T vector (Promega). Sequences encoding V5 were introduced in-frame immediately upstream of the *Clk* stop codon using the QuikChange site-directed mutagenesis kit (Stratagene) to create pGEM-T-*Clk*3′V5. The 3′ *Clk* genomic fragment in *attB*-P[acman]-*Clk* was swapped with the 3′ fragment in pGEM-T-*Clk*3′V5 using SphI and NotI to form *attB*-P[acman]-*Clk*V5. This transgene, which we refer to as *Clk*WT, was inserted into the VK00018 *attP* site on chromosome 2 via PhiC31-mediated transgenesis (32, 33). *Clk*WT was isogenized by backcrossing seven times to our w^{1118} control strain, and moved into Clk^{out} for behavioral and molecular analysis.

S2 Cell Culture and Generation of Stably Transfected S2 Cells—S2 cells were maintained in Schneider's *Drosophila* medium (Invitrogen) containing 10% fetal bovine serum with (100 units/ml) penicillin and streptomycin (100 μg/ml) (Invitrogen). The pMK33-*dClk*-6×His-3XFLAG-TAP plasmid was used to generate stably transfected S2 cells. This plasmid was derived from the pMK33-C-TAP tag vector (34). The 3×FLAG epitope was amplified from the Tetra-tag vector (35) with primers forward (5′-AATTCCCCCGGGCGGGACTACAAAGACCATGACGGTG-3′) and reverse (5′-CTATTCGCTAGCGCCCTTGTCATCGTCATCCTTGTAGTC-3′) and inserted as an XmaI-NheI fragment into the TA vector (Invitrogen) to form 3×FLAG-TA. The His_6 tag was generated by annealing oligos 5′ XbaI-SpeI (5′-CTAGTTCTAGACTGGTGCCGCGCCATCATCACCATCACCATGTCC-3′) and 3′ XmaI (5′-AAGATCTGACCACGGCGCGGTAGTAGTGGTAGTGGTACAGGGGCC-3′), and inserting into 3×FLAG-TA as an XbaI-XmaI fragment to form 6×His-3×FLAG-TA. A SpeI-NheI fragment from 6×His-3×FLAG-TA containing the His_6 and 3×FLAG epitope tags was then inserted into pMK33-C-TAP to form pMK33–6×His-3×FLAG-TAP. Finally, the *Clk* ORF was amplified from pAc-*dClk*-V5/His_6 (36) using XhoI *Clk* forward (5′-CTATTCCTCGAGATGGACGACGAGAGCGACGACAAG-3′) and SpeI *Clk* reverse (5′-AATTCCACTAGTTTGACTACTGCCTGGGGCTGTGTG-3′) primers, and subcloned into TA to form TA-*Clk*ORF (Invitrogen). The *Clk* ORF was excised from TA-*Clk*ORF with XhoI and SpeI and inserted into pMK33–6×His-3×FLAG-TAP to form pMK33-*dClk*-6×His-3×FLAG-TAP. To establish stably transfected S2 cells for *per*-luc reporter assays and CLK protein purification, pMK33-*dClk*-6×His-3×FLAG-TAP and pCoHygro (Invitrogen) or pMK33-*dClk*-6×His-3×FLAG-TAP, pMT-PER (37), pMT-DBT (37), and pCoHygro were co-transfected into S2 cells using the calcium phosphate transfection kit (Invitrogen) and selected for growth in 300 μg/ml of hygromycin.

Luciferase Assays in S2 Cells—Plasmids were introduced into S2 cells using the calcium phosphate transfection kit (Invitrogen), and luciferase assays were carried out as described (36), except that CLK or CLK, PER, and DBT expression were induced 24 h after transfection, and luciferase was assayed 24 h post-induction.

Purification of CLK for Phosphorylation Site Identification—2.5 Liters of S2 cells (3×10^6 cells/ml) stably transfected with pMK33-*dClk*-6×His-3×FLAG-TAP, pMT-PER, and pMT-DBT were induced with copper sulfate (500 μM), treated with MG132 (50 μM, Sigma) and cycloheximide (10 μg/ml, Sigma) 20 h after induction, and harvested 4 h later. Cells were then sonicated 15 times for 10 s using a Misonix XL2000 model sonicator at a setting of 6 in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, and protease inhibitor mixture (Roche Applied Science)). Cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The resulting supernatant was incubated with 200 μl of HisPur™ Cobalt resin (Thermo Scientific) for 2 h at room temperature and then loaded onto a column. The resin was washed with 10 ml of denaturing wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 10 mM imidazole), 10 ml of denaturing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 20 mM imidazole), and 10 ml of native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0) at 4 °C. Protein was eluted using 10 ml of cold elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0, plus protease inhibitor mixture) and mixed with 150 μl of anti-FLAG M2-affinity beads (Sigma) at 4 °C overnight. Beads were then washed with cold elution buffer three times for 10 min each time, and protein was eluted with an equal bead volume of modified SDS sample buffer (150 mM Tris-HCl, pH 6.8, 6 mM EDTA, 3% SDS, 30% glycerol) at 95 °C for 5 min. The eluate was resolved in 5% SDS-PAGE, and the excised CLK band was used for protease digestion and mass spectrometry.

Mass Spectrometry—Replicate gel segments were reduced using dithiothreitol, alkylated with iodoacetamide, and then subjected to digestion with trypsin (Promega), chymotrypsin (Worthington), elastase and pepsin (also Worthington). Digests were analyzed by nano LC/MS/MS with a NanoAcquity HPLC system (Waters, MA) interfaced to a LTQ Orbitrap Velos tandem mass spectrometer (ThermoFisher). Peptides were loaded on a trapping column and eluted over a 75-μm analytical column at 350 nl/min; both columns were packed with Jupiter Proteo resin (Phenomenex). A 30-min gradient was employed for each segment. The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 full width at half-maximum resolution and MS/MS was performed in the LTQ at unit resolution. The 15 most abundant ions were selected for MS/MS from each MS scan. Dynamic exclusion and repeat settings ensured each ion was selected only once and excluded for 30 s thereafter. Product ion data were searched against the combined forward and reverse Uniprot *Drosophila melanogaster* protein database using a locally stored copy of Mascot search engine version 2.3 (Matrix Science, London, UK) via Mascot Daemon version 2.3. Peak lists were generated using the Extract_MSn executable (ThermoFisher). The database was appended with common background proteins. Search parameters were precursor mass tolerance 10 ppm, product ion mass tolerance 0.5 Da, 2 missed cleavages allowed, fully tryptic peptides only, fixed modification of carbamidomethyl cysteine, variable modifications of oxidized methionine, protein N-terminal acetylation, pyroglutamic acid on N-terminal glutamine, and phosphorylation on serine, threonine, and tyrosine. Mascot search result flat files (DAT) were parsed to the Scaffold software version 3.1 (Proteome Software) with the following cutoff values: 90% protein and 50% peptide level probability (probabilities were assigned by the Protein Prophet algorithm.

TABLE 1

Primers used to generate phosphorylation site mutants

For each phosphorylation site or cluster of phosphorylation sites, forward and reverse primers were used to generate a mutant that replaces the native serine residue with a non-phosphorylatable alanine (see "Experimental Procedures").

Phosphorylation site	Primer	Sequence (5′ to 3′)
Ser^{258}	Forward	CCCAACTGATCAGGGAAATGGCCATTATTGATCCCACAAGTA
	Reverse	TACTTGTGGGATCAATAATGGCCATTTCCCTGATCAGTTGGG
Ser^{476}-Ser^{482}	Forward	GACATCGCGTCCCGCTGCGAGTTATGGCAATATCGCCTCCACAGGAATATCGCCG
	Reverse	CGGCGATATTCCTGTGGAGGCGATATTGCCATAACTCGCAGCGGGACGCGATGTC
Ser^{487}	Forward	AATATCAGCTCCACAGGAATAGCGCCGAAGGCC
	Reverse	GGCCTTCGGCGCTATTCCTGTGGAGCTGATATT
Ser^{525}	Forward	CATGATGACGCACGTCGCTTCGCAAAGCCAACGG
	Reverse	CCGTTGGCTTTGCGAAGCGACGTGCGTCATCATG
Ser^{645}	Forward	GGTGGCGCCAGTGCTTGCCCCACTTC
	Reverse	GAAGTGGGGCAAGCACTGGCGCCACC
Ser^{859}	Forward	GCCTTTCTCAATCTGGCACCCCTGCACTCATTG
	Reverse	CAATGAGTGCAGGGGTGCCAGATTGAGAAAGGC
Ser^{912}	Forward	CAAAATAATAACGAGGACGCGCTGTTGTCCTGCATGC
	Reverse	GCATGCAGGACAACAGCGCGTCCTCGTTATTATTTTG

Site-directed Mutagenesis—To analyze CLK phosphorylation in flies, phosphorylation site mutants except for S859A were generated in an NheI-NotI fragment from *attB*-P[acman]-*Clk*V5 that contains *Clk* genomic coding sequences inserted into TA (modified to include an NheI site) (Invitrogen). The resulting plasmid, *Clk*CDS-TA, was used to generate Ser to Ala phosphorylation site mutants via the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). For each CLK phosphorylation site, complementary forward and reverse oligonucleotides containing the desired mutation were synthesized for mutagenesis (Table 1). All mutations were confirmed by sequencing. Mutant NheI-NotI fragments were excised from *Clk*CDS-TA and used to replace the wild-type NheI-NotI fragment in *attB*-P[acman]-*Clk*V5, thereby forming *attB*-P[acman]-*Clk* phosphorylation mutant transgenes. To generate the S859A mutant, an NheI to SphI subfragment of *attB*-P[acman]-*Clk*V5 was first inserted into pSP72 (modified to include an NheI site) to form pSP72-*Clk*(NheI/SphI). An NcoI-SphI subfragment from pSP72-*Clk*(NheI/SphI) was then cloned into pSP72 (modified to include a NotI site) to generate pSP72-*Clk*(NcoI/SphI). Mutagenesis of serine 859 to alanine was then carried out using the indicated primers (Table 1) in pSP72-*Clk*(NcoI/SphI) to generate pSP72-*Clk*(NcoI/SphI)-S859A. The NcoI-SphI fragment from pSP72-*Clk*(NcoI/SphI)-S859A was used to replace the NcoI-SphI fragment in pSP72-*Clk*(NheI/SphI) to generate pSP72-*Clk*(NheI/SphI)-S859A, and the NheI-SphI from pSP72-*Clk*(NheI/SphI)-S859A was used to replace the NheI-SphI fragment in *attB*-P[acman]-*Clk*V5 to generate the *attB*-P[acman]-*Clk*S859A transgene. Transgenes bearing phosphorylation site mutants were injected (BestGene) into the VK00018 *attB* site (32). Transgenic inserts that were not homozygous viable were isogenized by backcrossing seven times to w^{1118} and moved into Clk^{out} for behavioral and molecular analysis.

To analyze CLK phosphorylation in S2 cells, we generated the pMT-CLK-V5-His parent plasmid by replacing a SacI-HindIII fragment containing the HA-tagged *Clk* ORF from pMT-HA-CLK-V5-His (19) with the corresponding fragment from TA-*Clk*ORF. All phosphorylation site mutants were generated in pMT-CLK-V5-His with the same oligonucleotides used above (Table 1) via the QuikChange II XL site-directed mutagenesis kit (Stratagene).

Western Blot Analysis and Phosphatase Treatment—S2 cells and fly head extracts from control and all phosphorylation site mutants were prepared using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05 mM EGTA, 10% glycerol, 1% Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS) containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM Na_3VO_4, and 1 mM NaF) and complete EDTA-free protease inhibitor mixture (Roche Applied Science). This homogenate was sonicated on ice 5 times for 10 s each time using a Misonix XL2000 model sonicator at a setting of 5 and then centrifuged at 20,000 × *g* for 10 min. The supernatant was collected, resolved in 5% gel, transferred, and probed with rabbit anti-V5 (Sigma, 1:5,000), mouse anti-FLAG (Sigma, 1:5,000), rabbit anti-PER (a gift from Dr. Michael Rosbash, 1:65,000), guinea pig anti-PER (gp73; a gift from Dr. Joanna C. Chiu, 1:2,000), guinea pig anti-CLK (gp50, 1:3,000), and mouse anti-β actin (Abcam, 1:2,000) antibodies. For phosphatase treatment, RIPA extract lacking 1 mM Na_3VO_4, and 1 mM NaF was treated with λ phosphatase (New England Biolabs) for 30 min at 30 °C. Horseradish peroxidase-conjugated secondary antibodies (Sigma) were diluted 1:1,000. Immunoblots were visualized using ECL plus reagent (GE Life Sciences). The ImageJ program was used to quantify protein abundance. CLK mobility was measured by placing a rectangle of the same size over each gel lane covering the region containing hypophosphorylated and hyperphosphorylated CLK bands and quantifying the signal via densitometric analysis using the ImageJ program. The quantified signal depicts the relative mobility of CLK in each lane.

Quantitative PCR Analysis—Quantitative PCR was carried out on fly heads as described (20). The following gene-specific primer pairs were used to amplify *rp49* (5′-TACAGGCCCAAGATCGTGAA-3′ and 5′-GCACTCTGTTGTCGATACCC-3′) and *per* (5′-TGATGGGCGACTACAACTCC-3′ and 5′-GTCGCTATTCCCATTGCTGT-3′).

Immunostaining—Brains were dissected from Clk^{out} and w^{1118};*Clk*WT;Clk^{out} rescue flies collected at ZT1, dissected, fixed, immunostained with guinea pig CLK GP50 primary and goat anti-guinea pig Cy-3 (Jackson ImmunoResearch Labs) secondary antibodies, and imaged by confocal microscopy as described (38).

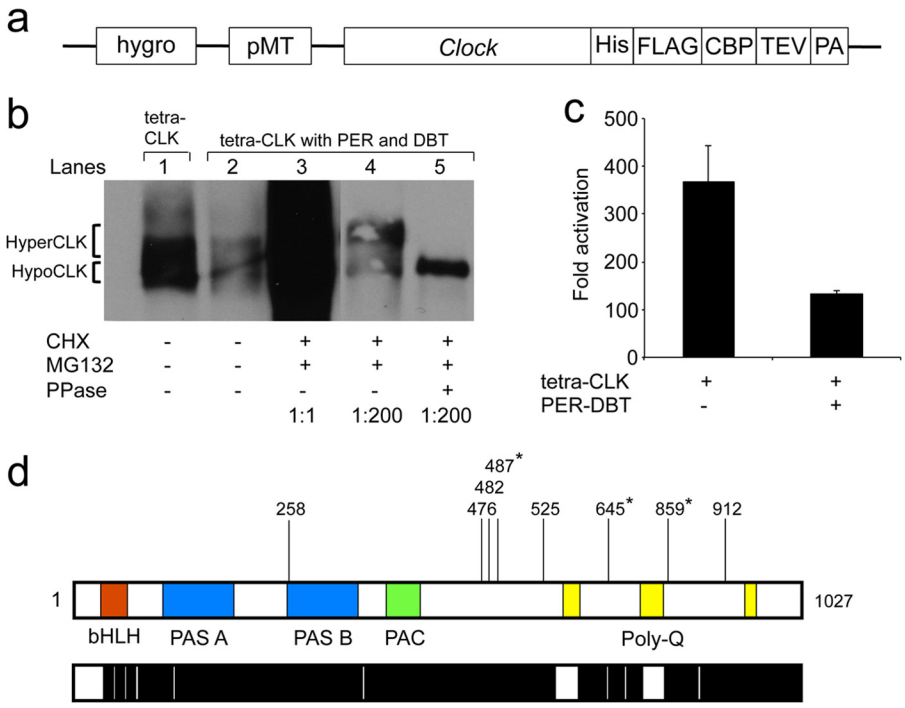

FIGURE 1. **Production of CLK in S2 cells for the identification of phosphorylation sites.** *a,* plasmid used to produce tetra-CLK in S2 cells. *Clk* expression was induced from a metallothionine promoter (*pMT*). Selection with hygromycin (*hygro*) was used to select S2 cells stably transfected with this plasmid. CLK expressed from this plasmid was epitope tagged with His_6 (*His*), 3×FLAG (*FLAG*), calmodulin-binding protein (*CBP*), and protein A (*PA*) that could be cleaved using a Tobacco etch virus protease cleavage site (*TEV*). *b*, Western blot of CLK protein expressed in S2 cells stably transfected with tetra-CLK alone or co-transfected with tetra-CLK, pMT-PER, and pMT-DBT. S2 cells were treated (+) or not treated (−) with cyclohexamide (*CHX*), MG132, or λ phosphatase (*PPase*). The sample from cells co-transfected with tetra-CLK, pMT-PER, and pMT-DBT and treated with cyclohexamide and MG132 was either not diluted (1:1) or diluted 1:200. Lanes on the Western blot are numbered *1–5*. *c*, bioluminescence assays of *per*-luc expression in S2 cells stably transfected with tetra-CLK or tetra-CLK, pMT-PER, and pMT-DBT. The fold-activation of *per*-luc expression is shown after the metallothionine promoter was induced. *d, top*, diagram of the CLK protein sequence that denotes the basic helix-loop-helix (*bHLH*) domain, the PER-ARNT-SIM A (*PAS A*) and PAS B domains, the PAC motif, poly(Q)-rich regions (*Poly-Q*), and the positions of serine residues that are phosphorylated in S2 cells. *Asterisks* denote proline-directed phosphorylation sites. *Bottom*, peptide coverage map of CLK where *black* denotes regions covered by mass spectrometry and *white* denotes regions not detected.

Circadian Locomotor Activity Monitoring—Two to 3-day-old male flies were entrained for 3 days in 12-h light:12-h dark cycles, and then kept in constant darkness for 7 days at 25 °C. Locomotor activity was monitored using the *Drosophila* Activity Monitor (DAM) system (Trikinetics). Complete darkness activity data were analyzed using ClockLab (Actimetrics) software as described (39).

RESULTS

Identification of CLK Phosphorylation Sites—To understand the function of CLK phosphorylation, we first determined which CLK residues are phosphorylated. CLK is maximally phosphorylated around dawn, when PER-TIM-DBT complexes repress transcription (18). Because CLK is also hyperphosphorylated in cultured Schneider 2 (S2) cells when co-expressed with DBT (19), S2 cells were used to produce sufficient quantities of hyperphosphorylated CLK for mass spectrometry. Four different epitope tags were added to the carboxyl terminus of CLK (referred to as tetra-CLK) to aid in purification (Fig. 1*a*). Stably transfected S2 cell lines that inducibly express tetra-CLK alone or co-express tetra-CLK, PER, and DBT were established to assess the stability, phosphorylation state, and transcriptional activity of tetra-CLK. Tetra-CLK accumulates to higher levels when it is expressed alone than when it is co-expressed with PER and DBT (Fig. 1*b*, compare *lanes 1* and *2*), consistent with previous studies showing CLK is less stable in the presence of PER and/or DBT (18, 19). When cells co-expressing tetra-CLK, PER, and DBT were treated with proteasome inhibitor MG132 and translation inhibitor cyclohexamide, high levels of hyperphosphorylated tetra-CLK preferentially accumulate based on the faster mobility of tetra-CLK after phosphatase treatment (Fig. 1*b*, compare *lanes 4* and *5*). S2 cells that express only tetra-CLK drive ~3-fold higher levels of *per* promoter-driven luciferase (*per*-luc) activity than cells that co-express tetra-CLK, PER, and DBT (Fig. 1*c*), demonstrating that epitope-tagged CLK transcriptional activity is subject to the same regulation as native CLK.

To identify CLK phosphorylation sites by mass spectrometry, stably transfected S2 cells expressing tetra-CLK, PER, and DBT were treated with MG132 and cyclohexamide, and tetra-CLK was isolated by sequential affinity chromatography using the His_6 and 3×FLAG epitope tags. Purified tetra-CLK was size separated by SDS-PAGE, and a Coomassie Blue-stained band comprised mainly of hyperphosphorylated tetra-CLK was excised, digested with proteases, and the resulting peptides were analyzed by mass spectrometry. Mass spectrometry identified peptides covering >85% of tetra-CLK (Fig. 1*d*). The regions of tetra-CLK not identified in this analysis were largely Q-rich and contain few potential phosphorylation sites. Eight

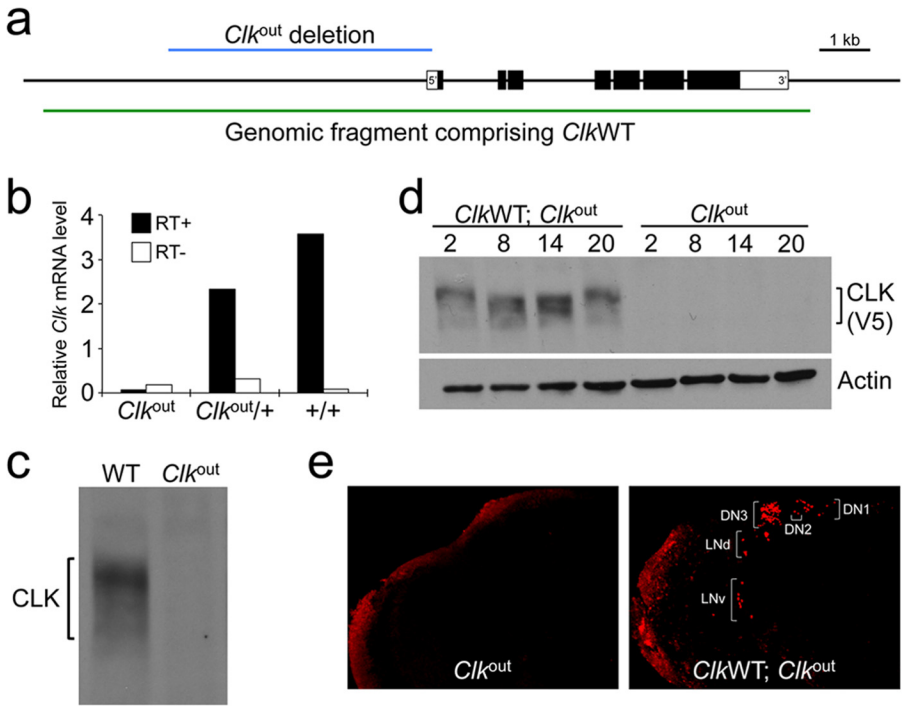

FIGURE 2. **Generation, analysis, and rescue of the *Clk*^{out} null mutant.** *a,* diagram of the *Clk* genomic region (*solid black line*) including exons (*boxes*) containing 5′ and 3′ UTR sequences (*white boxes*) and coding sequences (*black boxes*). The region deleted in the *Clk*^{out} mutation (*blue line*) and included in the *Clk*WT rescue transgene (*green line*) are shown. *b*, quantitative PCR analysis of *Clk* mRNA levels from head RNA that was (*black bars*) or was not (*white bars*) reverse transcribed in *Clk*^{out}, *Clk*^{out}/+, and +/+ flies. *c*, Western blot of head extracts from wild-type (*WT*) and *Clk*^{out} flies collected at ZT2 was probed with CLK antiserum to detect CLK levels. *d*, Western blot of head extracts from *Clk*WT rescued *Clk*^{out} flies and *Clk*^{out} flies collected at the indicated ZT was probed with V5 antiserum to detect CLK levels. β-Actin (*Actin*) served as a loading control. *e*, dissected brains from *Clk*^{out} and *Clk*WT rescued *Clk*^{out} flies immunostained with CLK antiserum. Punctate staining was detected in canonical small and large ventral lateral neuron (*LNv*), dorsal lateral neuron (*LNd*), dorsal neuron 1 (*DN1*), dorsal neuron 2 (*DN2*), and dorsal neuron 3 (*DN3*) brain pacemaker neuron clusters.

phosphorylated Ser residues were identified, including one in the PAS B domain and seven in the C-terminal half of the protein (Fig. 1*d*). Three of these sites, Ser^{487}, Ser^{645}, and Ser^{859}, are proline-directed phosphorylation sites, which are often targets of MAP kinases and cyclin-dependent kinases (40). Ser^{487} is clustered with two other phosphorylation sites at Ser^{482} and Ser^{476}, reminiscent of phosphorylation site organization in the per-short domain (8). Given that the C-terminal half of CLK contains the transcriptional activation domain (27, 28), phosphorylation in this region could impact CLK transcriptional activity.

Generation and Rescue of the Clk^{out} Null Mutant—To determine the physiological function of CLK phosphorylation, the standard approach is to test *Clk* transgenes bearing mutant phosphorylation sites for their ability to rescue behavioral and molecular rhythms of a *Clk* null mutant. However, the only *Clk* mutants available are the dominant-negative *Clk*^{Jrk} mutant (27), which has not been rescued, and the hypomorphic *Clk*^{ar} mutant (41), which expresses substantial CLK protein, but at subthreshold levels for behavioral rhythmicity. Because neither of these mutants was ideal for testing the function of CLK phosphorylation, we employed FLP-FRT recombination between PBac inserts in *Clk* exon 1 (PBac{WH}*Clk*[f06808]) and *Clk* upstream sequences (PBac{WH}*Clk*[f03095]) to generate a 5398-bp deletion that removed the *Clk* transcription start site and regulatory sequences that direct spatial and circadian expression (29, 31, 42) (Fig. 2*a*). The PBac insert remaining after FRT recombination was removed via PBac excision, producing white-eyed flies containing the deleted *Clk* sequences. These *Clk* deletion flies, which we call *Clk*^{out}, are viable as homozygotes, and show no obvious developmental defects (data not shown). Three lines of evidence demonstrate that *Clk*^{out} is a null mutant. First, *Clk*^{out} flies are behaviorally arrhythmic as homozygotes (Table 2, Fig. 3) or in heterozygous condition with *Clk*^{Jrk} or a *Clk* deficiency (Table 2). Second, *Clk* mRNA is undetectable in *Clk*^{out} flies (Fig. 2*b*). Third, CLK protein is not detectable in homozygous *Clk*^{out} flies on Western blots of fly head extracts (Fig. 2*c*) or in circadian clock neurons in the brain (Fig. 2*e*).

TABLE 2

Activity rhythms in *Clk*^{out} mutants and *Clk*^{out} or *nmo*^{P1} *Clk*^{out}/*nmo*Df *Clk*^{out} double mutants expressing WT *Clk* or *Clk* phosphorylation site mutant transgenes

Genotype	*n*	Rhythmic	Period ± S.E.	Power ± S.E.
		%		
Clk^{out}	16	0	NA	NA
Clk^{out}/*Clk*^{Jrk}	17	0	NA	NA
Clk^{out}/*Clk*Df[a]	15	0	NA	NA
*Clk*WT; *Clk*^{out}	32	93.5	24.2 ± 0.05	84.1 ± 7.1
*Clk*S258A; *Clk*^{out}	16	62.5	24.5 ± 0.10[b]	53.9 ± 10.3
*Clk*S476A/S482A; *Clk*^{out}	16	93.8	23.7 ± 0.09[c]	57.1 ± 2.4
*Clk*S487A; *Clk*^{out}	56	82.1	23.7 ± 0.04[c]	85.7 ± 6.5
*Clk*S476A/S482A/S487A; *Clk*^{out}	16	87.5	23.7 ± 0.10[c]	85.2 ± 13.3
*Clk*S525A; *Clk*^{out}	16	62.5	23.5 ± 0.05[c]	50.8 ± 10.9
*Clk*S645A; *Clk*^{out}	16	87.5	24.2 ± 0.09	39.6 ± 9.9
*Clk*S859A; *Clk*^{out}	32	87.5	23.5 ± 0.07[c]	99.4 ± 3.0
*Clk*S912A; *Clk*^{out}	16	100	24.6 ± 0.08[c]	86.0 ± 10.2
*Clk*WT; *nmo*^{P1} *Clk*^{out}/*nmo*Df *Clk*^{out}	20	75.0	22.3 ± 0.08	62.8 ± 10.3
*Clk*S859A; *nmo*^{P1} *Clk*^{out}/*nmo*Df *Clk*^{out}	32	71.8	21.6 ± 0.08[d]	55.1 ± 5.7

[a] The *Clk*Df genotype is Df(3L)DSC631.
[b] Significantly longer period than *Clk*WT ($p < 0.04$).
[c] Significantly shorter period than *Clk*WT ($p < 0.0001$).
[d] Significantly shorter period than *Clk*WT; *nmo*^{P1} *Clk*^{out}/*nmo*Df *Clk*^{out} ($p < 0.0001$).

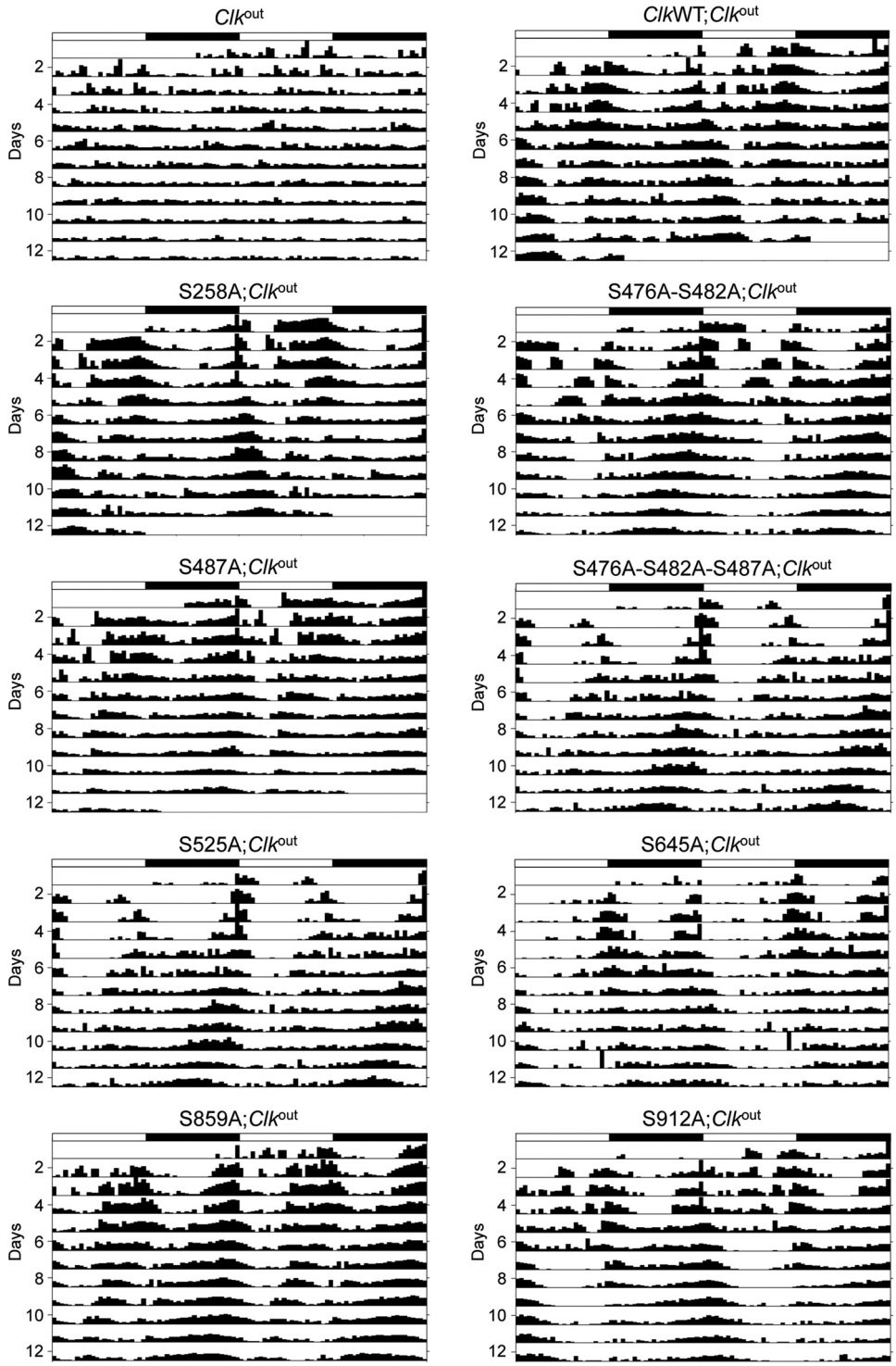

FIGURE 3. **Locomotor activity rhythms in *Clk*^{out} flies that express WT CLK and CLK phosphorylation mutants.** *Clk*^{out}, *Clk*WT;*Clk*^{out}, *Clk*S258A;*Clk*^{out}, *Clk*S476A/S482A;*Clk*^{out}, *Clk*S487A;*Clk*^{out}, *Clk*S476A/S482A/S487A;*Clk*^{out}, *Clk*S525A;*Clk*^{out}, *Clk*S645A;*Clk*^{out}, *Clk*S859A;*Clk*^{out}, and *Clk*S912A;*Clk*^{out} (S912A) flies were entrained in light/dark cycles and then placed in complete darkness on day 4 for 7 additional days. Representative examples of actograms for single flies of each genotype are shown.

To rescue *Clk*^{out}, a wild-type *Clk* transgene, *Clk*WT, was generated that contains *Clk* genomic sequences beginning ~8 kb upstream of the start of transcription and ending ~1.6 kb beyond the 3′-untranslated region (Fig. 2*a*). The transformation vector carrying the *Clk*WT genomic fragment contains an attB integration site for PhiC31 site-directed chromosomal insertion, thus enabling comparison of transgenes inserted at the same site by eliminating position effects on transgene expression (32). The *Clk*WT transgene was integrated into the VK00018 *attP* site on chromosome 2, and crossed into a homozygous *Clk*^{out} genetic background to determine whether it could rescue behavioral and molecular rhythms. To analyze locomotor activity rhythms, flies were entrained for 3 days in 12-h light:12-h dark cycles followed by 7 days in complete dark-

ness. When present in two copies, *Clk*WT rescued robust activity rhythms in >90% of *Clk*^{out} flies with periods of ~24.2 h (Table 2, Fig. 3). Consistent with this behavioral rescue, rhythms in CLK protein phosphorylation were detected as changes in mobility in Western blots of *Clk*WT;*Clk*^{out} fly head extracts collected at different times during a light:dark cycle (Fig. 2*d*). More relevant to behavioral rescue is expression in the ~150 circadian clock neurons in the adult brain, which can be subdivided into several anatomically and functionally distinct groups of cells including the small and large ventral lateral neurons, dorsal lateral neurons, and three subgroups of dorsal neurons (DN1, DN2, and DN3) (43). As expected, there was no detectable CLK-immunoreactive signal in the *Clk*^{out} flies, whereas *Clk*WT;*Clk*^{out} flies showed a similar spatial pattern as that observed for wild-type flies (38) (Fig. 2*e*). From these behavioral and molecular results, we conclude that *Clk*WT effectively rescues clock function and locomotor activity rhythms in *Clk*^{out} flies. Thus, the *Clk*WT transgene can be used as a template for generating *Clk* phosphorylation site mutants, and the *Clk*WT insert can also serve as a control for *Clk* phosphorylation site mutants inserted into the VK00018 *attP* site.

Generation and Analysis of CLK Phosphorylation Site Mutants—To determine the function of CLK phosphorylation sites, phosphorylation site mutants that convert Ser to non-phosphorylatable Ala were incorporated into the *Clk*WT transgene, which was inserted into the VK00018 *attP* site and tested for rescue of locomotor activity and molecular rhythms in *Clk*^{out} flies. Single phosphorylation site mutants were made for all three proline-directed sites and for the other three sites outside the Ser^{476}-Ser^{482}-Ser^{487} cluster. Within the Ser^{476}-Ser^{482}-Ser^{487} cluster, a double mutant for the non-proline-directed sites (Ser^{476} and Ser^{482}) and a triple mutant for the entire Ser^{476}-Ser^{482}-Ser^{487} cluster were generated. We will refer to transgenes bearing CLK phosphorylation site mutants by the amino acid number of the Ser to Ala substitution, thus S258A refers to the *Clk* S258A transgene. Analysis of locomotor activity rhythms showed that the free-running period of all mutants except S645A showed significant ($p < 0.05$), but small (<0.8 h), changes in period (Table 2, Fig. 3). Two of the mutants, S258A and S912A, had longer periods than *Clk*WT by 0.3 and 0.4 h, respectively. In contrast, S525A, S859A, and all three mutants in the Ser^{476}-Ser^{482}-Ser^{487} cluster had ≥0.5 h shorter periods than *Clk*WT. The proline-directed S487A and non-proline-directed S476A/S482A mutant shortened the period to the same extent as the S476A/S482A/S487A triple mutant, suggesting that phosphorylation in this region is tied to the same function within the clock mechanism. It is worth noting that most mutants shortened the circadian period, consistent with the hypothesis that PER-DBT-dependent phosphorylation of CLK compromises CLK-CYC activity (18–20).

We next determined the impact of CLK phosphorylation on the molecular clock mechanism. Interactions between BMAL1 and CLOCK in mice promote CLOCK post-translational regulatory events that enable CLOCK-BMAL1 transactivation of target genes (44). In contrast, CYC is always bound to limiting amounts of CLK independent of the CLK phosphorylation state and CLK-CYC transcriptional activity (18, 45), thus CYC binding to CLK phosphorylation mutants is not expected to alter CLK-CYC function. Because each CLK phosphorylation site mutant rescued locomotor activity rhythms with periods close to *Clk*WT, any differences in the phase of clock component cycling would not likely be detectable. However, changes in the abundance or activity of CLK could account for period shortening; increasing the *Clk* gene copy number shortens period (46), increasing the *per* gene copy number (as an direct output of CLK-CYC activity) shortens period (47), increasing CLK-CYC activity by adding an activation domain to CYC shortens period (46), and increased CLK phosphorylation correlates with reduced CLK-CYC activity (18). To determine whether CLK phosphorylation site mutants alter CLK abundance and/or activity, the levels and phosphorylation state of CLK and PER were examined in head extracts from mutant and control flies collected at Zeitgeber time 2 (ZT2) and ZT14 (note: ZT0 is lights on and ZT12 is lights off in light/dark cycles), times of maximal or minimal CLK and PER phosphorylation, respectively (17, 18). The abundance of CLK at ZT2 and ZT14 in the CLK phosphorylation site mutants are similar to those in *Clk*WT (Fig. 4, *a* and *b*), although CLK abundance at ZT14 is somewhat lower on average in S859A and S912A, but not significantly so ($p > 0.05$). The migration of CLK bands at ZT2 and ZT14 in every CLK phosphorylation site mutant strain except S859A were similar to those in *Clk*WT (Fig. 4*a*). In S859A, the migration of CLK at both ZT2 (Fig. 4*c*, compare *peaks with asterisks* in each column) and ZT14 (Fig. 4*c*, compare *peaks with diamonds* in each column) are faster than that in *Clk*WT, indicating that CLK is less phosphorylated. Given that CLK is hypophosphorylated when it is transcriptionally active, decreased CLK phosphorylation in S859A would predict a higher level of CLK-CYC transcription, consistent with S859A period shortening.

As expected, none of the CLK phosphorylation site mutants altered PER mobility at ZT2 or ZT14 (Fig. 4*a*). Although PER abundance in different CLK phosphorylation site mutants showed variations among different samples collected at the same time point, the only consistent difference in PER levels was seen in S859A, which had ~50% higher levels of PER at ZT2 and ~30% higher PER levels at ZT14 than *Clk*WT flies, although this trend was not significant (Fig. 4, *a* and *d*). If the high levels of PER in S859A mutant flies result from increased CLK-CYC transcription, then *per* mRNA levels should be high. Indeed, *per* mRNA levels at ZT14 are ~2-fold higher in S859A flies than in *Clk*WT flies (Fig. 5*a*). These results suggest that CLK protein is more transcriptionally active in S859A than *Clk*WT flies because CLK abundance in these strains is similar (see Fig. 4, *a* and *b*). This increase in CLKS859A activity could account for its short period phenotype.

CLK transcriptional activity can be sensitively and quantitatively assayed in S2 cells using a *per*-luc reporter gene (28). To determine whether CLKS859A activity is higher than WT CLK or other CLK phosphorylation site mutants, we compared *per*-luc reporter gene activity in S2 cells expressing WT CLK or different CLK phosphorylation site mutants. CLK phosphorylation site mutants activated *per*-luc to the same extent as WT CLK with the exception of CLKS859A, which activated higher *per*-luc levels than WT (Fig. 5*b*). The increased activity of CLKS859A in S2 cells is consistent with increased *per* mRNA

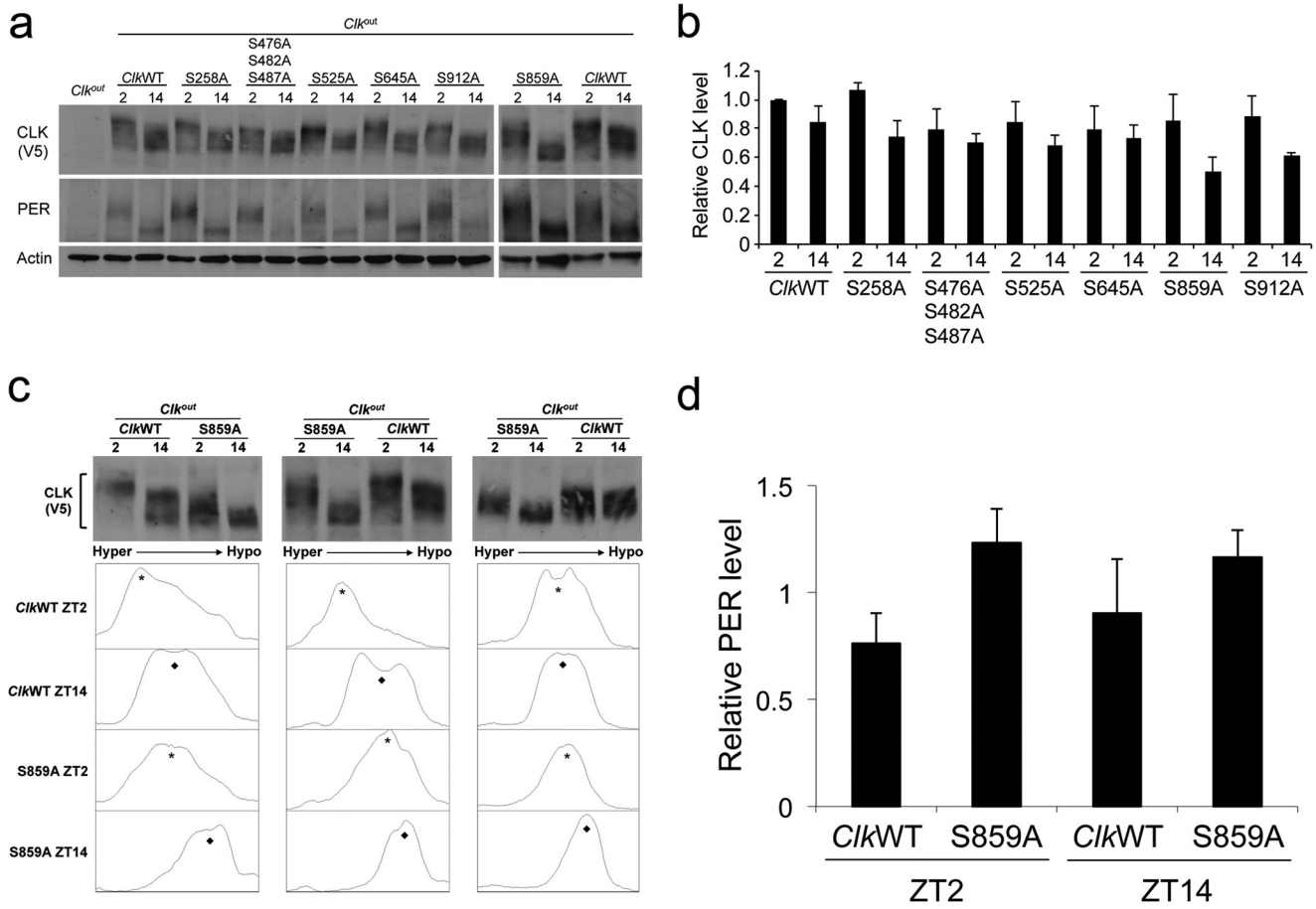

FIGURE 4. **CLK and PER expression and function in CLK phosphorylation site mutants.** *a,* Western blots of fly head extracts from Clk^{out}, *Clk*WT;Clk^{out} (*Clk*WT), *Clk*S258A;Clk^{out} (S258A), *Clk*S476A/S482A-S487A;Clk^{out} (S476A/S482A/S487A), *Clk*S525A;Clk^{out} (S525A), *Clk*S645A;Clk^{out} (S645A), *Clk*S859A;Clk^{out} (S859A), or *Clk*S912A;Clk^{out} (S912A) flies collected at ZT2 and ZT14 were probed with V5 and PER antisera to detect CLK and PER, respectively. β Actin (*Actin*) served as a loading control. *b,* quantification of CLK levels in the blot from *panel a* and two additional Western blots containing samples from independent collections. *c,* densitometric analysis of CLK mobility differences on three independent Western blots of head extracts from *Clk*WT and *Clk*S859A (S859A) flies collected at the indicated times. Densitometry traces are from higher mobility (*hyper*) on the left to lower mobility (*hypo*) on the right. *Black arrows* denote the bands to compare for a shift in mobility. *d,* quantification of PER levels in the samples shown in *panel a* and four additional samples from independent collections. The higher PER levels in S859A *versus Clk*WT at ZT2 are not significant ($p = 0.058$).

and protein expression in flies (Figs. 4*d* and 5*a*), and indicates that phosphorylation at this site inhibits CLK activity. PER and DBT repressed transcription to similar extents in WT CLK and all of the CLK phosphorylation site mutants (Fig. 5*b*), demonstrating that these mutants do not compromise PER-DBT-dependent repression. Thus, our results suggest that even though higher transcriptional activity may explain the period shortening in *Clk*S859A flies, neither increased CLK abundance (see Fig. 4*b*) nor activity (see Fig. 5*b*) appear to account for the period shortening in *Clk*S476A/S482A, *Clk*S487A, *Clk*S476A/S482A/S487A, and *Clk*S525A mutants.

Because CLK Ser^{859} is a proline-directed phosphorylation site, it is possible that this site is targeted by NMO kinase, which shortens the circadian period and alters CLK stability and phosphorylation state (22). A genetic approach was taken to determine whether *nmo* and *Clk*S859A functioned in one pathway or in different pathways to determine the circadian period. Locomotor activity rhythms in *Clk*WT; nmo^{P1} Clk^{out}/*nmo*Df Clk^{out} mutants had a period of 22.3 h, consistent with the period in nmo^{P1}/*nmo*Df flies (22), whereas the period of activity rhythms in *Clk*S859A;nmo^{P1} Clk^{out}/*nmo*Df Clk^{out} double mutants was 21.6 h (Table 2). The additive effect of the *nmo* and *Clk*S859A mutants on the circadian period suggests that NMO does not phosphorylate CLK Ser^{859}.

DISCUSSION

During the circadian cycle, CLK is hypophosphorylated when CLK-CYC transcription is high and hyperphosphorylated when CLK-CYC transcription is low (18, 20). CLK hyperphosphorylation requires PER and DBT (18–20), but the identity of phosphorylation sites in CLK and the role they play in regulating transcription has not been explored. Phosphorylation of CLK could regulate many processes that impact transcription including subcellular localization, activity, and stability of CLK. Although the subcellular localization of CLK in cultured S2 cells can be altered by mutating consensus nuclear localization and export sequences or co-expressing PER (19, 48), CLK appears to be predominantly localized to the nucleus at all times of day in brain neurons and photoreceptors of flies (38, 49), thus in this study we did not evaluate phosphorylation-dependent changes in subcellular localization. However, repression of CLK-CYC transcription is strongly correlated with CLK phosphorylation *in vivo* and in S2 cells (18–20, 50), and phosphorylation destabilizes CLK in S2 cells (18, 19),

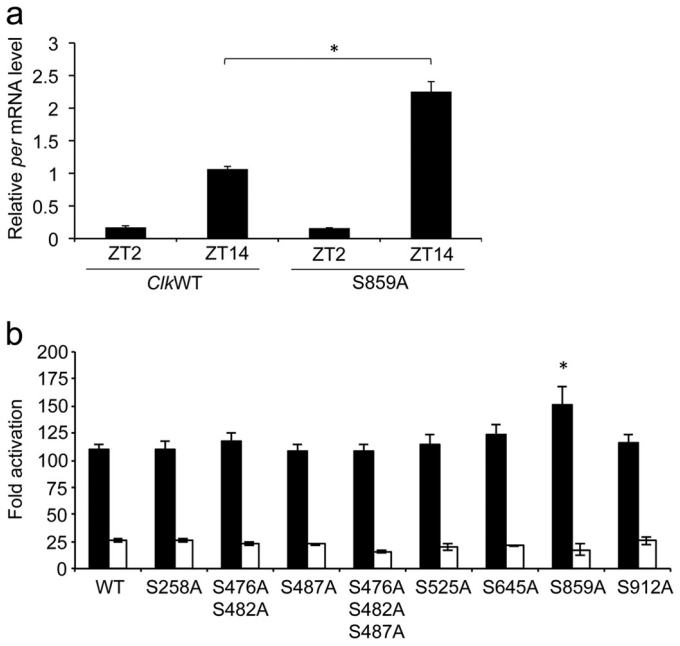

FIGURE 5. **Analysis of transcriptional activity of CLK phosphorylation site mutants in S2 cells and S859A *in vivo*.** *a*, quantitative PCR quantification of *per* mRNA levels from three independent collections of *Clk*WT and *Clk*S859A (S859A) flies at ZT2 and ZT14. *Asterisk* denotes significantly higher ($p < 0.0001$) *per* mRNA levels in S859A than *Clk*WT at ZT14. *b*, luciferase assays showing the fold-activation of *per*-luc expression in S2 cells expressing wild-type CLK (WT), CLK S258A (S258A), CLK S476A/S482A (S476A/S482A), CLK S487A (S487A), CLK S476A/S482A/S487A (S476A/S482A/S487A), CLK S525A (S525A), CLK S645A (S645A), CLK S859A (S859A), or CLK S912A (S912A) alone (*black bars*) or with PER and DBT (*white bars*). *Asterisk* denotes significantly higher ($p < 0.05$) *per*-luc activation in S859A than WT.

although the relevance of altered CLK stability is not well understood because CLK levels show little variation in fly heads, photoreceptor cells, and brain neurons over a circadian cycle (18, 38, 49).

To examine the function of CLK phosphorylation, we first used mass spectrometry to identify 8 phosphorylation sites on CLK expressed in S2 cells. A *Clk* transgene that eliminated phosphorylation at Ser^{645} rescued rhythms in Clk^{out} flies with a period similar to *Clk*WT, whereas eliminating phosphorylation at Ser^{258} and Ser^{912} rescued long period rhythms. At the molecular level, none of these three mutants noticeably altered the level or phosphorylation state of CLK and PER *in vivo*, or the activation or repression of *per*-luc transcription in S2 cells. The lack of a behavioral or molecular circadian phenotype shows that these phosphorylation sites have little to no impact on CLK levels, activity, or ability to keep time. It is possible that these three sites act in concert with others, and altered timekeeping may only be manifest when multiple sites are altered (Fig. 6). Alternatively, phosphorylation at these sites may alter circadian phenotypes other than period determination, such as entrainment to environmental cues or temperature compensation (51). We tested whether eliminating all seven C-terminal CLK phosphorylation sites produced a more robust circadian phenotype, but this mutant produced very low levels of CLK protein *in vivo* and in S2 cells (data not shown), which contrasted with previous results showing that phosphorylation destabilizes CLK (18, 19). Because eliminating 15 CLK phosphorylation sites identified in another study (including three in common with this study) increases CLK abundance,[3] it is likely that the particular combination of 7 Ser to Ala substitutions destabilizes CLK, perhaps due to misfolding.

Mutants that eliminated phosphorylation at Ser^{476}-Ser^{482}, Ser^{487}, Ser^{525}, and Ser^{859} rescued locomotor activity rhythms with periods ≥0.5 h shorter than *Clk*WT. The large proportion of CLK phosphorylation site mutants that shortend period is consistent with phosphorylation acting to inhibit CLK; loss of inhibition should increase CLK-CYC activation of target genes and decrease the time required for feedback repression (46, 52). The small magnitude of period shortening is similar to that seen when *Clk* or *per* gene copy number is increased, where period decreases by ~0.5 h for an extra copy of *Clk* or *per* up to a maximum of ~2 h period shortening (46, 47). Both the non-proline-directed S476A/S482A mutant and the proline-directed S487A mutant rescue behavioral rhythms that are ~0.5 h shorter than *Clk*WT. Eliminating phosphorylation at all three of these sites does not shorten the period of behavioral rhythms further, suggesting that this cluster of phosphorylation sites function together. Despite the short period behavioral rescue by S476A/S482A, S487A, S476A/S482A/S487A, and S525A CLK mutants, we did not detect changes in CLK levels or phosphorylation state in fly heads or CLK-CYC activation or repression in S2 cells. Although we do not understand how these mutants shorten period, the inability to phosphorylate these sites somehow accelerates or bypasses a step in the feedback loop, which implies that phosphorylating these sites imposes a delay (Fig. 6). Such a delay could occur by prolonging the time it takes for events that either repress or activate transcription (or both). This would be analogous to eliminating NMO or DBT phosphorylation sites in the per-short region of PER (8), although the period change due to loss of CLK phosphorylation at these sites is much less severe.

Loss of phosphorylation at Ser^{859} also shortens the period of behavioral rhythms by ~0.5 h. However, unlike the other CLK phosphorylation site mutants that shorten period, *Clk*S859A expresses higher levels of PER in fly heads than *Clk*WT and drives higher *per*-luc levels in S2 cells than WT CLK, yet S859A is repressed to the same extent as WT CLK. Because the levels of CLK are the same in *Clk*S859A and *Clk*WT fly heads, these results suggest that loss of phosphorylation at Ser^{859} increases activation. Increased CLK activity is consistent with the observation that CLK S859A protein migrates as a faster, less phosphorylated form. Because the S859A mutant enhances transcriptional activation without altering repression, phosphorylation of Ser^{859} likely occurs independent of PER-DBT repression and persists during CLK-CYC transcription (Fig. 6).

Of the 8 phosphorylation sites identified here, none appear to control PER-DBT-dependent processes such as transcriptional repression or the destabilization of CLK. Although hyperphosphorylated CLK was isolated from S2 cells that co-expressed PER and DBT, not all phosphorylation events alter gel migration, and S2 cells may lack kinases that are present in clock cells. Thus, we do not know if hyper-

[3] E. Y. Kim and I. Edery, personal communication.

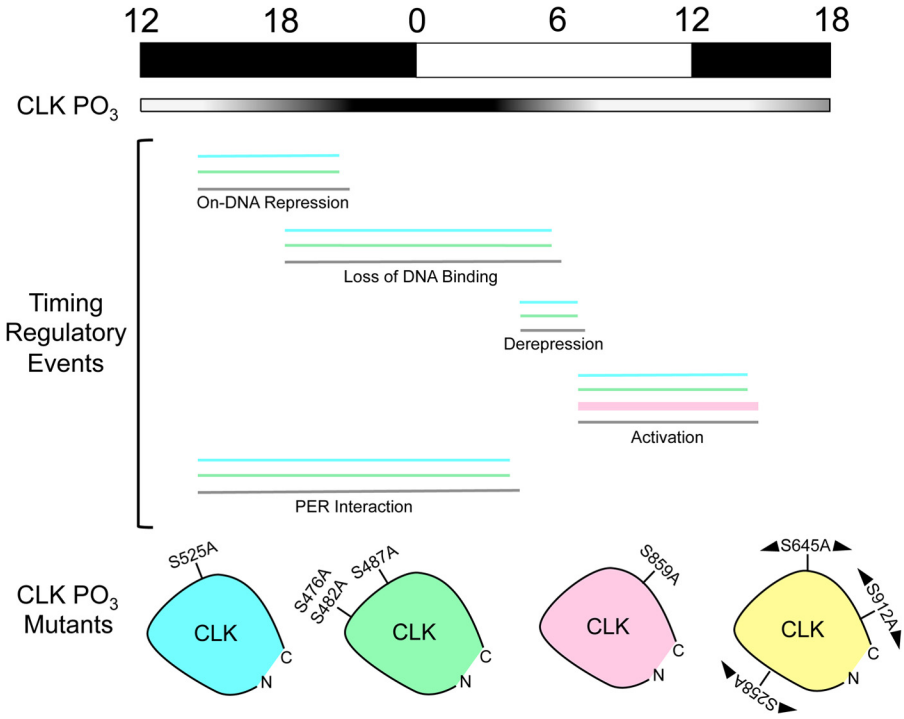

FIGURE 6. **Model for how CLK phosphorylation mutants impact circadian clock function.** The affects of CLK phosphorylation mutants (CLK PO_3 mutants) on regulatory events that control circadian timekeeping (Timing Regulatory Events) are shown in relationship to the CLK phosphorylation cycle (*CLK PO_3*) under light/dark conditions. The CLK phosphorylation mutants are: S525A (*blue*), the S476A/S482A/S487A group (*green*), S859A (*pink*), and the S258A, S645A, and S912A sites (*yellow*), where the S476A/S482A/S487A group affects the same timekeeping event and the S258A, S645A, and S912A sites may impact timekeeping in conjunction with other phosphorylation sites (*arrowheads*). *N* represents the amino terminus of CLK and *C* represents the carboxyl terminus of CLK. The indicated Timing Regulatory Events in wild-type flies (*gray line*) are compared with the S525A mutant (*blue line*), the S476A/S482A/S487A mutant group (*green line*), and the S859A mutant (*red line*). The *length* of the lines represent the time span of the regulatory event, and the *thickness* of the lines denote the strength of the event, with *thicker lines* representing increased strength. CLK PO_3 is highest when *shading is darkest* and lowest when *shading is lightest*. ZT (in hours): *white box*, light phase; *black box*, dark phase. Period shortening by S525A and the S476A/S482A/S487A group result from advancing any Timing Regulatory Event due to skipping phosphorylation at these sites, whereas period shortening by S859A results from increased CLK-CYC dependent activation. The S258A, S645A, and S912A mutants have little impact on circadian timekeeping by themselves, but may play a role in other important clock processes (see text for details).

phosphorylated CLK produced in S2 cells is phosphorylated at all the same sites as hyperphosphorylated CLK at ZT2 in clock cells from flies. Co-expressing high levels of PER and DBT in S2 cells may also impact the CLK phosphorylation pattern because many non-overlapping phosphorylation sites were identified in S2 cells that only overexpressed CLK, including sites that destabilize CLK.[3] It is difficult to estimate the extent to which CLK phosphorylation sites identified from S2 cell experiments match those from clock cells. Given that the sites identified in S2 cells do not account for processes dependent on phosphorylation by PER-DBT, it is likely that important sites were not identified and await *in vivo* analysis of CLK phosphorylation.

The CLK phosphorylation sites that we identified are conserved among different *Drosophila* species, but are less well conserved among other insects and are not conserved in mammals. This lack of conservation is not surprising because all but one site is in the C-terminal half of CLK, which contains no conserved domains and is rich in polyglutamine sequences (27, 28). The lack of conservation among phosphorylation sites is also seen for PER (8, 11, 14). Phosphorylation sites that promote nuclear entry of PER in *Drosophila*, the Ser^{149}-Ser^{151}-Ser^{153} cluster and Ser^{657} (4, 53), are not conserved in mammals. However, kinases that phosphorylate the Ser^{149}-Ser^{151}-Ser^{153} cluster and Ser^{657} in flies, casein kinase 2, and SGG/glycogen synthase kinase 3β, phosphorylate different sites that control PER2 nuclear localization and degradation in mammals (54, 55). Likewise, sites within the per-short domain of *Drosophila* PER are not conserved in mammals (8, 56, 57), and a phosphorylation site altered in patients with familial advanced sleep phase syndrome that controls hPER2 nuclear retention and/or transcriptional activity (Ser^{662}) is not conserved in *Drosophila* (14, 15, 58). These results suggest that even though phosphorylation impacts many aspects of CLK and PER ortholog function (*e.g.* subcellular localization, stability, and activity), and in many cases the kinases that regulate these processes are conserved, the phosphorylation sites that control how and when these proteins function within the clock are species-specific.

Ultimately, determining which sites on CLK are phosphorylated *in vivo*, when they are phosphorylated during the circadian cycle, what processes different phosphorylation sites control, and which kinases phosphorylate these sites will provide a detailed understanding of how phosphorylation controls CLK protein function. Moreover, other post-translational modifications of CLK such as ubiquitylation control CLK-CYC transcription (59), thus it will be important to determine how post-translational modifications are integrated to control CLK function. A detailed understanding of CLK modifications will

likely reveal conserved mechanisms that control the pace of the circadian oscillator.

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Gene Regulation:

Phosphorylation of the Transcription Activator CLOCK Regulates Progression through a ~24-h Feedback Loop to Influence the Circadian Period in *Drosophila*

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