TRITHORAX-dependent arginine methylation of HSP68 mediates circadian repression by PERIOD in the monarch butterfly

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Transcriptional repression drives feedback loops that are central to the generation of circadian (24–h) rhythms. In mammals, circadian repression of circadian locomotor output cycles kaput, and brain and muscle ARNT-like 1 (CLOCK:BMAL1)-mediated transcription is provided by a complex formed by PERIOD (PER) and CRYPTOCHROME (CRY) proteins. PER initiates transcriptional repression by binding CLK:BMAL1, which ultimately results in their removal from DNA. Although PER’s ability to repress transcription is widely recognized, how PER binding triggers repression by removing CLK:BMAL1 from DNA is not known. Here, we use the monarch butterfly as a model system to address this problem because it harbors a simplified version of the CLK:BMAL1-activated circadian clock present in mammals. We report that an intact CLOCK mouse exon 19 homologous region (CLKe19r) and the histone methyltransferase TRITHORAX (TRX) are both necessary for mammalian-CLOCK:BMAL1-mediated transcriptional activation, CLK–PER interaction, and PER repression. Our results show that TRX catalytic activity is essential for CLK–PER interaction and PER repression via the methylation of a single arginine methylation site (R45) on heat shock protein 68 (HSP68). Our study reveals TRX and HSP68 as essential links between circadian activation and PER-mediated repression and suggests a potential conserved clock function for HSPs in eukaryotes.

PERIOD | heat shock protein | arginine methylation | TRITHORAX | insect

Significance

Circadian repression drives the transcriptional feedback loops that keep circadian (24–h) time and synchronize an animal’s physiology and behavior to the daily environmental changes. Although PERIOD (PER) is known to initiate transcriptional repression by displacing the transcription activator CLOCK:BMAL1 from DNA, the underlying mechanism remains unknown. Using the monarch butterfly as a model harboring a simplified version of the mammalian circadian clock, we demonstrate that the binding of heat shock protein 68 (HSP68) to a region homologous to CLOCK mouse exon 19 is essential for CLOCK–PER interaction and PER repression. We further show that CLOCK–PER interaction and PER repression are promoted by the methylation of a single arginine methylation site (R45) on HSP68 via TRITHORAX catalytic activity.

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copies of PER and mammalian-like CRY, makes the monarch butterfly an attractive model system for mechanistic studies of circadian repression by PER.

In the present study, we demonstrate that, similar to mammals, an intact CLK mouse exon 19 homologous region (CLKe19r) is required for circadian transcriptional activity and behavioral circadian rhythms in vivo in the monarch butterfly. We show that CLKe19r and the histone methyltransferase TRITHORAX (TRX), the invertebrate homolog of MLL1, are both necessary for monarch CLK-BMAL1-mediated transcriptional activation, CLK–PER interaction, and PER repression in Drosophila S2 cells. Unexpectedly, we found that the catalytic activity of TRX is essential for PER repression and formation of the CLK–PER complex via the direct or indirect methylation of a single arginine methylation site (R45) on heat shock protein 68 (HSP68). Based on the functional conservation of CLKe19r between species, we speculate that this mechanism may be conserved in mammals.

Results

CLK Exon 19 Homologous Region Is Necessary for CLK-BMAL1-Mediated Transcriptional Activation and Repression by PER. To assess whether the monarch butterfly would be a relevant model to test if PER repression occurs through the homologous region of CLK exon 19 (CLKe19r), we started by testing whether this domain was necessary for circadian activation and behavioral rhythms in monarch butterflies, as is the case in mice (6–9). Using CRISPR-Cas9–mediated targeted mutagenesis, we generated a monarch mutant bearing a 287-bp deletion that eliminated the entire conserved CLK exon 19 located on monarch CLK exon 12 (hereafter called CLKΔ19r) (Fig. 1 A and B). To measure circadian behavior, we used pupal eclosion as our behavioral readout because no assays have been developed to track behavior in monarchs. Similar results were reported in mice (7), monarch CLKΔ19r hemizygous female mutants exhibited a long-period phenotype in circadian eclosion behavior when compared with wild-type sibling female controls (Fig. 1C). Moreover, circadian eclosion of CLKΔ19r heterozygous male mutants was also lengthened but to intermediate levels compared with hemizygous female mutants (Fig. 1C), indicative of a semidominant function for the mutant allele as previously shown in mice (8). CLKΔ19r hemizygous female mutants also showed disrupted molecular rhythms in the brain with constitutive low expression levels of period (per) and timeless (tim) messenger RNA (mRNA) under constant conditions (Fig. 1D). Together, these results show that monarch CLKΔ19r mutants are defective in circadian activation and phenocopy CLK19r mutant mice.

To determine whether CLKe19r is necessary for PER repression, we next used luciferase reporter gene assays in Drosophila S2 cells in which endogenous proteins were coexpressed with a reporter construct containing a tandem repeat of the proximal CACGTG E-box enhancer from the monarch gene promoter (15), CR Y2 was able to repress CLKΔ19r:BMAL1-mediated transcription, demonstrating that CLKe19r is necessary for PER repression but not for CR Y2 repression (Fig. 2B). To bolster this finding, we performed luciferase assays in S2 cells using the CLKe19r domain as a sponge. We reasoned that if PER represses CLK-BMAL1 through its interaction with CLKe19r, coexpressing the CLKe19r domain should displace PER away from CLK-BMAL1, thereby releasing repression. In line with our hypothesis, we found that coexpressing increasing doses of the CLKe19r domain fused to the Gal4 DNA binding domain (Gal4DBD), used to ensure folding of the short pentameric peptide, caused a dose-dependent inhibition of PER repression of CLKΔ19r by PER (Fig. 2 B, Left). Expressing high doses of Gal4DBD alone did not inhibit PER repression (Fig. 2 B, Right), showing that the effect of the sponge was solely due to the presence of CLKe19r.

We further tested whether CLKe19r would be sufficient for PER repression using a transactivation assay where a luciferase reporter gene under the control of a six tandem repeats of the yeast GAL4 upstream activator sequence (6X UAS) promoter (the DNA binding site of Gal4DBD) was cotransfected with either the strong herpes simplex virion protein 16 (VP16) TAD directly fused to Gal4DBD or the Gal4DBD-CLKΔ19r fusion protein (Fig. 2C). We found that Gal4DBD-VP16 and Gal4DBD-FLAG-CLKe19r-VP16 both potently activated transcription but that only Gal4DBD-FLAG-CLKe19r-VP16 was repressible by PER in a dose-dependent fashion (Fig. 2C), further demonstrating that CLKe19r is sufficient for PER repression. We noted that in the absence of VP16, Gal4DBD-FLAG-CLKe19r did not activate transcription (SI Appendix, Fig. S1B), suggesting that CLKe19r is necessary, but not sufficient, for transcriptional activation; thus, transactivation requires other domains on CLK.

The Histone Methyltransferase TRX Is Necessary for CLK-BMAL1-Mediated Transcriptional Activation and Repression by PER. Because CLKe19r is necessary for the rhythmic binding of the histone methyltransferase MLL1 that deposits H3K4me3 marks at core clock gene promoters during CLOCK:BMAL1 activation in mammals (10), we tested whether its insect homolog, TRX (16), was also necessary for both CLK:BMAL1 activation and PER repression. To this end, we coexpressed CLK and BMAL1 in the absence or presence of increasing doses of PER in S2 cells in which endogenous Trx was knocked down by RNA interference (RNAi). RNAi-mediated knockdown of endogenous Trx caused a significant decrease in CLK-BMAL1 activation and eliminated repression by PER but not by CR Y2 (Fig. 2D and SI Appendix, Fig. S1B), similar to that observed with CLKΔ19r:BMAL1 (Fig. 2A). These results suggest that both CLKΔ19r and TRX are required not only for CLK:BMAL1-mediated transcriptional activation but also, for PER repression. We confirmed that TRX action occurs through the CLKΔ19r domain by showing that Gal4DBD-FLAG-CLKΔ19r-VP16-mediated transcriptional activation is severely blunted, and its repression by PER is abolished when Trx is knocked down (SI Appendix, Fig. S1C).

To test whether CLKe19r and TRX were also necessary for CLK–PER interaction, we performed communoprecipitations (co-IPs) of FLAG-tagged PER coexpressed in S2 cells with either V5-tagged CLKΔ19r or V5-tagged CLK in the presence or absence of endogenous TRx. While PER and CLK interacted in the presence of TRX, we found that the interaction was abolished in the absence of CLKe19r or TRX (Fig. 2E). Taken together, our results demonstrate that both CLKe19r and TRX are required not only for CLK:BMAL1-mediated transcriptional activation and repression by PER but also, for CLK–PER interaction.

The Catalytic Activity of TRX Mediates PER Repression and CLK–PER Interaction. Based on the previously reported binding of MLL1 to CLOCK exon 19 in mammals (10), our results suggested that TRX could function as a scaffold protein for CLK–PER
interaction. Full MLL1 catalytic activity on H3K4 methylation occurs through the assembly of its cleaved N-terminal and catalytically active C-terminal SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domains to a WRAD multiprotein complex composed of WD repeat-containing protein 5 (WDR5), retinoblastoma-binding protein 5 (RbBP5), absent, small, or homoeotic discs 2-like protein (Ash2L), and dumpy-30 (Dpy-30) (17, 18). Importantly, WDR5 was previously shown to function as a modulator of PER activity that helps PER repression of TIM. Similar to mammalian MLL1, Trithorax possesses a cleavage site for the production of N-terminal and C-terminal subunits, and the SET domain is located in the C-terminal subunit (Fig. 3A).

We first tested whether the SET domain of TRX was necessary for PER repression by cotransfecting S2 cells in which endogenous Trx was knocked down with CLK, BMAL1, increasing doses of PER, the N-terminal domain of TRX (TRX-NT), and either the wild-type C-terminal domain of TRX (TRX-CT) or a truncated version lacking the SET domain (TRX-CTΔSET). Knockdown of endogenous Trx was achieved by using double-stranded RNAs (dsRNAs) targeting the 5' and 3' untranslated regions, which are absent on the TRX variants containing plasmids. Strikingly, we found that while coexpression of TRX-NT and TRX-CT restored both CLK:BMAL1 activation and its repression by PER, coexpression of TRX-NT and TRX-CTΔSET restored activation levels but not PER repression (Fig. 3B). Furthermore, coexpression of TRX-NT in these experimental conditions marginally contributed to the restoration of activation to wild-type levels but was dispensable for PER repression (SI Appendix, Fig. S2A).

Because the SET domain of TRX appeared to be necessary for PER repression of CLK:BMAL1-mediated transcription,
Fig. 2. CLKe19r and the histone methyltransferase TRX are necessary for CLK:BMAL1-mediated transcriptional activation, PER repression, and CLK–PER interaction. (A, Upper) The monarch per E-box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in Drosophila S2 cells in the presence of a dpBMAL1 expression plasmid and either dpCLK or dpCLKΔ19r (5 ng each), with increasing doses of dpPER or dpCRY2 (amounts are given in nanograms). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean ± SEM of three replicates. For each condition, one-way ANOVA, Tukey post hoc: *P < 0.05, **P < 0.01, and ns is nonsignificant (in black for repression); +P < 0.05, ++P < 0.01, and ns is nonsignificant (in blue for activation). (A, Lower) Western blots of V5-tagged dpCLK, dpCLKΔ19r, and dpCRY2. +, presence; −, absence. (B) dpPerEp-Luc (10 ng) was used in the presence of dpCLK, dpBMAL1 (5 ng each) and dpPER, without or with either Gal4DBD or Gal4DBD-dpCLKe19r (amounts are given in nanograms). Quantification of luciferase activity and statistics were performed as in A. (C) A UAS-driven luciferase reporter (UAS-Luc; 10 ng) was used in the presence or absence of plasmids expressing Gal4DBD-FLAG or dpCLKe19r fused to an N-terminal Gal4DBD-FLAG and a C-terminal VP16 transactivation domain (Gal4DBD-FLAG-dpCLKe19r-VP16; 5 ng each) and increasing doses of dpPER (amount is given in nanograms). Quantification of luciferase activity and statistics were performed as in A. Western blots were performed using the indicated antibodies. (D) dpPerEp-Luc (10 ng) was used in the presence of dpCLK and dpBMAL1 expression plasmids (5 ng each) with increasing doses of dpPER or dpCRY2 (amounts are given in nanograms) and in the presence or absence of dsRNA against egfp or the 5' and 3' untranslated regions (UTRs) of dTrx (7.5 μg each). Quantification of luciferase activity and statistics were performed as in A. Western blots were performed using the indicated antibodies. (E) Co-IPs from S2 cells transfected with FLAG-dpPER and either dpCLKΔ19r-V5- or dpCLK-V5-expressing plasmids (500 ng each) in the presence or absence of dsRNA against dTrx 5' and 3' UTRs (7.5 μg each). Co-IPs were probed with an anti-FLAG antibody, and western blots (WBs) were performed using the indicated antibodies. Red asterisk, dpPER protein. IP, immunoprecipitation.
we examined whether this domain could also be important for CLK–PER interaction. Co-IPs of FLAG-tagged PER coexpressed with V5-tagged CLK alone or in combination with either V5-tagged TRX-CT or TRX-CTΔSET in S2 cells in which endogenous Trx was knocked down revealed that CLK and TRX-CT were pulled down with PER only in the presence

![Diagram](https://www.pnas.org/content/119/15/e2115711119/Fig3.large.jpg)

**Fig. 3.** The catalytic activity of TRX is required for PER repression of CLK:BMAL1 and for CLK–PER interaction. (A) Schematic depicting conservation of the SET domains (purple boxes) and catalytic residues of human MLL1 (N3906) and its Drosophila ortholog TRX (N3665; yellow stars). Black stars indicate protease cleavage sites; colored ovals indicate other functional domains. (B) The monarch per E box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in S2 cells in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each) with increasing doses of dpPER (amounts are given in nanograms), in the absence of dsRNA against endogenous dTrx untranslated regions (UTRs), or in its presence along with the N-terminal (NT) fragment of dTRX and either the wild-type or a catalytically dead C-terminal (CT) fragment of dTRX lacking the SET domain (50 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean ± SEM of three replicates. For each set of conditions: one-way ANOVA, Tukey post hoc: **P < 0.05, ***P < 0.001, and ns is nonsignificant (in black for repression); ++P < 0.01, and ns is nonsignificant (in blue for activation). (C) Co-IPs from S2 cells transfected with FLAG-dpPER, dpCLK-V5 expressing plasmids (150 ng each) without or with dsRNA targeting dTrx 50 and 30 UTRs (7.5 μg each) and plasmids expressing either the wild-type dTRX-CT or catalytically inactive (dead) dTRX-CTΔSET (150 ng each). Co-IPs were probed with an anti-FLAG antibody, and western blots (WBs) were performed using the indicated antibodies. (D) dpPerEp-Luc (10 ng) was used in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each) with increasing doses of dpPER (amounts are given in nanograms) without or with dsRNA targeting endogenous dTrx and plasmids expressing dTRX-NT, the wild-type dTRX-CT, or the mutant dTRX-CTN3665A (50 ng each). Quantification of luciferase activity and statistics were performed as in B. **P < 0.01, and ns is nonsignificant. (E) Co-IPs from S2 cells transfected with FLAG-dpPER, dpCLK-V5 expressing plasmids (150 ng each) without or with dsRNA targeting dTrx 50 and 30 UTRs (7.5 μg each) and plasmids expressing either the wild-type dTRX-CT or catalytically inactive (dead) dTRX-CTΔSET (150 ng each). Co-IPs were probed with an anti-MYC antibody, and western blots (WBs) were performed using the indicated antibodies.

Asterisks: black, dpCLK; red, dpCLKΔ19r; gray, dTRX-CT; purple, dTRX-CTN3665A. IP, immunoprecipitation.
of the SET domain on TRX-CT (Fig. 3C). These results suggested that the catalytic domain and/or activity of TRX was necessary for both CLK–PER interaction and PER repression.

To distinguish between these two possibilities, we generated a catalytically inactive TRX and tested its involvement in CLK:BMAL1-mediated transcription, PER repression, and CLK–PER interaction. Asparagine 396 within the SET domain of human MLLI1 is conserved and located at position 3,665 of Drosophila TRX (Fig. 3D), was previously shown to be required for binding of the methyl donor S-adenosyl methionine but not for the overall structure of MLL1 (18). To generate catalytically inactive TRX, asparagine 3665 of TRX-CT was mutated to alanine to form TRX-CTN3665A. We performed luciferase reporter assays in S2 cells cotransfected with CLK, BMAL1, increasing doses of PER, RNAi against endogenous TRx, and either TRX-NT, TRX-CT, or TRX-CTN3665A. Results showed that, similar to what we found with TRX-CTΔSET, TRX-CTN3665A restored most CLK:BMAL1 transcriptional activity but failed to rescue PER repression, even at high doses of per, as compared with control TRX-CT (Fig. 3D and SI Appendix, Fig. S2 B and C). In addition, co-IPs of FLAG-tagged PER coexpressed with V5-tagged CLK alone or in combination with V5-tagged TRX-CT or V5-tagged TRX-CTN3665A in cells in which endogenous TRx was knocked down showed that the PER–CLK–TRX interaction was maintained with TRX-CT but abolished with the TRX-CTN3665A mutant (Fig. 3E). We verified that the lack of PER–CLK–TRX interaction with the TRX-CTN3665A mutant was not due to TRX-CTN3665A misfolding by showing that both TRX-CT and TRX-CTN3665A were able to interact with the SET domain of a previously described interactor, the absent, small, and homeotic-1 (ASH1) protein (20) (SI Appendix, Fig. S2D).

Moreover, we showed that in the absence of CLK, PER failed to interact with TRX-CT, regardless of whether it is catalytically active or inactive, suggesting that the formation of the complex requires CLK (Fig. 3E).

To test whether TRX-CT interacts with CLK through CLK2e19r as in mammals, we then performed co-IPs of either MYC-tagged CLK or CLK2a19r, each coexpressed with either V5-tagged TRX-CT or V5-tagged TRX-CTN3665A. We found that the interaction between CLK and TRX-CT was abolished in the absence of CLK2e19r or TRX catalytic activity (Fig. 3F), demonstrating that TRX interacts with CLK through CLK2e19r and that the catalytic activity of TRX is required for the CLK–TRX interaction. Altogether, these results demonstrate that TRX promotes CLK–PER interaction and PER repression via its methyltransferase catalytic function rather than as a scaffold protein.

TRX-Dependent Arginine Methylation of HSP68 Is Necessary for PER Repression and for CLK–PER Interaction. Given the established catalytic role of TRX in monomethylating lysine 4 of histone 3 (21), we hypothesized that TRX may function in the methylation of yet unknown proteins on lysines that would be required for the CLK–TRX–PER interaction. To test this possibility, we repeated co-IPs from S2 cells cotransfected with MYC-tagged CLK and FLAG-tagged PER in the presence or absence of endogenous TRx with coexpressed V5-tagged TRX-CT or TRX-CTN3665A and immunoblotted for lysine-methylated proteins using a pan anti-methylated lysine antibody. We predicted that lysine-methylated proteins would be present in conditions where CLK–PER interacts in the presence of endogenous TRX or exogenous TRX-CT but not when CLK–PER interaction is abolished in the absence of endogenous TRX or exogenous catalytically inactive TRX-CTN3665A. Unexpectedly, we found that lysine-methylated proteins were present in all conditions (SI Appendix, Fig. S3A), suggesting that the catalytic activity of TRX serves another function. Because protein methylation can occur on lysine and arginine residues, we tested whether proteins were methylated on arginine by probing the pull down with a pan anti-methylated arginine antibody. To our surprise and consistent with our original prediction, we found that arginine-methylated proteins pulled down only in the presence of endogenous TRX or exogenous TRX-CT (Fig. 4A). These results suggest that the histone methyltransferase TRX may function either as a protein arginine methyltransferase (PRMT) or in the recruitment of such an enzyme to methylate a protein necessary for the formation of the CLK–TRX–PER complex.

To identify candidate proteins methylated on arginine in the presence of TRX, we performed co-IPs in S2 cells cotransfected with MYC-tagged CLK and FLAG-tagged PER in the presence or absence of endogenous TRx (SI Appendix, Fig. S3B), followed by mass spectrometry. We identified 1,158 and 888 protein-unique peptides in the presence and absence of TRX, respectively, with 792 present in both conditions (Fig. 4B and Dataset S1). Among the 792 common to both conditions, 29 were enriched by fourfold or greater in the presence of TRX, of which 3 were methylated on arginine (Fig. 4B and Dataset S1). Of the 366 uniquely identified peptides in the presence of TRX, 7 of 97 protein-unique peptides with more than four spectral counts were also methylated on arginine (Fig. 4B and Dataset S1). We focused on the chaperone protein HSP68 (HSP of 68 kDa), which is methylated on a conserved arginine residue due to position 45. Interestingly, methylation of another conserved arginine residue on its mammalian homolog HSP70 has recently been reported to regulate HSP70 function in transcription on chromatin (22).

We first assessed HSP68 function in CLK:BMAL1-mediated transcription and PER repression using luciferase reporter assays. Similar to what we found with Trx knockdown, CLK:BMAL1-mediated activation was significantly reduced and PER repression was abolished when endogenous Hsp68 was knocked down (Fig. 4C and SI Appendix, Fig. S3C). Coexpressing exogenous wild-type HSP68 rescued both CLK:BMAL1 activation and PER repression. In contrast, coexpression of HSP68 bearing an alanine point mutant on R45 (HSP68R45A) rescued activation but not PER repression (Fig. 4C), demonstrating that methylation of HSP68 on R45 is necessary for repression of CLK:BMAL1 by PER but not for transcriptional activation. Using co-IPs, we also showed that, similar to what we found for TRX catalytic activity, methylation of HSP68 on R45 was necessary for CLK–PER interaction, as wild-type HSP68 but not HSP68R45A rescued the otherwise disrupted interaction when endogenous Hsp68 was knocked down (Fig. 4D). To unambiguously demonstrate that TRX-dependent methylation of HSP68 on R45 is required for CLK–PER interaction, we performed similar co-IPs but in cells where both endogenous Trx and Hsp68 were knocked down and cotransfected with V5-tagged TRX-CT and either FLAG-tagged wild-type HSP68 or the HSP68R45A mutant. We found that the CLK–HSP68–TRX–PER complex was reconstituted only in the presence of wild-type HSP68 (Fig. 4E).

We also tested the impact of methylation of HSP68 on R45 on the CLK–HSP68 interaction as well as the presence/absence of HSP68 for CLK–TRX binding in the absence of PER. We found that while HSP68 methylation on R45 was necessary for CLK–HSP68 interaction, HSP68 was dispensable for CLK–TRX binding (Fig. 4F and G), suggesting that TRX binds to CLK independently of HSP68. Both CLK–TRX and CLK–HSP68 interactions require, however, the presence of CLK2e19r (Fig. 4F and G). Taken together, these results are consistent with the idea that TRX binding to CLK occurs through CLK2e19r and that HSP68 binds to TRX. Furthermore, our findings that HSP68R45A is able to rescue to a great extent activation by CLK:BMAL1 in luciferase reporter assays (Fig. 4C) despite the lack of CLK–HSP68R45A interaction suggest that HSP68 likely plays two roles: one in
**Fig. 4.** TRX-dependent arginine methylation of HSP68 mediates circadian repression by PERIOD in the monarch butterfly

(A) Co-IPs from S2 cells transfected with MYC-tagged dpCLK and FLAG-dpPER expressing plasmids (500 ng each) without or with dsRNA targeting dTrx 5' and 3' untranslated regions (UTRs) (15 μg each) and plasmids expressing either the wild-type or N3665A mutant dTRX-CT (500 ng each). Co-IPs were probed with an anti-MYC antibody, and western blots (WBs) were performed using the indicated antibodies, including an antimethylated arginine antibody. (B) Venn diagram of proteins identified using mass spectrometry that pulled down with MYC-dpCLK coexpressed with FLAG-dpPER in S2 cells in the presence or absence of endogenous dTRX. Among peptides pulled down in the presence (+) of TRX with spectral counts greater than four in each category (unique to +TRX or present in both conditions but enriched in +TRX), the ones methylated on arginine are listed. (C) The monarch per E box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each), increasing doses of dpPER (amounts are given in nanograms) without or with dsRNA targeting endogenous dHsp68 5' and 3' UTRs (7.5 μg each), and plasmids expressing either wild-type or R45A mutant dHSP68 (50 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean ± SEM of three replicates. For each panel, one-way ANOVA, Tukey post hoc: *P < 0.05, **P < 0.01, and ns is nonsignificant (in black for repression); +P < 0.05 and ++P < 0.01 (in blue for activation). (D) Co-IPs from S2 cells transfected with MYC-dpCLK and FLAG-dpPER expressing plasmids (500 ng each) without or with dsRNA targeting dHsp68 5' and 3' UTRs (15 μg each) and with either wild-type or mutant R45A FLAG-tagged dHSP68 (500 ng each). Co-IPs were probed with anti-MYC antibody, and WBs were performed using the indicated antibodies. (E) Co-IPs from S2 cells transfected with dsRNA against the 5' and 3' UTRs of both dTrx and dHsp68 (15 μg each); plasmids expressing MYC-dpCLK, FLAG-dpPER, or dTRX-CT-V5 (500 ng each); and either wild-type or mutant R45A FLAG-tagged dHSP68 (400 ng each). Co-IPs were probed with anti-MYC antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dHSP68; red, dHSP68R45A. (G) Co-IPs from S2 cells transfected with FLAG-dpPER (500 ng), dsRNA against dHsp68 5' and 3' UTRs (15 μg each), and either wild-type or mutant R45A V5-tagged dHSP68 (500 ng each). Co-IPs were probed with anti-FLAG antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dHSP68; red, dHSP68R45A. IP, immunoprecipitation.
The repressive function of methylated HSP68 on R45 is conserved in light-driven monarch DpN1 cell rhythms. (A) Bioluminescence recordings from dpPER::LUC DpN1 cells in which repression by the major repressor CRY2 was attenuated via RNAi knockdown of endogenous Bmal1 (Bmal1i), overexpression of a Bmal1ΔCter mutant lacking an α-helix involved in CRY2 repression (Bmal1Δ), and knockdown of endogenous CRY2 (Cry2i; hereafter called sensitized cells) in the presence of either dsRNA against Trx (Trxi) or increasing doses of dsRNA against Hsp68s orthologs (Hsp68s 1Xi and 2Xi). RNAi knockdown of eGFP (eGFPi) was used as a control. Values are represented by mean ± SEM of three replicates. (B) Quantification of dpPER::LUC bioluminescence from A at troughs and peaks of days 2 and 3 posttransfection. One-way ANOVA, Tukey post hoc: **P < 0.01. (C) Bioluminescence recordings from sensitized dpPER::LUC DpN1 cells in the presence of increasing doses of dsRNA against Hsp68s orthologs without or with overexpression of wild-type or R45A mutant dHSP68. (D) Quantification of dpPER::LUC bioluminescence from C at troughs and peaks of days 2 and 3 posttransfection. One-way ANOVA, Tukey post hoc: **P < 0.01, and ns is nonsignificant.

Methylated HSP68 on R45 Functions in Repression in Light-Driven Monarch DpN1 Cell Rhythms. To confirm the repressive function of HSP68 in the monarch system, where circadian repression of CLK:BMAL1-dependent transactivation is mediated by PER and CRY2, we constructed a stable dpPER::LUCIFERASE (dpPER::LUC) reporter in the monarch DpN1 cell line by knocking in luciferase in frame with the 3′ end of endogenous per using CRISPR-Cas9–mediated homology-directed repair (Fig. 5). The resulting cell line exhibited PER::LUC rhythms in phase with those of endogenous PER (13) (Fig. 5A) in light-dark cycles, demonstrating proper functionality of the fusion dpPER::LUC protein. Of note, this cell line does not cycle under constant dark conditions (DD), and thus, circadian rhythms could not be observed in DD.

Because CRY2 acts as the major circadian repressor, we reasoned that weakening repression by CRY2 may be necessary to reveal the effects of HSP68 levels on PER repression, as these could be otherwise masked in the presence of a potent repression by CRY2. We thus sought to decrease CRY2 repression without completely abrogating the dpPER::LUC rhythms to uncover effects of Hsp68 knockdown on PER repression. To this end, we knocked down endogenous Bmal1 and overexpressed a Bmal1 variant lacking the C-terminal α-helix necessary for CRY2 repressive action [Bmal1ΔCter (15)] without or
with additional Cry2 knockdown. As expected, knocking down Bmal1 significantly decreased dpPER::LUC activation levels, and overexpression of Bmal1ΔCter increased activation, which was further elevated by knockdown of Cry2, albeit to a significantly lesser extent than in control cells (Fig. 5 A and B). In these sensitized cells, we confirmed the role of TRX in clock function by showing that additional knockdown of monarch Trx led to a significant decrease in activation levels at both trough and peak of the rhythm (Fig. 5 A and B). Consistent with our hypothesis that HSP68 is involved in repression, we also demonstrated that increasing doses of dsRNA against the three endogenous Hsp68 orthologs led to a dose-dependent increase in activation levels (Fig. 5 A and B and SI Appendix, Fig. S4). Importantly, overexpression of Drosophila HSP68 in sensitized DpN1 cells with the lowest dose of dsRNA against endogenous Hsp68 restored low activation levels, while overexpression of Hsp68R45A has no effect (Fig. 5 C and D), demonstrating that the critical role of methylation of R45 on HSP68 for PER repression initially observed in S2 cells is conserved in monarch DpN1 cells.

Discussion

Circadian repression, which relies in most animals on the interaction of PER and CRY complexes with circadian activators, is central to the generation of 24-h rhythms. While the mechanisms by which CRY exerts its transcriptional repressive function are increasingly understood, the modalities of PER repressive action are still unclear. To date, the most compelling mechanistic evidence comes from studies in Drosophila and in mammalian cells, which demonstrated that PER represses by displacing CLK:BMAL1 from DNA (23–26). In Drosophila, PER represses in two phases, first by binding the activator complex on DNA and then by recruiting TRX and HSP90, in a manner that requires the catalytically active TRX (23). In mammals, replication also occurs in two consecutive phases, with a displacement-type repression mediated by PER–CRY followed by a blocking-type repression independent of PER (24–26). How PER initiates repression has remained an open question. Our study in the monarch butterfly uncovers the histone methyltransferase TRX and HSP68 as key molecules required for PER repression and shows that TRX is not necessary for CLK:BMAL1-mediated transcriptional repression but is also crucial for PER repression via a previously unknown TRX-dependent arginine methylation of HSP68. Based on our data, we propose a model where the catalytically active TRX binds to CLK via the CLK19r domain to promote CLK:BMAL1-mediated transcription. TRX then enzymatically contributes to the methylation of HSP68 on arginine 45 either directly or indirectly by promoting the activity of a yet unknown PRMT that methylates HSP68. Binding of methylated HSP68 on R45 to the CLK–TRX complex ultimately permits the recruitment of PER to the activator complex to initiate repression (Fig. 6).

Although our model is not based on interactions of chromatin-bound proteins, our functional luciferase transcriptional assays support the idea that it likely reflects events that occur on DNA. Consistent with the finding that mammalian MLL1 binds to CLOCK19 on DNA (10), we showed that TRX binds to CLK through CLK19r and contributes to transcriptional activation, suggesting that the function of TRX/MLL1 is conserved within animal clocks. However, in our study, the catalytic function of TRX seems to primarily affect CLK:BMAL1 repression by PER rather than transcriptional activation as previously shown in mammals (10), as both ΔSET and N3665A TRX catalytically inactive mutants restore activation to wild-type levels in luciferase assays. Although our data unambiguously show that TRX catalytic function is necessary for the methylation of HSP68 on R45, whether TRX acts directly or indirectly remains to be determined. The ability of TRX to directly methylate arginines remains a formal possibility, but it seems rather unlikely as no evidence exists, to our knowledge, to that effect. Instead, TRX may be involved in the recruitment of a PRMT. The fact that Drosophila Ash2L, a component of the multiprotein complex that assembles to TRX/MLL1 for its catalytic function (17, 18), is itself methylated at a single arginine residue by PRMT1 (27) supports this idea.

Regardless of the nature of the protein responsible for methylating HSP68, our finding that HSP68 methylation on R45 is required for transcriptional repression by PER raises the intriguing possibility that arginine methylation of HSPs could play an important role in the regulation of gene transcription. The impact of HSP methylation in the regulation of gene transcription is not without precedent, as methylation of another highly conserved arginine residue at position 469 of HSP70, the mammalian homolog of HSP68, has been shown to modulate the recruitment of a key component of the transcription complex and thus, transcription initiation (22). Our findings that methylation of HSP68 R45, which is a conserved residue across species, is necessary for binding to a transcriptional repressor favor the idea that HSP methylation may have a broader role in the regulation of gene transcription than previously assumed. In the context of time-keeping mechanisms by the circadian clock, the discovery that HSP68 plays a critical role in PER repression within the monarch clockwork extends the possible key functions of HSPs in circadian clocks. In plants, the adenosine triphosphate (ATP)–dependent molecular chaperone HSP90, in complex with HSP70 and HSP70/HSP90 organizing protein, functions within the core oscillator by facilitating the folding and maturation of one of its target proteins, the F-box-type E3 ubiquitin ligase ZEITLUPE (28). Similarly, in mammals, HSP90 stabilizes BMAL1 through its ATP-dependent chaperone activity (29, 30). Given that proteins comprising the negative arm of the circadian clock network, including PERs from humans, mice, and insects, display a significant amount of predicted sequence disorder (31, 32), it is tempting to speculate that HSPs could also function to ensure the proper folding of PER for its binding to CLK to function as a circadian repressor. Whether this is the case and which role, if any, methylation of R45 on HSP68 plays in this process warrant further studies. Determining whether HSP68’s function in PER repression is conserved in other species will also be important.

Finally, in mammals, CLK19 has previously been shown to be required for cooperative binding of CLOCK:BMAL1 heterodimers to tandem E boxes (33), subsequent recruitment of MLL1 (10), and the binding of the repressor CLOCK-interacting protein circadian (CIPC) that occurs independently of PER (9, 34, 35). Our finding that CLK19 is required for indirect PER binding to...
CLK and PER-mediated circadian repression adds further support to the emerging notion that the CLK/Per domain may act as a hub for the assembly of larger macromolecular complexes on DNA for both circadian activation and repression, as previously suggested (36). The parallel between the convergence on CLK/Per for PER and CIPC repression and the convergence on the BMAL1 TAD for CRY and CHRONO repression (1, 37–39) may suggest the existence of a greater number of repressive complexes acting coordinately during the repressive phase of the circadian cycle than previously envisioned.

Materials and Methods
Detailed material and methods are provided in SI Appendix, Materials and Methods.

Monarch BUTTERFLY CLKp19p Mutant Line Generation and Husbandry. Monarch CLKp19p mutants were generated using CRISPR-Cas9-targeted mutagenesis and maintained as described in SI Appendix.

Eclosion Behavior. Eclosion behavior assays were performed as described in ref. 15.

S2 Cell Culture, Transfections, Transcriptional Assays, and Western Blotting. Conditions of culture and transfections of Drosophila S2 cells, protein extractions, procedures for luciferase assays, and western blotting, including antibodies and dilutions used, are described in SI Appendix.

Real-Time qRT-PCR. Quantification of circadian expression of per and tim in the monarch brain was performed as previously described (40). Procedures for quantification of dTrx and dHsp68 levels in RNAi experiments in S2 cells, including RNA extraction and primers used, are described in SI Appendix.

Co-IPs and Mass Spectrometry. Conditions for protein extractions, antibodies/dilutions, and procedures used for co-IPs and mass spectrometry are described in SI Appendix.

Monarch DpN1 PER::LUC Cells. Procedures to insert a luciferase reporter in frame with per in monarch DpN1 cells using CRISPR/Cas9-assisted homology-directed repair and establish a stable cell line are described in SI Appendix.

Data Availability. All data are included in the manuscript and/or supporting information.

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