Vertebrate-like CRYPTOCHROME 2 from monarch regulates circadian transcription via independent repression of CLOCK and BMAL1 activity

Ying Zhang[a,b], Matthew J. Markert[a,b], Shayna C. Groves[a,b], Paul E. Hardin[a,b], and Christine Merlin[a,b,1]

[a]Department of Biology, Texas A&M University, College Station, TX 77843; and [b]Center for Biological Clocks Research, Texas A&M University, College Station, TX 77843

Edited by Joseph S. Takahashi, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, and approved July 28, 2017 (received for review February 13, 2017)

Circadian repression of CLOCK-BMAL1 by PERIOD and CRYPTOCHROME (CRY) in mammals lies at the core of the circadian timekeeping mechanism. CRY repression of CLOCK-BMAL1 and regulation of circadian period are proposed to rely primarily on competition for binding with coactivators on an α-helix located within the transactivation domain (TAD) of the BMAL1 C terminus. This model has, however, not been tested in vivo. Here, we applied CRISPR/Cas9-mediated mutagenesis in the monarch butterfly (*Danaus plexippus*), which possesses a vertebrate-like CRY (dpCRY2) and an ortholog of BMAL1, to show that insect CRY2 regulates circadian repression through TAD α-helix–dependent and –independent mechanisms. Monarch mutants lacking the BMAL1 C terminus including the TAD exhibited arrhythmic eclosion behavior. In contrast, mutants lacking the TAD α-helix but retaining the most distal C-terminal residues exhibited robust rhythms during the first day of constant darkness (DD1), albeit with a delayed peak of eclosion. Phase delay in this mutant on DD1 was exacerbated in the presence of a single functional allele of *dpCry2*, and rhythmicity was abolished in the absence of dpCRY2. Reporter assays in *Drosophila* S2 cells further revealed that dpCRY2 represses through two distinct mechanisms: a TAD-dependent mechanism that involves the dpBMAL1 TAD α-helix and dpCLK W328 and a TAD-independent mechanism involving dpCLK E333. Together, our results provide evidence for independent mechanisms of vertebrate-like CRY circadian regulation on the BMAL1 C terminus and the CLK PAS-B domain and demonstrate the importance of a BMAL1 TAD-independent mechanism for generating circadian rhythms in vivo.

circadian clock | CRYPTOCHROME 2 | BMAL1 C terminus | CLOCK | CRISPR

Circadian timing enables organisms to coordinate their physiology and behavior with the daily cycle by anticipating fluctuating environmental changes (1, 2). At the core of the timekeeping mechanism in animals is a cell-autonomous molecular transcriptional/translational feedback loop that controls the rhythmic expression of clock-controlled genes with a period close to 24 h. In mammals, the heterodimeric basic helix–loop–helix (bHLH) transcription factor CLOCK:BMAL1 initiates feedback loop function by activating transcription of the *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) genes (3–5). Accumulating levels of PER-CRY then form complexes that translocate to the nucleus and interact with CLOCK:BMAL1 to repress the transcription of their own genes. Once the repressors are degraded, CLOCK:BMAL1 activity is restored to initiate a new cycle of transcription. Despite the central importance of circadian repression for generating 24-h rhythms, the molecular mechanisms underlying PERs' and CRYs' repressive function are not fully understood.

PERs are essential for the nuclear translocation of PER-CRY complexes (6) and for rhythmic PER-CRY-CLK-BMAL1 interactions (6, 7). CRYs, on the other end, are essential for CLK:BMAL1 transcriptional repression (5, 8, 9). The potent inhibitory effect of CRYs on CLOCK:BMAL1-mediated transcription has been observed in vitro in the absence of PERs (10), suggesting that CRYs can interact directly with CLOCK:BMAL1. Residues that are important for the CRY1-CLOCK-BMAL1 interactions have been identified in both the PAS-B domain of CLOCK and the BMAL1 C terminus (11). The crystal structure of a complex containing the mouse CLOCK:BMAL1 bHLH-PAS domains has revealed that five of these residues localized on the CLOCK PAS-B HI loop form a finger accessible for CRY1 binding (12). However, the CRY1-CLOCK interaction is thought to provide stability only to the ternary complex, while CRY1-BMAL1 C terminus interaction is proposed to mediate repression (13–15). Based on in vitro cell culture and biophysical experiments, CRY1 has recently been found to dock on CLOCK PAS-B and to regulate circadian cycling by competing for binding with coactivators, such as p300, on an α-helix located in the transactivation domain (TAD) of the BMAL1 C terminus (13–17). Despite these important advances, it is unknown if CRY1-BMAL1 C terminus interaction constitutes the main mechanism driving circadian rhythms in vivo. The genetic dissection of this mechanism has been hampered by the lack of a BMAL1 C-terminal mutant mouse that retains transcriptional activity and by the functional redundancy of the two mouse CRY paralogues.

The monarch butterfly, *Danaus plexippus*, is uniquely suited to determine the importance of the CRY-BMAL1 C terminus for generating circadian rhythms in vivo. Not only does the monarch butterfly possess a single copy of a mammalian-like repressive CRY (designated "CRY2") (18–20) and an ortholog of mammalian

Significance

Daily rhythms in animal behavior, physiology, and metabolism are driven by cell-autonomous mechanisms that keep time and control overt rhythms via transcriptional feedback loops, making it fundamental to define the mechanisms driving rhythmic transcription. In mammals, PERIOD and CRYPTOCHROME (CRY) rhythmically repress CLOCK:BMAL1 transcriptional activity, but the mechanisms by which CRY represses CLOCK:BMAL1 activity are not fully understood. Using CRISPR/Cas9 for in vivo genetic manipulations in the monarch, we show that repression of circadian transcription by vertebrate-like CRY is mediated primarily by a BMAL1 transactivation domain (TAD)-independent mechanism involving the CLK-PAS B domain, while repression on the BMAL1 TAD is dispensable for the generation of rhythms but alters circadian phase during the first day of constant darkness by affecting activation levels.

Author contributions: Y.Z., M.J.M., and C.M. designed research; Y.Z., M.J.M., and S.C.G. performed research; P.E.H. contributed new reagents/analytic tools; Y.Z., M.J.M., P.E.H., and C.M. analyzed data; and Y.Z. and C.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: cmerlin@bio.tamu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702014114/-/DCSupplemental.

BMAL1 with a highly conserved TAD in the last 30 amino acids of its C terminus, but its genome can also be targeted with CRISPR/Cas9 (21). Here, we generated domain-specific mutations in the monarch (dp)BMAL1 in vivo using CRISPR/Cas9. We found that while the dpBMAL1 C terminus lacking in its *Drosophila* ortholog (dCYCLE; dCYC) is required for transcriptional activity, the TAD α-helix located on this domain plays a role in regulating circadian phase or period but is not required for the generation of circadian rhythms. Using cell-based reporter assays in *Drosophila* Schneider 2 (S2) cells, we showed that dpCRY2 represses through two distinct mechanisms: a TAD-dependent mechanism that involves the dpBMAL1 TAD α-helix and dpCLK W328 located in the dpCLK PAS-B domain and a TAD-independent mechanism involving dpCLK E333. These findings provide insights into the mechanisms of repression by vertebrate-like dpCRY2 and demonstrate that a BMAL1 TAD-independent mechanism plays a major role in the generation of circadian rhythms.

Results

Monarch dpCLK:dpBMAL1 Transcriptional Activity Requires the dpBMAL1 C-Terminal Domain Lacking in *Drosophila* CYCLE. To begin to genetically define the mechanisms by which circadian activation is mediated in the monarch molecular clockwork, we first sought to determine if transactivation function was mediated by monarch dpCLK or the C terminus of dpBMAL1 in vivo. To this end, we generated a monarch dpBMAL1-deletion mutant lacking the C-terminal domain, which is lacking in its *Drosophila* ortholog CYCLE (dCYC) (Fig. 1*A*). Using CRISPR/Cas9, we introduced frameshift mutations at a location of the coding region that would result in the production of *Drosophila*-like dpCYC (Fig. 1*B*). From 150 embryos injected, 6 of 22 surviving larvae were mosaic at the targeted site based on a Cas9-based in vitro cleavage assay (Fig. 1*B* and Fig. S1 *A* and *B*). We selected an adult male butterfly bearing somatic mutations at about 50% for backcrosses (Fig. 1*B* and Fig. S1*C*), and progeny were screened for germline transmission of the targeted lesions. Six of ten genotyped larvae carried a mutated allele, which was a single 13-bp deletion in all germline transformants (Fig. 1*B* and Fig. S1*C*). We found sex-based segregation for the mutation in the corresponding adults, with females being hemizygous and males heterozygous, demonstrating that *dpBmal1* is located on the Z sex chromosome along with monarch *Clk* (21) [in lepidopterans, females are heterogametic (ZW), and males are homogametic (ZZ)]. Importantly, the 13-bp deletion germline mutation resulted in a frameshift leading to the truncation of the *dpBmal1* C terminus and was designated "*dpCyc-like*."

To test whether the dpCLK:dpCYC-like heterodimer retained transcriptional activity, we assessed the effect of the dpCYC-like truncation on circadian behavior and the molecular clockwork. We first examined the timing of pupal eclosion (i.e., the emergence of the adult from its pupal case), a robust and quantifiable behavior under the control of the brain circadian clock (18, 22, 23), focusing our analysis on female *dpCyc-like* knockouts, heterozygous males, and wild-type siblings of both sexes (*Materials and Methods*). We found that the circadian timing of adult eclosion was abolished in *dpCyc-like* hemizygous butterflies, while heterozygous and wild-type siblings eclosed rhythmically with a similar peak of eclosion during the early subjective day ($P > 0.05$, one-way ANOVA followed by Tukey's post hoc test) (Fig. 1*C* and Fig. S2*A*). To assess if arrhythmicity in *dpCyc-like* mutants resulted from a defect in the transcriptional activity of dpCLK:dpCYC-like, we examined the expression levels of two well-characterized CLK:BMAL1/CYC direct target genes that are core clock components, *period* (*per*) and *timeless* (*tim*), in the brain of *dpCyc-like* hemizygous and wild-type siblings by qPCR (Fig. 1*D*). Consistent with our behavioral data, *per* and *tim* circadian rhythms were abolished in brains of dpCYC-like hemizygous mutants, and their expression levels were constitutively low (Fig. 1*D*). Together, these data demonstrate that the BMAL1 C-terminal domain containing the G and TAD regions described in mammals (14) is required for transcriptional activation in the monarch.

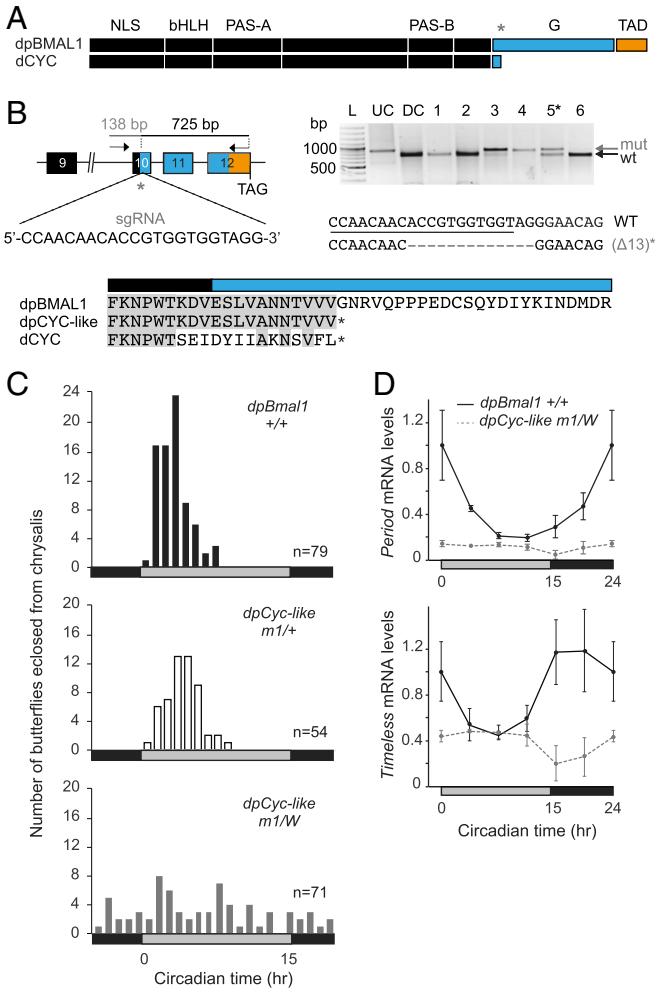

Fig. 1. Monarch dpCLK:dpBMAL1 transcriptional activity requires the dpBMAL1 C-terminal domain lacking in *Drosophila* CYC. (*A*) Schematic representation of monarch dpBMAL1 and its C-terminal domain (G and TAD) conserved with mammalian BMAL1, lacking in its *Drosophila* ortholog dCYC. The gray star indicates the position of the single-guide RNA (sgRNA) used to introduce indels. (*B*, *Upper Left*) *DpBmal1* genomic locus with the sgRNA and the primers used to amplify the 863-bp targeted region for analysis of mutagenic lesions. (*Upper Right*) Detection of mutagenic lesions (mut) in somatic cells of a subset of potential founder G0 butterflies using a Cas9-based in vitro cleavage assay. DC, digested control; L, ladder; UC, undigested control. The black star indicates the somatic mutant selected for backcrossing to generate a monarch dpCYC-like mutant lacking the BMAL1 G and TAD regions. DpCYC-like mutants carry a 13-bp deletion. (*Lower*) Partial alignment of dpBMAL1, dpCYC-like mutant, and dCYC proteins showing the position of the truncation in dpCYC-like relative to the C terminus of dCYC. (*C*) Profiles of adult eclosion in DD of wild-type (black bars), heterozygous (white bars), and hemizygous mutant (gray bars) siblings of the *dpCyc-like* mutant line (designated "m1") entrained to 15 h light/9 h dark (LD 15:9) throughout the larval and pupal stages. Data from DD1 and DD2 are pooled and binned in 1-h intervals. The horizontal bars at the bottom of the graphs show subjective day (gray) and night (black). $P < 0.0001$ (one-way ANOVA); $dpBmal1^{+/+}$ vs. $dpCyc\text{-}like^{m1/+}$, $P > 0.05$; $dpBmal1^{+/+}$ vs. $dpCyc\text{-}like^{m1/W}$, $P < 0.01$; $dpCyc\text{-}like^{m1/+}$ vs. $dpCyc\text{-}like^{m1/W}$, $P < 0.01$ (Tukey's post hoc test). (*D*) Circadian expression of *period* and *timeless* in brains of wild-type (solid black lines) and hemizygous mutant (dashed gray lines) siblings of the *dpCyc-like* mutant line. Values are mean ± SEM of three animals. The horizontal bars at the bottom of the graphs show subjective day (gray) and night (black). Interaction genotype × time: *per*, $P < 0.01$; *tim*, $P < 0.05$ (two-way ANOVA).

The dpBMAL1 TAD Helix Is Dispensable for Generating Behavioral Rhythms. Previous in vitro studies have shown that an α-helix within the last 30 amino acids of the mouse (m)BMAL1 C terminus plays an important role in circadian cycling by acting as the site for coactivator binding and repression by mCRY1 (13, 14). Whether this mechanism is crucial in vivo for generating circadian rhythms has never been tested.

We therefore sought to generate a dpBMAL1 mutant butterfly carrying a partially truncated C-terminal TAD lacking the α-helix using CRISPR/Cas9, by targeting the region upstream of the TAD with a single guide RNA (Fig. 2*A*). From the 25 larvae surviving the injection (of 188 injected embryos), eight exhibited somatic mosaicism at the targeted site, and we selected the most highly targeted of the ones surviving to adulthood for backcrosses (Fig. S1 *A* and *D*). Half of their progeny were either heterozygous or hemizygous for a targeted lesion, and sequencing of the mutated alleles identified two microdeletions of 6 bp or 7 bp, respectively, which were reminiscent of the only two somatic mutations also identified in the founder (Fig. S1 *D* and *E*). The 7-bp deletion introduced a frameshift which, by chance, resulted in the coding of three of the last six residues (i.e., LxWPxx) found within the most distal dpBMAL1 C terminus and introduced a stop codon immediately thereafter (Fig. 2*B* and Fig. S1*F*). This mutation thus eliminated the TAD α-helix and adjacent sequences but retained some of the most distal C-terminal residues that are conserved in mBMAL1.

To explore the relative contribution of the TAD α-helix and the most distal C-terminal region to the generation of circadian rhythms in vivo, we therefore established a mutant monarch line carrying this 7-bp deletion, hereafter named "*dpBmal1ΔCter*." To our surprise, we found that *dpBmal1ΔCter* hemizygous mutants exhibited a robust circadian rhythm of eclosion (Fig. 2*C* and Fig. S2*B*), demonstrating that the TAD α-helix is dispensable for behavioral rhythms in vivo. However, we found that the distribution in eclosion time was significantly affected in a dose-dependent manner, with a mean of eclosion time for hemizygous mutant butterflies occurring ∼1.5 h later than for wild-type siblings ($P < 0.05$, one-way ANOVA followed by Tukey's post hoc test) (Fig. 2 *C* and *D*). This result demonstrates that lack of the TAD α-helix causes a delay in eclosion behavior on the first day of constant darkness (DD), consistent with its previously reported role in mCRY1 and p300 binding and in regulating circadian rhythms in vitro (14). Although these data show that the mutation alters the phase of the rhythm, it is equally likely that this phase difference results from an altered circadian period.

To determine the effect of the *dpBmal1ΔCter* deletion on the brain molecular clockwork, we quantified *per* and *tim* expression in both wild-type and hemizygous mutants (Fig. 2*E*). As expected based on our behavioral data, both clock genes were cycling in DD in *dpBmal1ΔCter* hemizygous mutants, albeit with a decreased amplitude compared with the rhythms observed in wild-type butterflies. Notably, peak levels of *tim* were significantly reduced, while trough levels were not significantly altered in *dpBmal1ΔCter* hemizygous mutants [$P < 0.05$ at circadian time 0 (CT0) and CT20; Student's *t* test], and a similar trend was observed for *per*. This decrease in activation levels is consistent with the delayed eclosion observed in heterozygous and hemizygous *dpBmal1ΔCter* mutants and with a role of the TAD α-helix in coactivator binding (14). Together, these data demonstrate that the monarch BMAL1 C-terminal TAD α-helix and downstream adjacent sequences are dispensable for circadian rhythms in vivo but contribute to the amplitude of the molecular rhythms.

Repression by dpCRY2 on the dpBMAL1 TAD Helix Regulates Circadian Phase. To determine if circadian repression in our *dpBmal1ΔCter* monarch mutant was mediated by dpCRY2, we generated homozygous *dpBmal1ΔCter* monarch mutants carrying two, one, or no functional *dpCry2* alleles through two subsequent interbreeding

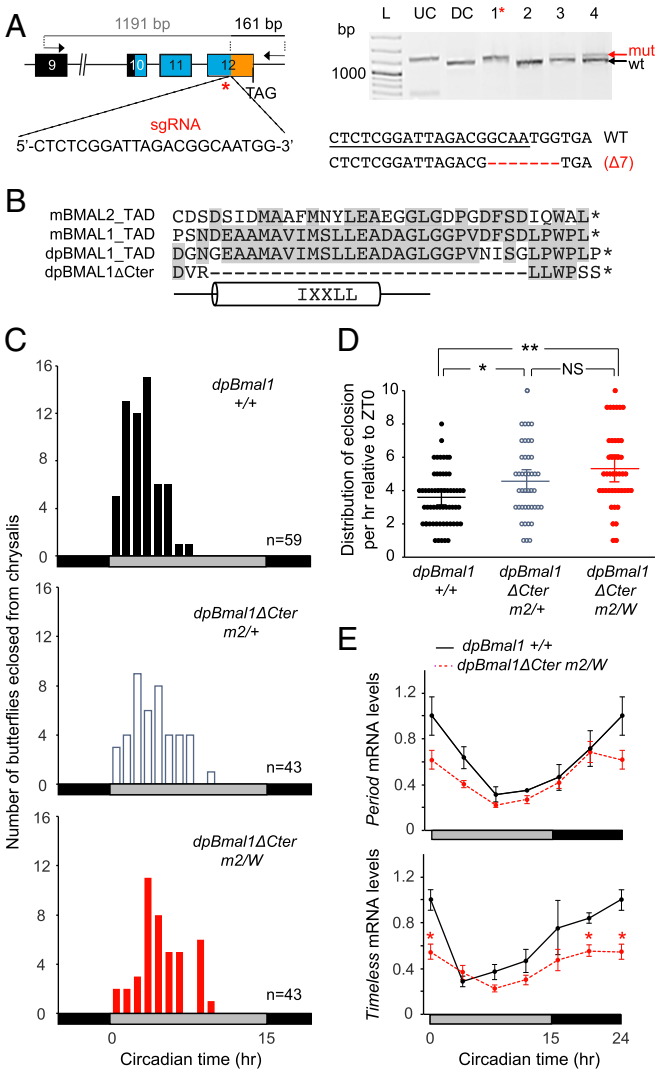

Fig. 2. The dpBMAL1 TAD helix is dispensable for the generation of circadian rhythm in vivo but regulates the phase by maintaining high activation levels. (*A*, *Left*) *DpBmal1* genomic locus showing positions of the sgRNA used to generate a TAD truncation mutant (dpBMAL1ΔCter) and primers used to amplify the 1,352-bp targeted region for analysis of mutagenic lesions. (*Right*) Detection of mutagenic lesions (mut) in somatic cells of a subset of potential founder G0 butterflies using a Cas9-based in vitro cleavage assay. DC, digested control; L, ladder; UC, undigested control. The red star indicates the somatic mutant selected for backcrossing to generate a dpBMAL1ΔCter mutant lacking most of the TAD but retaining three of the most distal amino acids. DpBMAL1ΔCter mutants carry a 7-bp deletion. (*B*) Sequence alignment showing TAD regions of BMAL1 and BMAL2 proteins from mouse (m), dpBMAL1, and the dpBMAL1ΔCter mutant. The TAD helix region is shown below the alignment. (*C*) Profiles of adult eclosion in DD of wild-type (black bars), heterozygous (white bars), and hemizygous mutant (red bars) siblings of the *dpBmal1ΔCter* mutant line (designated "m2") entrained to LD 15:9 throughout the larval and pupal stages. Data from DD1 and DD2 are pooled and binned in 1-h intervals. Horizontal bars at the bottom of the graphs indicate subjective day (gray) and night (black). (*D*) Distribution of eclosion during the subjective day for each genotype. Dots indicate the number of butterflies eclosed at each time interval relative to subjective lights on. $P < 0.0001$ (one-way ANOVA); $^*P < 0.05$, $^{**}P < 0.01$, NS, nonsignificant (Tukey's post hoc test). (*E*) Circadian expression of *period* and *timeless* in brains of wild-type (solid black lines) and hemizygous mutant (dashed red lines) siblings of the *dpBmal1ΔCter* mutant line. Values are the mean ± SEM of three animals. Horizontal bars below the graphs show subjective day (gray) and night (black). *per*, $P > 0.05$; *tim*, $P < 0.02$ (two-way ANOVA, interaction genotype × time); $^*P < 0.05$ (Student's *t* test between each genotype at each time point).

crosses (*Materials and Methods* and Fig. 3). At the behavioral level, we found that *dpBmal1ΔCter* homozygous mutants with no functional allele of *dpCry2* (*dpBmal1 m2*; $dpCry2^{-/-}$) lost circadian rhythmicity of eclosion (Fig. 3*A* and Fig. S2*C*), as expected based on the previously reported circadian arrhythmicity of *dpCry2* knockouts (18). However, to our surprise, *dpBmal1ΔCter* homozygous mutants carrying a single functional allele of *dpCry2* (*dpBmal1 m2*; $dpCry2^{+/-}$) eclosed significantly later during the circadian cycle than *dpBmal1ΔCter* homozygous mutants carrying two functional alleles of *dpCry2* (*dpBmal1 m2*; $dpCry2^{+/+}$) ($P < 0.05$, one-way ANOVA followed by Tukey's post hoc test) (Fig. 3*A* and Fig. S2*C*). These results stand in sharp contrast with previous work showing no difference in the timing of eclosion between monarchs carrying either two or a single functional allele of *dpCry2* in a wild-type dpBMAL1 background (18).

To examine the consequences of this interaction on the molecular clock, we quantified *per* and *tim* mRNA levels in the brains of *dpBmal1ΔCter* homozygous mutants with two, one, or no functional alleles of *dpCry2* (Fig. 3*B*). Consistent with our behavioral analysis, we found that in the absence of dpCRY2 the circadian rhythms of *per* and *tim* mRNA in the brains of *dpBmal1ΔCter* homozygous mutants were abolished, with *per* and *tim* mRNA expression being constitutively high over the circadian cycle. In contrast, *per* and *tim* mRNA in the brains of *dpBmal1ΔCter* homozygous mutants with a single functional allele of *dpCry2* were rhythmic, but the phase of the rhythm was significantly delayed compared with butterflies with two functional alleles of *dpCry2*. *Per* and *tim* mRNAs have previously been shown to cycle in a circadian manner with the same phase in brains of wild-type monarchs and heterozygous CRY2 mutants (18). Therefore, the dose-dependent effect of dpCRY2 we observed in *dpBmal1ΔCter* homozygous mutants suggests a dose-dependent effect of dpCRY2 for repression on a domain in dpCLK or outside the dpBMAL1 TAD helix that is masked in the presence of the BMAL1 TAD helix.

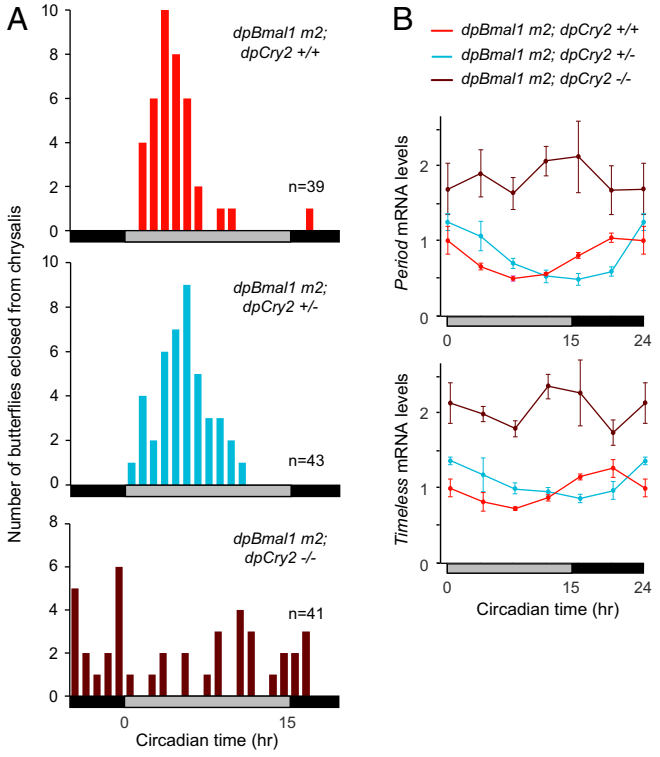

Fig. 3. Behavioral and molecular rhythms in *dpBmal1ΔCter* mutants are driven by dpCRY2 repression. (*A*) Profiles of adult eclosion in DD of *dpBmal1ΔCter* mutants (m2, containing both homozygous males and hemizygous females) in a wild-type background for *dpCry2* (red bars), in a heterozygous background for *dpCry2* (blue bars), and in a *dpCry2*-null background (brown bars). Data collected in DD1 and DD2 are pooled and binned in 1-h intervals. The horizontal bars below the graphs indicate subjective day (gray) and night (black). *dpBmal1 m2*; $dpCry2^{+/+}$ vs. *dpBmal1 m2*; $dpCry2^{-/-}$, $P < 0.01$; *dpBmal1 m2*; $dpCry2^{+/+}$ vs. *dpBmal1 m2*; $dpCry2^{+/-}$, $P < 0.05$ (one-way ANOVA followed by Tukey's post hoc test). (*B*) Circadian expression of *period* and *timeless* in brains of *dpBmal1ΔCter* mutants in a wild-type background for *dpCry2* (red lines), in a heterozygous background for *dpCry2* (blue lines), and in a *dpCry2*-null background (brown lines). Values shown are the mean ± SEM of three animals. The horizontal bars below the graphs indicate subjective day (gray) and night (black). Interaction genotype × time, *dpBmal1 m2*; $dpCry2^{+/+}$ vs. *dpBmal1 m2*; $dpCry2^{-/-}$: *tim* and *per*, $P < 0.00001$; *dpBmal1 m2*; $dpCry2^{+/+}$ vs. *dpBmal1 m2*; $dpCry2^{+/-}$: *per*, $P < 0.001$; *tim*, $P < 0.005$ (two-way ANOVA).

Domains on dpCLK:dpBMAL1 Other than the dpBMAL1 C Terminus Contribute to dpCRY2-Dependent Transcriptional Repression. To determine whether circadian repression of *dpBmal1ΔCter* mutants by dpCRY2 could be mediated through the more distal C-terminal residues of BMAL1 retained in the *dpBmal1ΔCter*, we used a GAL4/UAS cell-based reporter assay to assess the transactivation capacity of different dpBMAL1 C-terminal domains and their ability to be repressed by dpCRY2. Because both the G and TAD regions in the C terminus of BMAL1 have been implicated in activation and repression by mCRY1, we included the G domain in our analysis. S2 cells were cotransfected with a luciferase reporter under the control of a 10×UAS promoter and either a wild-type dpBMAL1 C-terminal domain or a deletion mutant fused at the C terminus of the DNA-binding domain of the yeast transcription factor GAL4 (GAL4DBD) in the absence or presence of increasing doses of dpCRY2 (Fig. 4*A*). We found that the G and TAD regions fused to GAL4DBD elicited a large increase in transcriptional activity, while neither the G region nor the TAD region alone elicited substantial transcriptional activity, indicating that both regions are necessary for transcription. Consistent with the idea that dpCRY2 could bind to the TAD helix, transcription mediated by both the G and TAD regions was inhibited by dpCRY2 in a dose-dependent manner ($P < 0.05$; one-way ANOVA). We also found that the G and truncated TAD domain reminiscent of our *dpBmal1ΔCter* mutant elicited transcription but with levels reduced by approximately threefold compared with the full-length C-terminal domain, in agreement with a role for the TAD α-helix in enhancing transcriptional activation. However, transcription elicited by the C-terminal domain present in the *dpBmal1ΔCter* mutant was not inhibited by dpCRY2, even at high doses ($P = 0.81$; one-way ANOVA), demonstrating that neither the G domain nor the more distal C-terminal residues (i.e., LxWPxx) are sufficient to mediate dpCRY2 repression. These results suggested that the circadian rhythms observed in vivo in *dpBmal1ΔCter* mutants resulted from transactivation provided by the G and the most distal C-terminal residues of the TAD and repression by dpCRY2 on domains other than the TAD α-helix, on either dpCLK or dpBMAL1.

To test this hypothesis, we next tested the ability of dpCRY2 to repress dpCLK:dpBMAL1 C-terminal truncation mutants by cotransfecting S2 cells with a luciferase reporter construct containing a tandem repeat of the proximal CACGTG E-box enhancer from the monarch *per* promoter (20) and with constructs expressing dpCLK and either full-length dpBMAL1 or C-terminal truncation mutants, in the absence or presence of increasing doses of dpCRY2 (Fig. 4*B*). As previously shown (20), dpCLK:dpBMAL1 elicited an increase in transcriptional activity, which was inhibited by dpCRY2 in a dose-dependent manner ($P < 0.0001$; one-way ANOVA). No increase in transcriptional activity could be detected when dpCLK was cotransfected with either a truncated dpBMAL1 mutant lacking the TAD domain (*dpBmal1ΔTAD*) or a truncated dpBMAL1 mutant lacking the G and TAD domains (*dpCyc-like*), consistent with a

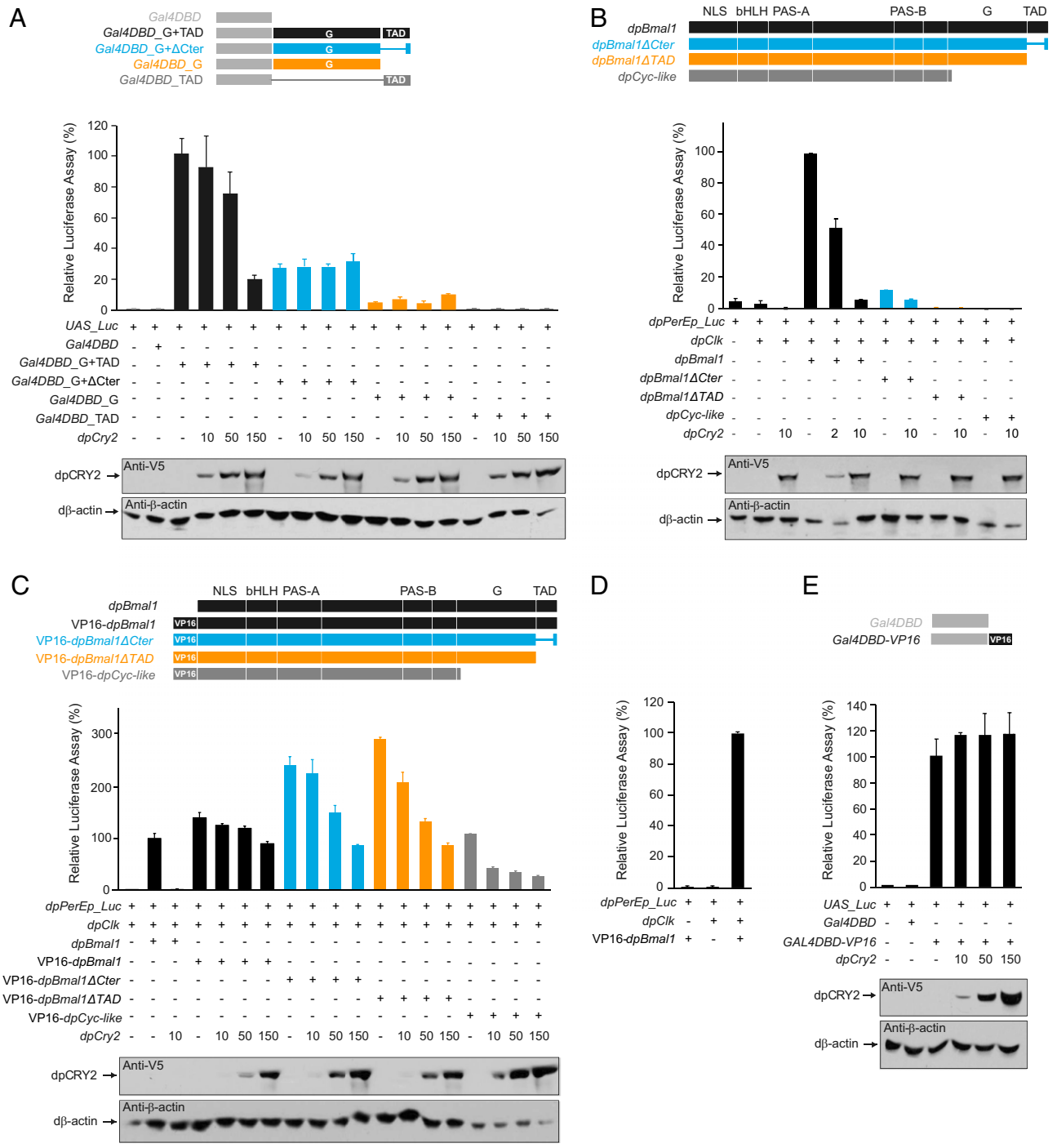

Fig. 4. Several domains on dpCLK and dpBMAL1 contribute to transcriptional repression by dpCRY2 in S2 cells. (*A*, *Upper*) DpCRY2 does not repress on the BMAL1 G and ΔCter mutant TAD domains. A UAS luciferase reporter (UAS_Luc; 10 ng) was used in the presence (+) or absence (–) of Gal4DBD, Gal4DBD_G+TAD, Gal4DBD_G+ΔCter, Gal4DBD_G, and Gal4DBD_TAD expression plasmids (5 ng each), and increasing doses of dpCRY2 (amounts are given in nanograms). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is the mean ± SEM of three replicates. (*Lower*) Western blots of V5-tagged dpCRY2 and *Drosophila* β-actin protein expression levels. (*B*) DpCRY2 inhibits dpCLK:dpBMAL1- and dpCLK:dpBMAL1ΔCter-mediated transcription. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence (+) or absence (–) of dpCLK, dpBMAL1, dpBMAL1ΔCter, dpBMAL1ΔTAD, and dpCYC-like expression plasmids (5 ng each) and increasing doses of dpCRY2 (amounts are given in nanograms). Quantification of luciferase activity, values, and Western blot analysis are shown as in *A*. (*C*) DpCRY2 dose-dependent inhibition of dpCLK:dpBMAL1 mutants fused to the VP16 transactivation domain in their N termini. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence (+) or absence (–) of dpCLK, dpBMAL1, VP16-dpBMAL1, VP16-dpBMAL1ΔCter, VP16-dpBMAL1ΔTAD, and VP16–dpCYC-like expression plasmids (5 ng each) and increasing doses of dpCRY2 (amounts are given in nanograms). Quantification of luciferase activity, values, and Western blot analysis are depicted as in *A*. One-way ANOVAs for dose-dependent repression by dpCRY2 on each BMAL1 variant: $P < 0.0001$ to $P < 0.005$. (*D*) DpCLK is required for VP16-dpBMAL1–mediated transcription. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence (+) or absence (–) of dpCLK and VP16-dpBMAL1 expression plasmids (5 ng each). Quantification of luciferase activity and values are depicted as in *A*. (*E*) DpCRY2 does not repress the VP16 activation domain. A UAS luciferase reporter (UAS_Luc; 10 ng) was used in the presence (+) or absence (–) of Gal4DBD and Gal4DBD-VP16 expression plasmids (5 ng each) and increasing doses of dpCRY2 (amounts are given in nanograms). Quantification of luciferase activity, values, and Western blot analysis are depicted as in *A*. $P = 0.79$ (one-way ANOVA).

transactivation function for these domains. However, cotransfection of dpCLK and dpBMAL1ΔCter elicited only a small but significant increase in transcriptional activity ($P < 0.05$; Student's t test), which, despite its low level, was inhibited by dpCRY2 ($P < 0.0001$; Student's t test). To unambiguously determine whether dpCRY2 was able to repress different dpCLK:dpBMAL1 deletion variants, we next enhanced transcription by fusing the strong viral transcriptional activator VP16 (24) to dpBMAL1 variants at either the N terminus (Fig. 4*C*) or the C terminus (Fig. S3). Regardless of the position of VP16 on the fusion proteins, all heterodimers of dpCLK-dpBMAL1 variants, including the dpCLK:dpCYC-like heterodimer, elicited a large increase in transcriptional activity and were inhibited in a dose-dependent manner by dpCRY2. Importantly, we verified that activation of these fusion proteins was not an artifact caused by the VP16 activation domain (Fig. 4*D*) and that repression by dpCRY2 was not caused by repression on the VP16 domain itself (Fig. 4*E*). Collectively, these data demonstrate that at least two sites for repression by dpCRY2 exist, one on the TAD α-helix of dpBMAL1 (Fig. 4*A*) and one on either dpCLK or another region of dpBMAL1 upstream of the G and TAD regions.

Given that CRY1-mediated circadian repression in mouse has been shown to occur via competition for binding with coactivators such as p300 to the BMAL1 C terminus (13, 14), we predicted that knocking down endogenous *Drosophila p300* (i.e., *nejire*) in S2 cells cotransfected with dpCLK:dpBMAL1 would not only decrease transcriptional activity but also facilitate transcriptional repression by dpCRY2. While dsRNA-mediated knockdown of eGFP had no effect on transcription, we found that knocking down *p300* by ∼60% led to a threefold decrease in transcriptional activity of dpCLK:dpBMAL1 (Fig. S4 *A* and *C*). In both cases, dpCRY2 inhibited dpCLK:dpBMAL1-mediated transcription in a dose-dependent manner, but we did not observe any difference in the amount of dpCRY2 necessary for transcriptional repression in untreated cells and cells treated with dsRNA against *p300* (Fig. S4 *A* and *B*). Although we cannot exclude the possibility that p300 has a higher affinity than dpCRY2 for the dpBMAL1 TAD, this finding is consistent with the idea that the dpBMAL1 C terminus is not the only site of repressive action by vertebrate-like CRY2. It may also suggest the intriguing possibility that dpCRY2 competes with other coactivators for dpCLK:dpBMAL1 binding either on the dpBMAL1 TAD or on yet unknown domains of dpCLK and dpBMAL1.

Both the CLK PAS-B Domain and the BMAL1 C Terminus Contribute to the Repressive Potency of Vertebrate-Like CRY2. To assess the relative contribution of the BMAL1 C terminus versus other domains of CLK and BMAL1 to repression by insect CRY2, we took advantage of *Drosophila* circadian transcriptional activators. Like many other dipterans, *Drosophila* has lost both CRY2 and the BMAL1 C terminus including the G and TAD regions on dCYC (Fig. 5*A*). However, dCLK has evolved a TAD domain that mediates dCLK:dCYC transcriptional activity in the absence of the BMAL1 C terminus (25), thereby allowing us to compare the strength of repression by insect CRY2 in the absence or presence of the BMAL1 C terminus by using a wild-type dCLK:dCYC or a dCLK:dCYC chimeric protein bearing the dpBMAL1 C terminus.

We first examined the ability of dpCRY2 to repress dCLK:dCYC in S2 cells and found that dpCRY2 strongly repressed dCLK:dCYC in a dose-dependent manner (Fig. 5*B*). However, to our surprise, this inhibition was accompanied by a dose-dependent decrease in *dcyc* expression levels, an effect that has not been observed with the *Drosophila* circadian repressor dPER (26) and which was not due to CLK destabilization (Fig. 5*B*). To eliminate a possible confounding effect of dpCRY2 on *dcyc* transcription or RNA stability, we knocked down endogenous *dcyc* mRNA levels via RNAi and expressed dCYC exogenously (Fig. 5*C*) and showed that overexpression of both dCLK:dCYC fully rescued the transcriptional activity that was otherwise efficiently reduced by the knock down of *dcyc* mRNA. In this condition, we found that dpCRY2 inhibited dCLK:dCYC in a dose-dependent manner, reaching ∼50% inhibition at the maximal dose tested ($P < 0.005$; one-way ANOVA), thereby demonstrating that dpCRY2 is able to repress dCLK:dCYC. In addition, fusing the dpBMAL1 C terminus to dCYC further enhanced transcriptional activity by approximately threefold as well as dose-dependent repression by dpCRY2 ($P < 0.0001$; one-way ANOVA), which repressed activation levels at maximum doses tested by ∼80% compared with the 50% observed in the absence of the BMAL1 C terminus (Fig. 5*D*). Together, these results show that both the BMAL1 C terminus and other domains on CLK or CYC/BMAL1 contribute to repression potency by vertebrate-like CRY2.

Because of its known role in mammalian CRY1 binding (11, 27), the CLOCK PAS-B domain, and in particular five of its residues (12), appeared to be an ideal candidate region for repression by vertebrate-like CRY2. While the five residues are fully conserved in monarch dpCLK (Fig. 5*E*), two of them are changed to conserved residues in *Drosophila* dCLK (W349F and E354D) (Fig. 5*E*). We thus reasoned that if dpCRY2 represses dCLK:dCYC on these five residues, mutating F349 and D354 on dCLK to vertebrate-like residues (i.e., F349W/D354E) should increase the potency of its repression. As predicted, we found that dpCRY2 represses dCLK F349W/D354E in a dose-dependent manner with higher potency than wild-type dCLK (Fig. 5*F*). However, the levels of activation of dCLK F349W/D354E:dCYC were reduced by ∼50% compared with dCLK:dCYC (Fig. S5), suggesting that the changes in residues 349 and 354 of dCLK may have arisen to maintain high activation levels. However, we did not observe a significant increase in dpCRY2 repression potency of the dCYC:dCLK F349W/D354E transcription factor when the BMAL1 C terminus was fused to dCYC (Fig. 5*F* and Fig. S5). Taken together, our results suggest that both the BMAL1 C terminus and the CLK PAS-B domain contribute to the repressive potency of vertebrate-like CRY2 and identify the amino acids tryptophan and glutamic acid in the CLK HI loop as residues important for this function.

DpCLK W328 and E333 Residues Independently Contribute to a TAD-Dependent and a TAD-Independent Repression by dpCRY2. To determine the relative contribution of the corresponding tryptophan and glutamic acid residues on dpCLK for dpCRY2-dependent repression in the monarch clock, we tested the effect of mutations on dpCLK (W328A/E333A, W328A, and E333A) in S2 cells (Fig. 6 and Fig. S6). Consistent with our hypothesis that residues in the dpCLK PAS-B domain contribute to dpCRY2-dependent repression, we found that W328A/E333A and W328A mutations in dpCLK significantly weakened dpCRY2-dependent repression of dpCLK:dpBMAL1 (Fig. 6*A*). However, the single E333A mutation, which significantly decreased activation levels, did not affect the potency of repression by dpCRY2 (Fig. 6*A*). These results suggest that dpCLK W328, but not dpCLK E333, plays an important role in dpCRY2-dependent repression when the dpBMAL1 C terminus is present, similar to results shown in mammals (15). Because our in vivo and in vitro data supported the existence of a TAD-independent mechanism of repression by dpCRY2, we also tested whether it could be mediated through dpCLK by assessing the effect of the same dpCLK mutations on the dpCLK:VP16-dpCYC-like transcription factor in which the dpBMAL1 C terminus was lacking. Surprisingly, in this context, we found that while the W328A mutation had no significant effect on the ability of dpCRY2 to repress the transcription factor, both W328A/E333A and E333A abolished dpCRY2-dependent repression (Fig. 6*B*). Together, these results demonstrate the existence of two independent mechanisms of repression by dpCRY2: a TAD-dependent mechanism that involves dpCLK W328 and the C terminus of dpBMAL1 and a TAD-independent mechanism involving the E333 residue on dpCLK that may be

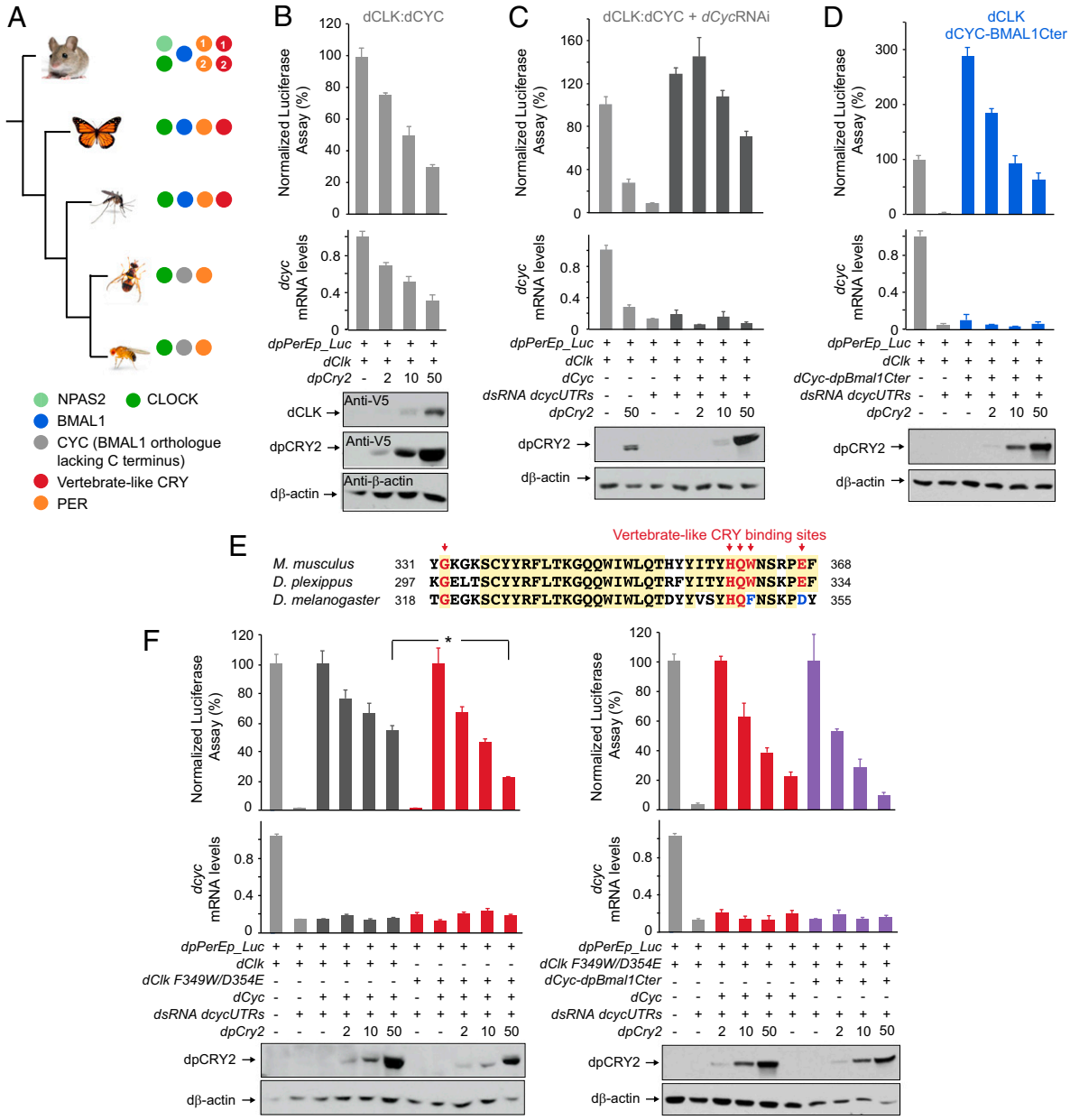

Fig. 5. Fusing the dpBMAL1 C terminus to *Drosophila* dCLK:dCYC or mutating dCLK F349W/D354E independently restores strong repression by dpCRY2 in S2 cells. (*A*) Evolutionary relationship of insect species representative of lepidopterans and dipterans (*Left*) with their respective core clock components (*Right*). Dipterans shown comprise the Nematoceran (mosquitoes) and the Brachyceran (flies) lineages with the melon fly and the fruit fly. The mouse is shown as a representation of vertebrate clocks and as an outgroup of the tree. (*B*, *Top*) dpCRY2 affects *Drosophila* dCLK:dCYC transcription by decreasing *dcyc* mRNA levels. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in presence of a dpCLK expression plasmid (5 ng each) and increasing doses of dpCRY2 (amounts are given in nanograms). Firefly luciferase activity was computed relative to renilla luciferase activity. $P < 0.0001$ (one-way ANOVA). (*Middle*) Endogenous *dcyc* mRNA levels were quantified using qPCR. $P < 0.0005$ (one-way ANOVA). Each value is the mean ± SEM of three replicates. (*Bottom*) Western blots of V5-tagged dCLK and dpCRY2 and *Drosophila* β-actin protein expression levels. (*C*) DpCRY2 weakly represses transcription by acting directly on *Drosophila* dCLK:dCYC proteins. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence of dCLK with (+) or without (–) exogenous dCYC expression plasmids (5 ng each) in the presence of dsRNA against the 5′ and 3′ UTR of endogenous *dcyc* (7.5 μg each). Increasing doses of dpCRY2 were provided; amounts are given in nanograms. Quantification of luciferase activity and endogenous *dcyc*, values, and Western blot analysis are depicted as in *B*. (*D*) Fusing a dpBMAL1 C terminus to dCYC rescues dpCRY2's strong repressive capability. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence of dCLK alone or in the presence of dCLK and dCYC-dpBMAL1 C terminus expression plasmid (5 ng each) and dsRNA against the 5′ and 3′ UTR of endogenous *dcyc* (7.5 μg each). Increasing doses of dpCRY2 were provided; amounts are given in nanograms. Quantification of luciferase activity and endogenous *dcyc*, values, and Western blot analysis are depicted as in *B*. (*E*) Alignment of partial CLK proteins from the mouse (*Mus musculus*), the monarch butterfly (*Danaus plexippus*), and the fruit fly (*Drosophila melanogaster*). The red arrows indicate the conservation of the five previously described mouse CRY1-binding amino acids (11, 12). (*F*) Mutating dCLK F349W/D354E increases dpCRY2's repressive capability on dCLK:dCYC-mediated transcription (*Left*), but fusing the dpBMAL1 C terminus to the mutant protein has no additional effect (*Right*). The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence of either wild-type dCLK or dCLK F349W/D354E expression plasmids (5 ng each) with (+) or without (–) exogenous dCYC or dCYC-dpBMAL1 C terminus expression plasmids (5 ng each) in the presence of dsRNA against the 5′ and 3′ UTR of endogenous *dcyc* (7.5 μg each). Increasing doses of dpCRY2 were provided; amounts are given in nanograms. Quantification of luciferase activity is presented as values relative to 100% for each set of conditions, and endogenous *dcyc* values and Western blot analysis are depicted as in *B*. *$P < 0.05$ (two-way ANOVA followed by Tukey's post hoc test).

responsible for the persistence of rhythms observed in the *dpBmal1ΔCter* mutants.

To determine whether deletion of the dpBMAL1 TAD α-helix impairs the interaction between dpBMAL1 and dpCRY2, we coimmunoprecipitated dpCRY2 with WT c-Myc-dpCLK and either WT VP16-dpBMAL1 or the VP16-dpBMAL1ΔCter mutant. Consistent with our finding that the dpBMAL1 TAD α-helix is dispensable for dpCRY2-dependent repression, dpBMAL1ΔCter and WT dpCLK coimmunoprecipitated with dpCRY2 to a similar extent as WT dpBMAL1 and dpCLK (Fig. 6 *C* and *D*). Because the E333A mutation in the dpCLK HI loop abolished TAD-independent repression by dpCRY2 (Fig. 6*B*), we also sought to determine whether this mutation also disrupts the dpCYC-like/dpCLK/dpCRY2 complex by coimmunoprecipitating dpCRY2 with dpCYC-like–VP16 and the c-Myc–dpCLK E333A mutant, the c-Myc–dpCLK W328A mutant, or WT c-Myc–dpCLK as a control. We showed that dpCYC-like and all dpCLK variants tested (WT, W328A, and E333A) coimmunoprecipitated with dpCRY2, demonstrating that none of these mutations disrupted the stable interaction of dpCLK and dpCYC-like with dpCRY2 (Fig. 6 *C* and *D*). However, we observed a decrease in the amount of coimmunoprecipitated dpCYC-like and dpCLK in the presence of the E333A mutation. Given the lack of repression by dpCRY2 on the E333A mutant but not the W328A mutant (Fig. 6*B*), our results suggest that dpCLK E333 supports either dpCYC-like–dpCRY2 binding or dpCLK-dpCRY2 binding, which is likely required for TAD-independent repression by dpCRY2.

Discussion

The mechanisms by which CRYs regulate the circadian activity of CLOCK-BMAL1 in mammals have been proposed, based on biophysical and cell-based assays, to primarily occur through dynamic interactions between CRY1 and the BMAL1 TAD α-helix (13, 14). In contrast, the CRY-interacting interface on the CLOCK PAS-B HI loop (12) is thought to play a role only in docking CRY onto CLOCK-BMAL1 (14). However, the relative importance of these two sites on CLOCK:BMAL1 for CRY1 repression has not been firmly established through in vivo experiments, because the only existing mouse mutant lacking the BMAL1 C-terminal TAD harbors compromised transcriptional activity (28). In this work, we leveraged the monarch butterfly as an alternative system to directly test in vivo the importance of the BMAL1 TAD for vertebrate-like CRY repressive function because it possesses mammalian-like clock components and is readily amenable to CRISPR-mediated targeted mutagenesis (21). Through the generation of a mutant lacking the BMAL1 TAD α-helix but retaining the most distal C-terminal residues sufficient to provide transcriptional activity, we present in vivo genetic evidence showing that, despite regulating the circadian phase or period, the BMAL1 TAD α-helix is not necessary for repression of CLOCK-BMAL1 transcriptional activity by insect CRY2. Using cell-based reporter assays, we show that monarch dpCRY2 can repress dpCLK:dpCYC (a BMAL1 mutant lacking the C terminus lost in *Drosophila*) in the presence of a VP16-activation domain as well as the *Drosophila* dCLK:dCYC heterodimer, which is transcriptionally active through the glutamine-rich region of dCLK (29). DpCRY2 repression of dCLK:dCYC can be enhanced by either fusing a dpBMAL1 C terminus to dCYC or mutating two of the vertebrate-like CRY1-binding sites on the dCLK PAS-B domain to mammalian-like residues. Conversely, mutating the corresponding residues on the monarch dpCLK PAS-B domain not only weakened dpCRY2-dependent repression of dpCLK:dpBMAL1 but also abolished that of the transcriptionally active dpCLK:VP16–dpCYC-like. Together, these results demonstrate that vertebrate-like CRY regulation of circadian rhythms occurs through two independent mechanisms on CLK and the BMAL1 C terminus.

Our discovery that mutant monarchs lacking the BMAL1 TAD α-helix but retaining transcriptional activity maintain robust behavioral and molecular rhythms (Fig. 2) challenges the idea that the BMAL1 TAD is key to circadian repression (13, 14, 30) and shows that, at least in the butterfly, the BMAL1 TAD α-helix is not required for circadian repression by vertebrate-like CRY. The delayed phase of adult eclosion (by ∼1 h on DD day 1) observed in butterfly mutants lacking the TAD α-helix is nevertheless consistent with the TAD α-helix having a role in phase or period determination, similar to its function in mammals (13, 14). Our results showed that both the G and TAD C-terminal regions of monarch BMAL1 are necessary for strong activation and that the TAD α-helix plays a dual role in activation and repression by insect CRY2 in vitro (Fig. 4*A*). The decreased activation levels of *per* and *tim* mRNA in the brain of the monarch mutant lacking the BMAL1 TAD α-helix are consistent with its function as an activation domain and may explain the behavioral phase delay observed in vivo, as activation levels have previously been correlated to phase or period determination (31). In mammals, transcriptional activation occurs through the recruitment of coactivators such as CBP/p300 to BMAL1 (14, 32, 33), and repression by CRY1 is thought to occur through competition for binding with CBP/p300 on the BMAL1 TAD α-helix and the most distal C-terminal residues (14). As expected if dpCLK:dpBMAL1 activates transcription through a conserved mechanism involving recruitment of p300, knocking down endogenous *Drosophila p300* in S2 cells substantially reduced dpCLK:dpBMAL1-mediated activation (Fig. S4). However, in contrast to what would be expected if repression by dpCRY2 is mediated solely through competition with p300 on the dpBMAL1 TAD, no decrease in the amount of dpCRY2 necessary for transcriptional repression was observed when *p300* levels were reduced. This finding does not necessarily contradict a model in which dpCRY2 and p300 compete for dpBMAL1 TAD binding in the monarch. Because the reduction of *p300* was only partial (∼60%) (Fig. S4) in our reporter assay, we cannot exclude the possibility that the remaining p300 could efficiently outcompete dpCRY2 for dpBMAL1 TAD binding, as previously observed for the mammalian p300 kinase-inducible domain interacting (KIX) domain (14). Alternatively, given that our results were obtained in a cellular context rather than in vitro chemical shift perturbation studies (14), it is possible that additional coactivators bound to dpCLK:dpBMAL1 also compete with dpCRY2 for binding. Histone-modifying enzyme orthologs of MLL and JARID1a, which are recruited at CLOCK:BMAL1, or proteins that recruit the transcriptional machinery are all potential candidates (34–37).

Regardless of the exact molecules with which dpCRY2 might compete on the dpBMAL1 TAD, our results provide strong evidence that this interaction is not sufficient for repression by dpCRY2. Generating monarch mutants lacking the dpBMAL1 TAD α-helix and harboring no functional allele or a single functional allele of *dpCry2* has allowed us to unambiguously demonstrate that repression in these mutants was mediated by dpCRY2 and not by other negative regulators (Fig. 3). Furthermore, using in vitro reporter assays, we have excluded the possibility that dpCRY2 repression could occur through the last, most distal amino acids (LxWPxx) of the dpBMAL1 TAD retained in our mutant. Importantly, we showed that dpCRY2 has the ability to repress a dpBMAL1 protein lacking the C terminus (i.e., the G and TAD regions lost in *Drosophila* dCYC) when transcriptional activity is provided by fusing a VP16 activation domain (Fig. 4). Together, these results demonstrate that a dpBMAL1 TAD-independent mechanism is critical for repression and rhythm generation by dpCRY2. They are also consistent with previous findings in mice showing that the C-terminal domain of CRY1, which interacts with the BMAL1 C terminus, regulates clock function but is not necessary for transcriptional repression (38, 39).

The CLOCK PAS-B domain HI loop has been shown to play a central role in the establishment of complexes with mammalian CRY1, where a single W at position 362 on CLOCK directly interacts

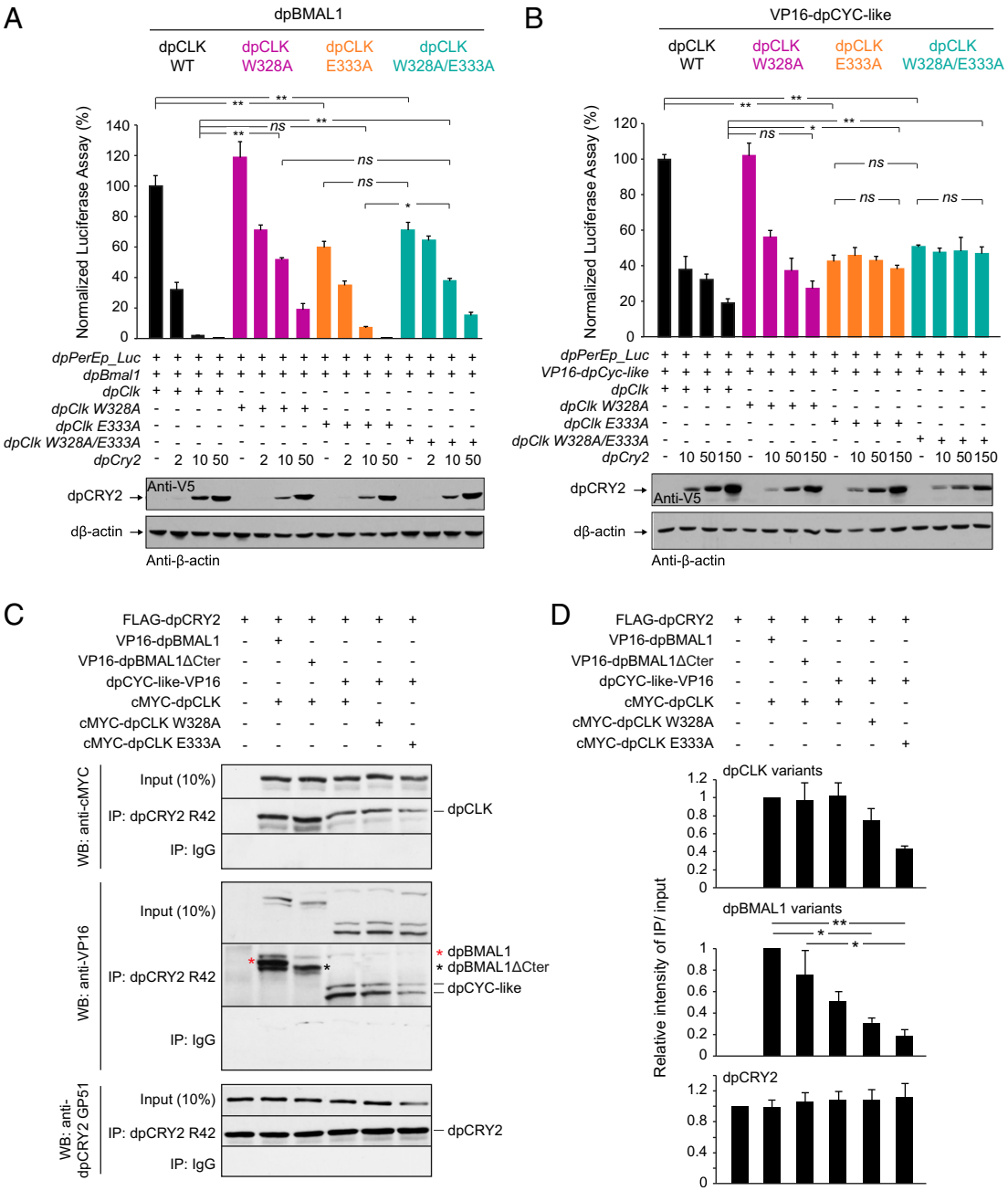

Fig. 6. The dpCLK W328 and E333 residues independently contribute to TAD-dependent and TAD-independent repression by dpCRY2. (*A* and *B*) Effects of dpCLK mutations (dpCLK W328A, dpCLK E333A, and dpCLK W328A/E333A) in the presence (*A*) or absence (*B*) of the dpBMAL1 C terminus. The monarch *per* E box luciferase reporter (dpPerEp_Luc; 10 ng) was used in the presence (+) of dpBMAL1 (*A*) or VP16–dpCYC-like (*B*) and dpCLK variants expression plasmids (5 ng each) with increasing doses of dpCRY2; amounts are given in nanograms. Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is the mean ± SEM of three replicates. Western blots of V5-tagged dpCRY2 and *Drosophila* β-actin protein expression levels are shown below the graphs. $*P < 0.05$; $**P < 0.01$; ns, not significant (two-way ANOVA followed by Tukey's post hoc test). (*C*) Coimmunoprecipitations (IP) of c-Myc–dpCLK and either VP16-dpBMAL1 or VP16-dpBMAL1ΔCter and of dpCYC-like–VP16 and c-Myc–dpCLK WT, c-Myc–dpCLK W328A, or c-Myc-dpCLK E333A by FLAG-dpCRY2 with anti-dpCRY2 R42 antibody from transfected S2 cells. Western blots (WB) were performed using the indicated antibodies. The double band for dpCYC-like–VP16 likely corresponds to alternatively used translation initiation sites. The top band was quantified in *D*. (*D*) Quantification of *C*. For each protein, the relative intensity corresponds to the intensity of IP over input signal measured using Image J in each condition, relative to the intensity of IP over input signal of dpCRY2, WT dpCLK, and WT dpBMAL1. Each value is the mean ± SEM of three independent experiments. dpCLK variants, $P < 0.05$; dpBMAL1 variants, $P < 0.005$; dpCRY2, $P = 0.96$ (one-way ANOVA). $*P < 0.05$; $**P < 0.01$ (Tukey's post hoc test).

with the photolyase homology region (PHR) of vertebrate-like CRY (15). Here, we identify a dual role for the monarch CLK PAS-B domain HI loop in both TAD-dependent and TAD-independent repressive mechanisms by dpCRY2 (Fig. 6). Similar to mammalian CLOCK W362 (15), dpCLK W328 plays a role in the TAD-dependent repression of dpCLK:dpBMAL1 by dpCRY2, contributing to strong repression presumably by facilitating sequestration of the BMAL1 TAD by dpCRY2, as proposed in mammals (14, 15). We also show that dpCLK E333, another residue of the CLK PAS-B domain HI loop, is necessary for both strong activation and

TAD-independent repression by dpCRY2. Based on both our in vivo and in vitro results, we propose that dpCRY2 represses dpCLK:dpBMAL1 primarily through a BMAL1 TAD-independent mechanism involving dpCLK E333, with the BMAL1 TAD modulating circadian rhythms only by modulating activation levels. We speculate that these two repressive mechanisms could represent two consecutive phases of repression, and, given the conservation of the BMAL1 TAD and the vertebrate-like CRY-binding sites on the CLK PAS-B between mammals and monarchs, could also apply to mammalian CRYs.

Our results underscore the relevance of the monarch butterfly, in which clock proteins are not duplicated, as a system for the in vivo genetic dissection of clockwork mechanisms that could have far-reaching implications for our understanding of how the mammalian clock works. Given the crucial role that the circadian clock plays in the remarkable navigational capabilities of the migratory monarch butterfly (40), understanding the intricate mechanisms by which the monarch circadian clock keeps time will also provide a molecular foundation for the identification of the neural clock circuits involved in flight orientation and migratory behavior.

Materials and Methods

For details on CRISPR/Cas9 targeted mutagenesis, genetic crosses, eclosion behavior assays, real-time PCR, S2 cell assays, and coimmunoprecipitations, see *SI Materials and Methods*. All primers and templates used for generating constructs are listed in Dataset S1.

ACKNOWLEDGMENTS. We thank Steve Reppert for the generous gift of the anti-dpCRY2 R42 and anti-dpCRY2 GP51 antibodies and for providing the pAC plasmids containing *dpClk*, *dpBmal1*, and *dpCry2*; Michael Rosbash for providing the pAC-*Renilla*-Luc– and VP16-containing plasmids and for discussions; Courtney Caster for information that neither CRY2 nor the BMAL1 C terminus is present in the genome of flies belonging to the Brachyceran lineage and for help in generating Fig. 5*A*; Justin Vann, Sarah Kenny, and Kendall Bowen for help with monarch husbandry; Aldrin Lugena and Samantha Iiams for help with the generation of a construct used in the study; Jerome Menet for discussions and suggestions; and Deborah Bell-Pedersen for comments on the manuscript. This work was supported by startup funds from Texas A&M University and by National Science Foundation Grant IOS-1456985 (to C.M.) and NIH Grant NS052854 (to P.E.H.).

1. Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* 418:935–941.
2. Young MW, Kay SA (2001) Time zones: A comparative genetics of circadian clocks. *Nat Rev Genet* 2:702–715.
3. Gekakis N, et al. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280:1564–1569.
4. King DP, et al. (1997) Positional cloning of the mouse circadian clock gene. *Cell* 89: 641–653.
5. Kume K, et al. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98:193–205.
6. Chen R, et al. (2009) Rhythmic PER abundance defines a critical nodal point for negative feedback within the circadian clock mechanism. *Mol Cell* 36:417–430.
7. Nangle SN, et al. (2014) Molecular assembly of the period-cryptochrome circadian transcriptional repressor complex. *eLife* 3:e03674.
8. Griffin EA, Jr, Staknis D, Weitz CJ (1999) Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science* 286:768–771.
9. van der Horst GT, et al. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398:627–630.
10. Ye R, et al. (2014) Dual modes of CLOCK:BMAL1 inhibition mediated by cryptochrome and period proteins in the mammalian circadian clock. *Genes Dev* 28:1989–1998.
11. Sato TK, et al. (2006) Feedback repression is required for mammalian circadian clock function. *Nat Genet* 38:312–319.
12. Huang N, et al. (2012) Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. *Science* 337:189–194.
13. Kiyohara YB, et al. (2006) The BMAL1 C terminus regulates the circadian transcription feedback loop. *Proc Natl Acad Sci USA* 103:10074–10079.
14. Xu H, et al. (2015) Cryptochrome 1 regulates the circadian clock through dynamic interactions with the BMAL1 C terminus. *Nat Struct Mol Biol* 22:476–484.
15. Michael AK, et al. (2017) Formation of a repressive complex in the mammalian circadian clock is mediated by the secondary pocket of CRY1. *Proc Natl Acad Sci USA* 114:1560–1565.
16. Czarna A, et al. (2011) Quantitative analyses of cryptochrome-mBMAL1 interactions: Mechanistic insights into the transcriptional regulation of the mammalian circadian clock. *J Biol Chem* 286:22414–22425.
17. Takahata S, et al. (2000) Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes Cells* 5:739–747.
18. Merlin C, Beaver LE, Taylor OR, Wolfe SA, Reppert SM (2013) Efficient targeted mutagenesis in the monarch butterfly using zinc-finger nucleases. *Genome Res* 23:159–168.
19. Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol Biol Evol* 24:948–955.
20. Zhu H, et al. (2005) The two CRYs of the butterfly. *Curr Biol* 15:R953–R954, and erratum (2006) 16:730.
21. Markert MJ, et al. (2016) Genomic access to monarch migration using TALEN and CRISPR/Cas9-mediated targeted mutagenesis. *G3 (Bethesda)* 6:905–915.
22. Froy O, Gotter AL, Casselman AL, Reppert SM (2003) Illuminating the circadian clock in monarch butterfly migration. *Science* 300:1303–1305.
23. Zhu H, et al. (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biol* 6:e4.
24. Wysocka J, Herr W (2003) The herpes simplex virus VP16-induced complex: The makings of a regulatory switch. *Trends Biochem Sci* 28:294–304.
25. Allada R, White NE, So WV, Hall JC, Rosbash M (1998) A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* 93:791–804.
26. Bae K, Lee C, Hardin PE, Edery I (2000) dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. *J Neurosci* 20:1746–1753.
27. Zhao WN, et al. (2007) CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nat Cell Biol* 9:268–275.
28. Park N, et al. (2015) A novel Bmal1 mutant mouse reveals essential roles of the C-terminal domain on circadian rhythms. *PLoS One* 10:e0138661.
29. Darlington TK, et al. (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* 280:1599–1603.
30. Gustafson CL, et al. (2017) A slow conformational switch in the BMAL1 transactivation domain modulates circadian rhythms. *Mol Cell* 66:447–457.
31. Kadener S, Menet JS, Schoer R, Rosbash M (2008) Circadian transcription contributes to core period determination in Drosophila. *PLoS Biol* 6:e119.
32. Hosoda H, et al. (2009) CBP/p300 is a cell type-specific modulator of CLOCK/BMAL1-mediated transcription. *Mol Brain* 2:34.
33. Lee Y, et al. (2010) Coactivation of the CLOCK-BMAL1 complex by CBP mediates resetting of the circadian clock. *J Cell Sci* 123:3547–3557.
34. DiTacchio L, et al. (2011) Histone lysine demethylase JARID1a activates CLOCK-BMAL1 and influences the circadian clock. *Science* 333:1881–1885.
35. Katada S, Sassone-Corsi P (2010) The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol* 17:1414–1421.
36. Lande-Diner L, Boyault C, Kim JY, Weitz CJ (2013) A positive feedback loop links circadian clock factor CLOCK-BMAL1 to the basic transcriptional machinery. *Proc Natl Acad Sci USA* 110:16021–16026.
37. Valekunja UK, et al. (2013) Histone methyltransferase MLL3 contributes to genome-scale circadian transcription. *Proc Natl Acad Sci USA* 110:1554–1559.
38. Czarna A, et al. (2013) Structures of Drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function. *Cell* 153:1394–1405.
39. Khan SK, et al. (2012) Identification of a novel cryptochrome differentiating domain required for feedback repression in circadian clock function. *J Biol Chem* 287: 25917–25926.
40. Reppert SM, Guerra PA, Merlin C (2016) Neurobiology of monarch butterfly migration. *Annu Rev Entomol* 61:25–42.
41. Hwang WY, et al. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 31:227–229.
42. Jao LE, Wente SR, Chen W (2013) Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA* 110:13904–13909.
43. Rohland N, Reich D (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res* 22:939–946.
44. McDonald MJ, Rosbash M, Emery P (2001) Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the Drosophila timeless promoter. *Mol Cell Biol* 21:1207–1217.
45. Kwan KM, et al. (2007) The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 236:3088–3099.
46. Chang DC, Reppert SM (2003) A novel C-terminal domain of drosophila PERIOD inhibits dCLOCK:CYCLE-mediated transcription. *Curr Biol* 13:758–762.
47. Hung HC, Maurer C, Kay SA, Weber F (2007) Circadian transcription depends on limiting amounts of the transcription co-activator nejire/CBP. *J Biol Chem* 282:31349–31357.