# CLOCK stabilizes CYCLE to initiate clock function in *Drosophila*

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Edited by Joseph S. Takahashi, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, and approved August 31, 2017 (received for review May 2, 2017)

**The *Drosophila* circadian clock keeps time via transcriptional feedback loops. These feedback loops are initiated by CLOCK-CYCLE (CLK-CYC) heterodimers, which activate transcription of genes encoding the feedback repressors PERIOD and TIMELESS. Circadian clocks normally operate in ∼150 brain pacemaker neurons and in many peripheral tissues in the head and body, but can also be induced by expressing CLK in nonclock cells. These ectopic clocks also require *cyc*, yet CYC expression is restricted to canonical clock cells despite evidence that *cyc* mRNA is widely expressed. Here we show that CLK binds to and stabilizes CYC in cell culture and in nonclock cells in vivo. Ectopic clocks also require the blue light photoreceptor CRYPTOCHROME (CRY), which is required for both light entrainment and clock function in peripheral tissues. These experiments define the genetic architecture required to initiate circadian clock function in *Drosophila*, reveal mechanisms governing circadian activator stability that are conserved in perhaps all eukaryotes, and suggest that *Clk*, *cyc*, and *cry* expression is sufficient to drive clock expression in naive cells.**

circadian clock | *Drosophila* | protein stability | CYCLE | cryptochrome

Circadian clocks drive daily rhythms in metabolism, physiology, and behavior in a wide array of organisms. The identification of "clock genes" in *Drosophila* revealed that the circadian timekeeping mechanism is based on transcriptional feedback loops (1), which are used to keep time in most, if not all, eukaryotes. Despite this mechanistic conservation, the core components of animal, plant, and fungal feedback loops differ (2). In the *Drosophila* feedback loop, CLOCK-CYCLE (CLK-CYC) heterodimers activate *period* (*per*) and *timeless* (*tim*) transcription, PER-TIM complexes feed back to repress CLK-CYC transcription, and degradation of PER-TIM complexes release CLK-CYC to initiate the next cycle of transcription (1). These feedback loops operate in only ∼150 brain neurons and many, but not all, peripheral tissues in adults (reviewed in refs. 3 and 4).

Because CLK-CYC initiates clock function as a differentiated feature of most, if not all, brain pacemaker neurons that control activity rhythms (5), activation of these two genes is thought to determine which cells and tissues will contain circadian clocks. The activation of *Clk* has been well documented in brain pacemaker neurons (5, 6), but comparatively little is known about *cyc* expression. We recently showed that a fully functional GFP-*cyc* transgene expresses GFP-CYC protein exclusively in circadian pacemaker neurons (5), suggesting that CYC expression is limited to clock cells. However, the lack of enrichment of *cyc* mRNA in brain pacemaker neurons suggests that *cyc* is broadly expressed (7).

During fly development *Clk* is activated in all cells that will ultimately contain circadian clocks, but expressing *Clk* in cells that normally lack clock function can generate ectopic clocks (8). Like canonical clock cells, these ectopic clocks require *cyc* and show robust rhythms in *per* and *tim* mRNA and protein cycling in light-dark (LD) cycles that dampen in constant darkness (DD) (8, 9). This result is consistent with the possibility that *cyc* mRNA is broadly expressed, yet CYC is detected only in canonical clock cells (5). These observations suggest that *Clk* is required for CYC expression to initiate clock function, but how *Clk* promotes CYC accumulation and whether these clock components are sufficient to initiate clock function is not known.

Here we show that *Clk* controls CYC accumulation by stabilizing CYC in cultured *Drosophila* Schneider 2 (S2) cells. Likewise, CYC accumulates specifically in ectopic cells expressing *Clk*, indicating that CLK also stabilizes CYC in vivo. CLK and CYC, however, are not sufficient for ectopic clock function; *cry* is also required to entrain and/or maintain these clocks. This work reveals genes that initiate circadian clock function, defines conserved mechanisms underlying the accumulation of activator complexes in eukaryotes, and suggests that *Clk*, *cyc*, and *cry* expression are sufficient to program clock function in naive *Drosophila* cells.

## Results

**CYC Protein Is Stabilized by CLK.** Previous work showing that *cyc* mRNA is not enriched in pacemaker neurons suggests that *cyc* is also expressed in nonclock cells (7). Broad *cyc* expression is consistent with the ability of *Clk* to generate clocks in nonclock brain neurons (8, 9), but contrasts with the pacemaker neuron-specific accumulation of GFP-CYC (5). To reconcile these data, we propose that *cyc* mRNA is broadly expressed and CYC accumulates only in cells that express *Clk*. If CYC accumulation is dependent on *Clk*, then loss of *Clk* in clock neurons should also eliminate CYC. Indeed, GFP-CYC was not detectable in pacemaker neurons from $Clk^{out}$ null mutant flies (10) (Fig. 1*A*). To determine if *Clk* is required for CYC accumulation in fly heads, where most clock gene expression emanates from retinal photoreceptors (11), we used a *cyc*-FLAG transgene that fully rescues clock function (12). The levels of CYC-FLAG in $Clk^{out}$ heads was reduced >10-fold compared with controls having intact clocks (Fig. 1 *B* and *C*). In

### Significance

The *Drosophila* circadian clock controls daily rhythms in physiology, metabolism, and behavior via ∼24-h transcriptional feedback loops. CLOCK requires its heterodimeric partner CYCLE to initiate clock function in canonical groups of brain neurons and peripheral tissues, but *Clock* expression can also induce clocks in ectopic locations. Here, we show that CLOCK stabilizes CYCLE in canonical and ectopic clock cells, where CYCLE is normally rapidly degraded, and that ectopic clocks also require CRYPTOCHROME. This work defines the genetic architecture and molecular mechanisms required for clock initiation that are likely conserved in other eukaryotes and suggests that ectopic clocks can be generated by *Clock*, *cycle*, and *cryptochrome* expression in naive cells.

Author contributions: P.E.H. conceived of and supervised the project; T.L. and P.E.H. designed research; T.L. performed research; G.M. and W.Y. contributed new reagents/analytic tools; T.L. and P.E.H. analyzed data; and T.L. and P.E.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707143114/-/DCSupplemental.

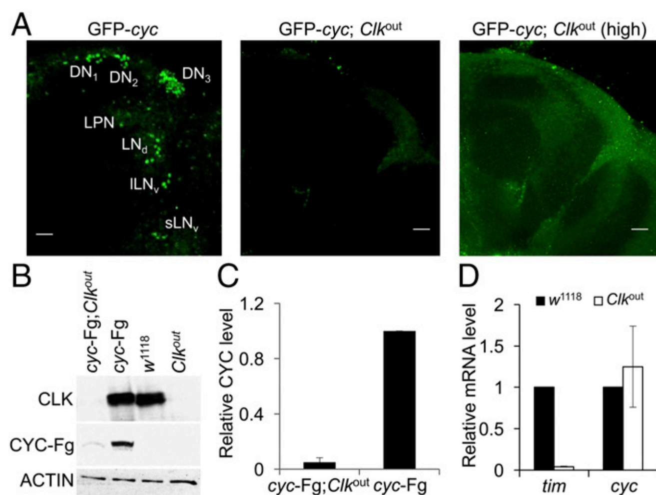


**Fig. 1.** CYC protein is expressed at low levels in *Clk*$^{out}$ flies. (*A*) GFP-CYC expression in brain pacemaker neurons was assessed in GFP-*cyc* and GFP-*cyc*; *Clk*$^{out}$ flies entrained in LD for at least 3 d and collected at ZT2. Immunostaining was performed on dissected brains using anti-GFP antibody and imaged by confocal microscopy as described (*Materials and Methods*). (*Left*) An 80-μm projected Z-series image of a right brain hemisphere from a GFP-*cyc* fly. (*Middle*) A 104-μm projected Z-series image of a right brain hemisphere from a GFP-*cyc*; *Clk*$^{out}$ fly. (*Right*) Same projected Z-series as *Middle* image with an increased laser power (high). Brains are oriented where lateral is to the right and dorsal is at the top. DN1, DN2, DN3, LPN, $LN_d$, $lLN_v$, and $sLN_v$ refer to pacemaker neuron groups as defined in the text. (Scale bar, 10 μm.) All images are representative of six or more brains. (*B*) Western blots containing proteins from the heads of control ($w^{1118}$), *cyc*-FLAG (*cyc*-Fg), *cyc*-FLAG;*Clk*$^{out}$ (*cyc*-Fg;*Clk*$^{out}$), and *Clk*$^{out}$ flies collected at ZT14 were probed with CLK, FLAG, and β-ACTIN antibodies to measure CLK, CYC-FLAG (CYC-Fg), and β-ACTIN (ACTIN), respectively. β-ACTIN was used as a loading control. (*C*) Relative CYC-FLAG levels on Western blots described in *B* were determined by measuring band intensities using Image J software (*Materials and Methods*). Values represent mean ± SEM from three independent experiments. (*D*) RT-qPCR analysis of *tim* and *cyc* mRNA levels in heads from control ($w^{1118}$) and *Clk*$^{out}$ flies collected at ZT14. Values represent mean ± SEM from five independent experiments.

contrast, *cyc* mRNA levels are the same in control ($w^{1118}$) and *Clk*$^{out}$ heads (Fig. 1*D*), indicating that *Clk* is not required for *cyc* transcription. These results show that *Clk* promotes CYC accumulation at the posttranscriptional level.

Reduced levels of CYC in *Clk*$^{out}$ flies could result from decreased synthesis or increased stability. Although there is evidence that transcription factors such as BMAL1 and HIF2α act in the cytoplasm to enhance translation (13, 14), we favor the possibility that CYC is stabilized by CLK as a product of heterodimer formation, which can stabilize other heterodimeric transcription factors (15, 16). To test whether CLK stabilizes CYC, we first determined the half-life of FLAG-tagged CYC in *Drosophila* S2 cells. S2 cells were transfected with pMK33-*cyc*-FLAG plasmid, CYC-FLAG expression was induced, translation was inhibited using cycloheximide (CHX), and samples were collected as described (*Materials and Methods*). The levels of CYC-FLAG declined rapidly after CHX addition, with a half-life of ∼1 h (Fig. 2 *A* and *D*). To identify the CYC degradation pathway, we measured the CYC-FLAG half-life after treatment with the 26S proteasome inhibitor MG132. CYC-FLAG levels were unchanged in the presence of MG132, indicating that CYC is degraded by proteasome (Fig. 2 *B* and *D*). To determine the impact of CLK on CYC protein stability, we measured CYC levels in the presence of V5-tagged CLK. CYC-FLAG was stabilized in the presence of CLK-V5 with a half-life of ∼9 h, demonstrating that CLK stabilizes CYC (Fig. 2 *C* and *D*). When CYC-FLAG and CLK-V5 were coexpressed in S2 cells, CLK-V5 was coimmunoprecipitated with CYC-FLAG, demonstrating that CLK and CYC are in the same complex (Fig. 2*E*). These results show that CLK stabilizes CYC by protecting CYC from proteasomal degradation.

***Clk*** **Promotes CYC Accumulation in Ectopic Cells, but Is Not Sufficient for Clock Function in All Ectopic Cells.** If CLK stabilizes CYC in vivo as it does in S2 cells, we predict that CYC will accumulate in cells that ectopically express CLK. To test this prediction, *Clk* was driven in *cry*-expressing clock and nonclock neurons using the 3.0*cry*-Gal4 driver (17) and in nonclock-expressing mushroom body neurons using the hormone-activated MB-GeneSwitch (MB-GS) driver (18). We first confirmed the spatial expression pattern of these drivers by using them to activate UAS-*lacZ*nls, which expresses nuclear-localized β-galactosidase. As expected, the 3.0*cry*-Gal4 driver is expressed in a subset of pacemaker neurons including ∼8 $DN_1$s, ∼2 $DN_3$s, $sLN_v$s, $lLN_v$s, and ∼6 $LN_d$s and in nonclock cell groups including the new 1, new 2, and dorsal optic lope (DOL) neurons (Fig. S1 *A*–*C*). Likewise, the MB-GS driver was strongly expressed in mushroom body neurons in the presence, but not the absence, of RU486 inducer (Fig. S1 *D*–*I*). We then determined whether CLK stabilizes CYC in nonclock cells by generating flies that contain the 3.0*cry*-Gal4 or MB-GS driver, a UAS-*Clk* responder, and the GFP-*cyc* transgene, collecting these flies at Zeitgeber Time 2 (ZT2, where ZT0 is lights on and ZT12 is lights off) and immunostaining them with GFP to detect CYC and PER to mark CLK-CYC–dependent gene expression.

When the 3.0*cry*-Gal4 driver was used to express *Clk*, GFP-CYC expression was expanded to include all endogenous pacemaker neurons and 3.0*cry*-Gal4 driver-specific nonclock cells (Fig. 3 *D*–*F*). Among the different nonclock cell groups, we focused on DOL cells since they comprise ∼20 cells that are spatially segregated from pacemaker neurons and other 3.0*cry*-Gal4–expressing cells. GFP-CYC was detected in DOL cells in the presence, but not in the absence, of *Clk* expression (Fig. 3 *A*–*C*), demonstrating that CLK promotes CYC accumulation in vivo.

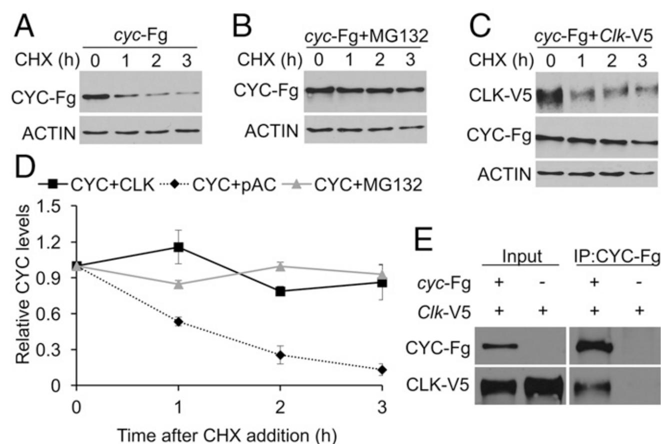


**Fig. 2.** CYC protein is stabilized when coexpressed with CLK. S2 cells transfected with pMK-*cyc*-FLAG (*cyc*-Fg) plasmid alone or in combination with pAc-*Clk*-V5 (*Clk*-V5) plasmid were incubated with $CuSO_4$ for 1 h to induce *cyc*-Fg expression and then treated with CHX to inhibit translation. (*A*) S2 cells transfected with *cyc*-Fg. (*B*) S2 cells transfected with *cyc*-Fg and treated with MG132 at 0 h. (*C*) S2 cells cotransfected with *cyc*-Fg and *Clk*-V5. Proteins were extracted from cells harvested at the indicated times after CHX addition and used to prepare Western blots that were probed with anti-FLAG, anti-CLK, and anti–β-ACTIN antibodies. (*D*) Relative CYC-Fg levels were quantified using Image J software as described (*Materials and Methods*) and plotted as the mean value ± SEM from four independent experiments. (*E*) Protein extracts from cells transfected with *Clk*-V5 alone or *Clk*-V5 and *cyc*-Fg were subjected to immunoprecipitation using anti-FLAG antibody. Western blots containing cell extracts (Input) or immune complexes (IP) were probed with anti-FLAG and anti-CLK antibodies.

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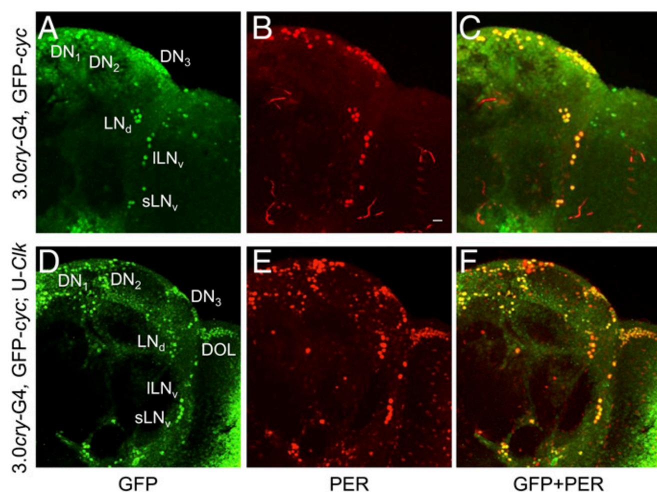


**Fig. 3.** CLK expression in DOL cells promotes GFP-CYC accumulation. The 3.0*cry*-Gal4, GFP-*cyc* (3.0*cry*-G4, GFP-*cyc*), and 3.0*cry*-Gal4, GFP-*cyc*; UAS-*Clk*/+ (3.0*cry*-G4, GFP-*cyc*; U-*Clk*) flies were entrained in LD for at least 3 d and collected at ZT2. Immunostaining with GFP and PER antibodies was performed on dissected brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in the text, and DOL cells are as defined in Fig. S1. Colocalization of GFP (green) and PER (red) is shown as yellow. (*A*–*C*) A 76-μm projected Z-series image of a 3.0*cry*-G4, GFP-*cyc* fly brain immunostained with GFP (*A*), PER (*B*), or GFP and PER (*C*). (*D*–*F*) An 86-μm projected Z-series image of a 3.0*cry*-G4, GFP-*cyc*; UAS-*Clk* fly brain immunostained with GFP (*D*), PER (*E*), or GFP and PER (*F*). GFP and PER immunostaining is detected in the indicated groups of pacemaker neurons and DOL cells, as well as in additional ectopic locations. (Scale bar, 10 μm.) All images are representative of six or more brains.

Moreover, PER also accumulates in pacemaker neurons and DOL cells (Fig. 3*E*), indicating that CLK-CYC activates downstream target genes. Consistent with previous results (8, 9), PER levels cycle in DOL cells during 12 h light:12 h dark (LD) cycles (Fig. 4 *B*, *E*, *G*, and *H*), although PER cycling amplitude in DOL cells is less robust than in $sLN_v$s (Fig. 4 *A*–*H*). These results show that *Clk* expression promotes CYC accumulation and PER cycling in DOL cells.

To determine if PER cycling in DOL cells is driven by LD cycles, we monitored PER rhythms in DOL cells and $sLN_v$s during DD. Flies containing 3.0*cry*-Gal4 and UAS-*Clk* were entrained in LD for 3 d, transferred to DD, and collected every 12 h for 2 d starting at circadian time 0 (CT0), which corresponds to subjective lights on. In $sLN_v$s, PER abundance showed significant ($P < 0.05$) circadian cycling with high levels at CT0, CT24, and CT48 and low levels at CT12 and CT36 (Fig. S2 *A* and *B*). In DOL cells, PER abundance was not significantly rhythmic, although PER levels at CT0 and CT24 were significantly ($P < 0.01$) higher than at CT12 (Fig. S2 *C* and *D*), indicative of a rapidly dampened rhythm.

When MB-GS was used to drive *Clk*, GFP-CYC was detected in all endogenous pacemaker neurons plus MB neurons (Fig. 5 *D*, *D1*, *F*, and *F1*), but only in endogenous pacemaker neurons in controls lacking MB-GS–driven *Clk* (Fig. 5 *A* and *C*). As in DOL cells, *Clk* expression supports PER accumulation in MB neurons (Fig. 5 *B*, *E*, and *E1*), indicating that CLK engages CYC to drive target gene transcription. However, PER levels were constant in MB neurons at ZT0 and ZT12 (Fig. 6 *B*, *E*, *G*, and *H*), in contrast to the robust rhythms of PER staining intensity seen in pacemaker neurons (Fig. 6 *A*–*H*) or in DOL cells during LD (Fig. 4 *B*, *E*, *G*, and *H*). From these results, we conclude that, even though *Clk* expression in MB neurons promotes CYC accumulation, it is not sufficient to support clock function.

**CRY Is Required for Ectopic Clock Function.** The ability of 3.0*cry*-Gal4–driven *Clk*, but not MB-GS–driven *Clk*, to generate ectopic clocks likely results from gene expression differences in these target cell populations. One obvious difference is that 3.0*cry*-Gal4 presumably drives expression only in CRY-positive cells, whereas no CRY is detected in MB neurons targeted by MB-GS (19–21). To confirm that CRY is expressed in DOL cells, we used a GFP-*cry* transgene to mark cells that endogenously express CRY with high sensitivity (19) and found that GFP-CRY is indeed expressed in DOL cells, albeit at lower levels than in pacemaker neurons (Fig. S3 *A* and *C*). Importantly, when 3.0*cry*-Gal4 was used to express UAS-*Clk* in GFP-*cry* flies, GFP-CRY levels increased substantially (Fig. S3*B*), suggesting that *Clk*-dependent factors enhance *cry* expression in DOL cells. Since *cry* expression is required for light entrainment and/or clock function in multiple peripheral tissues (22–24), *cry* may also be required for ectopic clock function. To

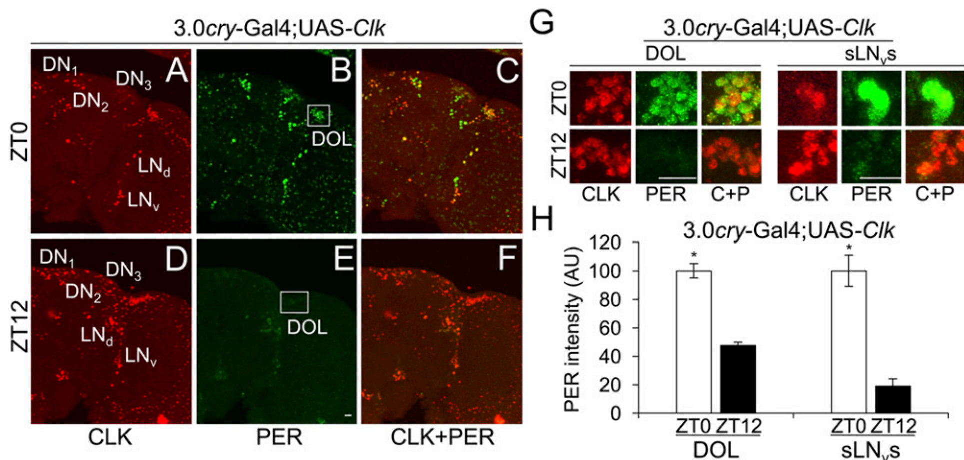


**Fig. 4.** *Clk* expression in DOL cells is sufficient for PER cycling in LD. The 3.0*cry*-Gal4/+; UAS-*Clk*/+ (3.0*cry*-Gal4; UAS-*Clk*) flies were entrained in LD for 3 d and collected at ZT0 and ZT12. Immunostaining with CLK and PER antibodies was performed on dissected brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in the text, and DOL cells are as defined in Fig. S1. Colocalization of CLK (red) and PER (green) is shown as yellow. (*A*–*C*) An 88-μm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (*A*), PER (*B*), or CLK and PER (*C*). (*D*–*F*) A 76-μm projected Z-series image from flies collected at ZT12 and immunostained with CLK (*D*), PER (*E*), or CLK + PER (*F*). (Scale bar, 10 μm). Panels *A*–*F* are the same magnification. (*G*) Magnified 24-μm projected Z-series images of DOL cells (*Left*) or magnified 12-μm projected Z-series images of $sLN_v$s (*Right*) from flies collected at ZT0 in *A*–*C* or ZT12 in *D*–*F*. (Scale bar, 10 μm.) C+P, CLK + PER. All images are representative of six or more brains. (*H*) PER immunostaining intensity was quantified in DOL cells and $sLN_v$s from flies collected at ZT0 and ZT12. AU, arbitrary units. Error bars indicate ±SEM. PER intensity was significantly (*$P < 0.001$) higher in both DOL cells and $sLN_v$s at ZT0 than at ZT12 by two-tailed Student's *t* test.

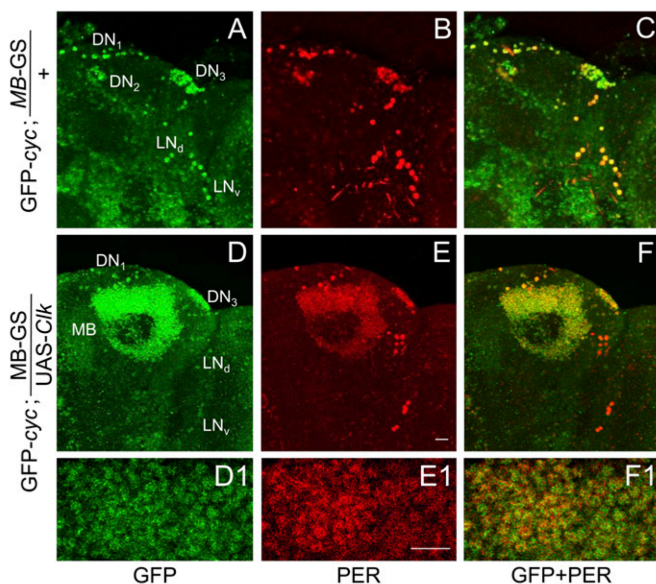


**Fig. 5.** CLK expression in MB neurons promotes GFP-CYC accumulation. GFP-*cyc*; MB-GS and GFP-*cyc*; *MB-GS*/UAS-*Clk* flies induced with RU486 were entrained and collected as described in Fig. 3. Immunostaining with GFP and PER antibodies was performed on dissected brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in the text, and MB neurons are as defined in Fig. S1. Colocalization of GFP (green) and PER (red) is shown as yellow. (*A*–*C*) A 106-μm projected Z-series image of a GFP-*cyc*; MB-GS fly brain immunostained with GFP (*A*), PER (*B*), or GFP and PER (*C*). (*D*–*F*) A 118-μm projected Z-series image of a GFP-*cyc*; MB-GS/UAS-*Clk* fly brain immunostained with GFP (*D*), PER (*E*), and GFP and PER (*F*). GFP and PER immunostaining are detected in the indicated groups of pacemaker neurons and in mushroom body neurons. (*D1*, *E1*, and *F1*) Magnified 2-μm image of MB neurons shown in *D*–*F*. (Scale bar, 10 μm.) All images are representative of six or more brains.

test this possibility, we used MB-GS to drive both *Clk* and *cry* expression in the presence of RU486 and assessed PER levels in MB neurons at ZT0 and ZT12. PER levels cycled robustly in MB neurons upon *Clk* and *cry* coexpression (Fig. 7), demonstrating that *cry* is indeed necessary for clock function in MB neurons.

We then determined whether PER cycling in MB neurons coexpressing *Clk* and *cry* persisted in DD. Although PER levels in $sLN_v$s showed significant ($P < 0.05$) circadian cycling with peaks at CT0, CT24, and CT48 and troughs at CT12 and CT36 (Fig. S4 *A* and *B*), the levels of PER in MB neurons did not show significant cycling (Fig. S4 *C* and *D*). However, PER levels in MB neurons at CT0 and CT24 were significantly ($P < 0.01$) higher than at CT12 (Fig. S4*D*), indicating that PER oscillations dampen rapidly in DD. Thus, ectopic clocks in MB neurons, like those in DOL cells, show a robust rhythm in PER cycling that quickly dampens in DD.

## Discussion

Here, we show that clock neuron-specific CYC accumulation in clock neurons and in *Clk*-dependent ectopic clocks in brain neurons is due to stabilization of CYC by CLK. In pacemaker neurons and whole fly heads, CLK is required for the accumulation of CYC (Fig. 1), demonstrating that *Clk* is required for CYC accumulation in clock cells. Experiments in S2 cells showed that CYC has a short ∼1-h half-life due to proteasomal degradation that is lengthened approximately ninefold when *Clk* is coexpressed (Fig. 2). Since CLK and CYC form complexes in S2 cells, the most parsimonious conclusion is that CLK-CYC heterodimerization stabilizes CYC via protection from proteasomal degradation.

Costabilization of heterodimeric transcription factors is not common, but two C/EBP family members, Ig/EBP and CHOP, are stabilized upon heterodimerization (16), and the *Neurospora* zinc-finger-PAS circadian activator White Collar 1 (WC1) is stabilized by White Collar 2 (WC2) upon WC1-WC2 heterodimerization (15). Our data account for CYC accumulation solely in *Clk*-expressing neurons and further define the first events required to initiate clock function in *Drosophila*. In mammals, *Bmal1* mRNA is expressed at high levels, but BMAL1 levels are low in $Clock^{-/-}$ animals (25). Given that *Clock* and *Bmal1* are orthologs of *Clk* and *cyc*, respectively (2), the stabilization of BMAL1 by CLOCK may be a conserved property of these proteins. Moreover, since WC2 stabilizes WC1 in *Neurospora*, stabilization of one circadian activator by its partner may be a conserved feature of eukaryotic clocks.

CLK likely binds CYC soon after CYC synthesis to produce stable CLK-CYC heterodimers. Since *cyc* mRNA does not cycle (26), CLK-CYC production is likely driven by rhythms in *Clk*

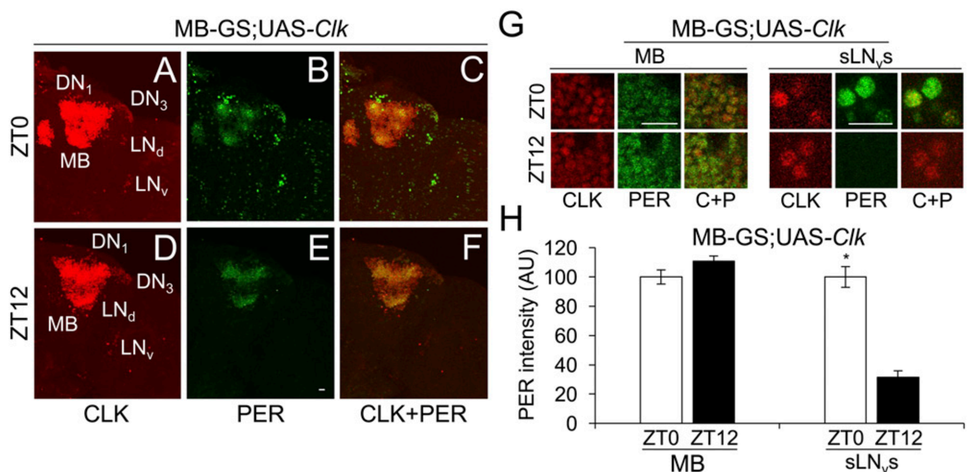


**Fig. 6.** *Clk* expression in MB neurons does not support PER cycling in LD. MB-GS/+; UAS-*Clk*/+ (MB-GS; UAS-*Clk*) flies were induced with RU486, entrained and collected as described in Fig. 4. Immunostaining with CLK and PER antibodies was performed on dissected adult brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in the text, and MB neurons are as defined in Fig. S1. Colocalization of CLK (red) and PER (green) is shown as yellow. (*A*–*C*) A 136-μm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (*A*), PER (*B*), or CLK and PER (*C*). (*D*–*F*) A 136-μm projected Z-series image of a brain from flies collected at ZT12 and immunostained with CLK (*D*), PER (*E*), or CLK and PER (*F*). (Scale bar, 10 μm). Panels *A*–*F* are the same magnification. (*G*) Magnified 2-μm images of MB neurons (*Left*) and magnified 18-μm projected Z-series images of $sLN_v$s (*Right*) from flies collected at ZT0 in *A*–*C* or at ZT12 in *D*–*F*. (Scale bar, 10 μm.) C+P, CLK + PER. All images are representative of six or more brains. (*H*) PER immunostaining intensity was quantified in MB neurons and $sLN_v$s from flies collected at ZT0 and ZT12. AU, arbitrary units. Error bars indicate ±SEM. PER intensity was significantly (*$P < 0.01$) higher in $sLN_v$s at ZT0 than at ZT12 by two-tailed Student's *t* test.

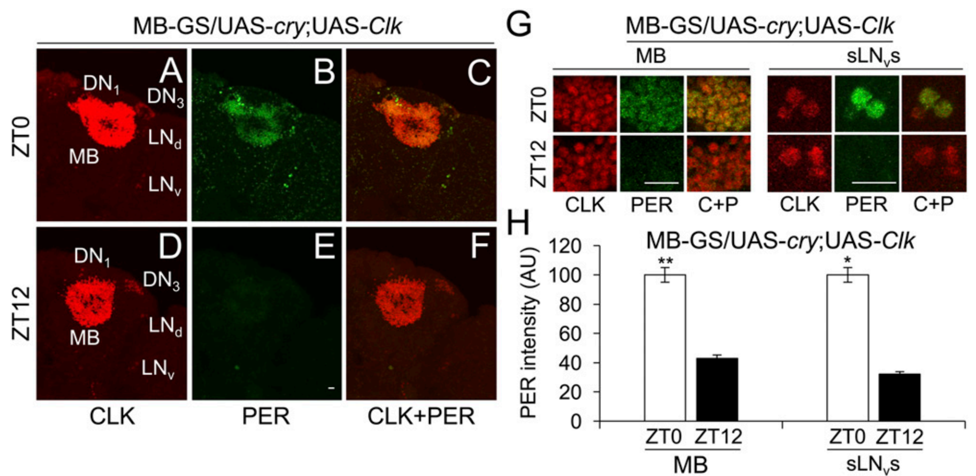


**Fig. 7.** *Clk* and *cry* expressions are required to support PER cycling in MB neurons during LD. MB-GS/UAS-*cry*; UAS-*Clk*/+ (MB-GS/UAS-*cry*; UAS-*Clk*) flies were induced with RU486, entrained and collected as described in Fig. 4. Immunostaining with CLK and PER antibodies was performed on dissected brains and imaged by confocal microscopy. Projected Z-series images of the right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in the text, and MB neurons are as defined in Fig. S1. Colocalization of CLK (red) and PER (green) is shown as yellow. (*A*–*C*) A 122-μm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (*A*), PER (*B*), or CLK and PER (*C*). (*D*–*F*) A 122-μm projected Z-series image of a brain from flies collected at ZT12 and immunostained with CLK (*D*), PER (*E*), or CLK and PER (*F*). (Scale bar, 10 μm). Panels *A*–*F* are the same magnification. (*G*) Magnified 2-μm images of MB neurons (*Left*) and magnified 16-μm projected Z-series images of $sLN_vs$ from flies collected at ZT0 in *A*–*C* or at ZT12 in *D*–*F*. (Scale bar, 10 μm.) C+P, CLK + PER. All images are representative of six or more brains. (*H*) PER immunostaining intensity was quantified in MB neurons and $sLN_vs$ from flies collected at ZT0 and ZT12. AU, arbitrary units. Error bars indicate ±SEM. PER intensity was significantly (**$P < 0.001$) higher in MB neurons and significantly higher in $sLN_vs$ (*$P < 0.01$) at ZT0 than at ZT12 by two-tailed Student's *t* test.

mRNA, which peak near dawn (27, 28). Increased CLK-CYC production near dawn apparently offsets degradation due to CLK phosphorylation early in the day, resulting in constant CLK (and thus CLK-CYC) levels (29, 30). Just as CYC levels are low in the absence of CLK, CLK levels decrease in the absence of CYC despite high levels of *Clk* mRNA (30, 31). Consequently, the vast majority of CLK and CYC are present as stable CLK-CYC heterodimers, which apparently accumulate at levels determined by CLK abundance. If CLK levels fall, as in the $Clk^{ar}$ mutant, then target gene cycling is diminished and rhythmic behavior is disrupted (32). Likewise, increased CLK activity, as seen in flies lacking *Clk* 3′UTR regulatory sequences, disrupts CLK-CYC target gene transcription and behavioral rhythms (33). The loss of *Clk* 3′UTR regulatory sequences causes ectopic *Clk* expression in the brain and production of additional PIGMENT DISPERSING FACTOR (PDF) neuropeptide-expressing neurons, which likely account for variable CLK-CYC target gene expression and arrhythmic behavior, respectively (33).

Because *cyc* mRNA expression is not restricted to pacemaker neurons in the brain (7), and ectopic clock generation by *Clk* is *cyc*-dependent (9), CLK is predicted to stabilize CYC in nonclock cells. Indeed, *Clk* expression promotes CYC accumulation in *cry*-expressing nonclock neurons and in MB neurons (Figs. 3 and 5). Although *cyc* mRNA can give rise to CYC when *Clk* is ectopically expressed, *cyc* mRNA function in nonclock cells is not known. CYC could be generated and rapidly degraded in many nonclock cell types, but protected from degradation by other binding partners that are induced, for example, in response to environmental stress. Alternatively, *cyc* mRNA may function directly, independent of producing CYC protein, in nonclock cells. Further studies are necessary to define *cyc* mRNA function in nonclock cells.

Once CYC is stabilized by CLK, CLK-CYC complexes can activate target gene transcription. In *cry*-expressing DOL cells, *Clk* expression induces ectopic clock function as measured by rhythms in PER accumulation that parallel those in pacemaker neurons during LD (Fig. 4). PER rhythms persist with a reduced amplitude on DD day 1 and are lost by DD day 2 (Fig. S2). This inability to maintain a robust rhythm is reminiscent of fly peripheral clocks that show lower amplitude rhythms than brain pacemaker neurons (34), which maintain high-amplitude rhythms via reinforcing neuronal signaling (34, 35). Nevertheless, *Clk*-induced ectopic clocks maintain high-amplitude PER rhythms under LD conditions (Fig. 4), indicative of a functional molecular clock. Although CLK-CYC activates feedback repressors to drive ectopic clock function, other clock components including posttranslational regulators (e.g., kinases, phosphatases) must be expressed in ectopic cells (1). These posttranslational clock regulators are likely widely expressed since they control many regulatory pathways, although some could be activated via ectopic *Clk* expression since they contain E-box regulatory elements bound by CLK-CYC (12).

Although *Clk* is sufficient to generate ectopic clocks in DOL cells, this was not the case in MB neurons, where *Clk* expression led to constant PER levels during LD (Fig. 6). One difference between DOL cells and MB neurons is that DOL cells express CRY (Fig. S3 *A* and *C*), while MB neurons lack CRY expression (20, 21). Since CRY mediates light entrainment in some pacemaker neurons and is necessary for light entrainment and clock function in peripheral tissues (19, 22, 23, 36–39), our inability to generate an ectopic MB clock may be due to the lack of CRY. Indeed, expressing both *Clk* and *cry* in MB neurons resulted in robust PER cycling in LD (Fig. 7), indicative of ectopic clock function. These *Clk*- and *cry*-induced PER rhythms in MB neurons mirror those in pacemaker neurons during LD, but rapidly dampen in DD (Fig. S4). The rapid dampening of PER rhythms in MB neurons is similar to that in DOL cells during DD (Fig. S2) and is faster than that in peripheral clocks using *per*-luciferase or *tim*-luciferase reporter assays (22, 23).

The inability of DOL cells and MB neurons to sustain clock function in DD likely stems from multiple factors including suboptimal or nonrhythmic expression of genes that contribute to timekeeping (e.g., *Clk*, *cry*, posttranscriptional regulators) and a lack of intercellular coupling that sustains robust rhythms in pacemaker neurons (34, 35). Although 3.0*cry*-Gal4–driven UAS-*Clk* enhances GFP-CRY expression in DOL cells (Fig. S3*B*), MB-GS–driven UAS-*Clk* is apparently unable to activate *cry* expression in MB neurons, suggesting that CLK can engage factors in DOL cells to increase *cry* expression, but cannot engage factors required to activate *cry* in MB neurons. These results suggest that the properties of molecular clocks in canonical and ectopic cells differ depending on the function and gene expression characteristics of the cell.

Our experiments demonstrate that *cry*, like *Clk*, is required for ectopic clock function. Since *cyc* is also necessary for ectopic clock function, it is possible that naive *Drosophila* cells can be programmed to express molecular clocks by expressing *Clk*, *cyc*, and *cry*. If such clock programming is possible, this work could

lead to the development of *Drosophila* cell lines having clocks that operate in LD. The resulting cell lines would be analogous to monarch DpN1 cells, which possess a robust molecular clock that operates only in LD (40), yet represent a valuable tool for understanding the molecular machinery required for feedback loop function.

## Materials and Methods

The following *Drosophila* strains were used in this study: $w^{1118}$, w; Cyo/Sco; TM2/TM6B, $cyc^{01}$ (26), $Clk^{out}$ (10), GFP-*cyc*; $cyc^{01}$ (5), w; *cyc*-Flag (12), 3.0*cry*-Gal4 (17), MB-GeneSwitch (18), UAS-*Clk* (8), UAS-*lacZ* (#3955; Bloomington Drosophila Stock Center), UAS-*cry* (37), and GFP-cry (19). The pMK33-TAP-3XFLAG-6XHis expression vector (10) was used to generate the pMK33-TAP-3XFLAG-6XHis-*dcyc* (pMK33-*cyc*-Flag) plasmid for inducing *cyc* expressing in S2 cells. S2 cells maintained in Schneider's *Drosophila* medium with 10% FBS and antibiotics were transfected, and gene expression was induced under conditions used to measure proteasomal degradation and protein half-life (10, 41). Western blots containing S2 cell and fly-head extracts and immunoprecipitates were prepared, probed with antisera, and quantified as described (10). RT-qPCR was carried out on fly heads as described (42). RU486 induction of MB-GS expression was carried out as described (18) with modifications. Antibody staining and imaging in adult brains was carried out as previously described (5, 43, 44). Immunostaining in clock cells was quantified from digital images of fly brains as described (9). For details concerning plasmid construction, S2 cell experiments, Western blot analysis, RT-qPCR analysis, RU486 induction, immunoprecipitations, and brain immunostaining, imaging, and quantification, please refer to *SI Materials and Methods*.

**ACKNOWLEDGMENTS.** We thank the following people for providing fly stocks for this study: Patrick Emery for UAS-*cry*, Michael Rosbash for w; *cyc*-Flag and $cyc^{01}$, Ravi Allada for UAS-*Clk*, and Gregg Roman for MB-GeneSwitch. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were also used in this study. We also thank Michael Rosbash for the pAC-*Clk*-V5 plasmid and Stan Vitha from the Texas A&M Microscopy and Imaging Center for help with confocal microscopy. This work was supported by NIH Grant NS094807.

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