Understanding the role of chromatin remodeling in the regulation of circadian transcription in Drosophila

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Circadian clocks enable organisms to anticipate daily changes in the environment and coordinate temporal rhythms in physiology and behavior with the 24-h day-night cycle. The robust cycling of circadian gene expression is critical for proper timekeeping, and is regulated by transcription factor binding, RNA polymerase II (RNAPII) recruitment and elongation, and post-transcriptional mechanisms. Recently, it has become clear that dynamic alterations in chromatin landscape at the level of histone posttranslational modification and nucleosome density facilitate rhythms in transcription factor recruitment and RNAPII activity, and are essential for progression through activating and repressive phases of circadian transcription. Here, we discuss the characterization of the BRAHMA (BRM) chromatin-remodeling protein in Drosophila in the context of circadian clock regulation. By dissecting its catalytic vs. non-catalytic activities, we propose a model in which the non-catalytic activity of BRM functions to recruit repressive factors to limit the transcriptional output of CLOCK (CLK) during the active phase of circadian transcription, while the primary function of the ATP-dependent catalytic activity is to tune and prevent over-recruitment of negative regulators by increasing nucleosome density. Finally, we divulge ongoing efforts and investigative directions toward a deeper mechanistic understanding of transcriptional regulation of circadian gene expression at the chromatin level.

Introduction

The circadian clock is an endogenous timer that enables organisms from all kingdoms of life to anticipate environmental changes associated with the 24-h rotation of the Earth and maintains daily rhythms in physiology and behavior. The molecular design of the circadian oscillator and the mechanisms that relay the temporal information to output molecular pathways underlying biological rhythms have continued to fascinate researchers and have been studied extensively over the past two decades.

As in other organisms studied to date, the Drosophila circadian oscillator relies on a transcriptional-translational feedback mechanism to maintain endogenous cycling. Two basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) transcription factors CLOCK (CLK) and CYCLE (CYC) are the key activators of circadian transcription. Within the core oscillator, they form heterodimers and bind to the E-box sequences of period (per) and timeless (tim) in a time-of-day specific manner, initiating transcription of these negative elements in the late day. CLK-activated gene expression subsequently peaks in the early evening. After a time-delay established by post-transcriptional and post-translational mechanisms, which are critical to extend the oscillatory cycle to a full 24 hour, PER and TIM enter the nucleus and inhibit the transcriptional activity of CLK-CYC later in the evening. In a second interlocked feedback loop, CLK-CYC heterodimers activate PAR Domain Protein 1ε (PDP1ε) and VRILLE (VRI), two transcription factors responsible for activating and repressing Clock expression, respectively. As PDP1ε and VRI both bind to D-box elements (also called V/P box) on the clock promoter, the interplay between these two factors, and the delayed expression of PDP1ε relative to that of VRI, restrict the timing of prominent Clock expression between...
early to mid-day. Two other transcription factors, *clockwork orange* (*cwo*)[^10]-[^13] and *nejire* (*nej*) CREB-binding protein (*CBP*)[^14,15] have also been observed to modulate CLK-activated transcription and affect the core oscillator function, but their roles still need to be further clarified. Outside of the core oscillator, CLK has been observed to bind to at least 800 loci within the *Drosophila* genome based on chromatin immunoprecipitation (ChIP)-seq analysis, illustrating the mechanism by which the oscillator relays temporal cues and controls circadian cycling of output genes.[^16]

While the interactions between the key transcription factors of the *Drosophila* circadian oscillator, namely PER, TIM, CLK, CYC, and their rhythmic association with target circadian gene promoters are well characterized,[^6]-[^15] much less is known about the regulation at the chromatin level to facilitate daily rhythms in transcription until recently. Using ChIP followed by quantitative PCR (qPCR) and ChIP-seq analysis in *Drosophila*, mammalian systems, as well as in other clock models, a number of targeted and genome-wide studies have shown that clock-regulated genes undergo extensive daily chromatin remodeling with respect to histone modifications as well as nucleosome condensation.[^18]-[^22] In *Drosophila*, daily rhythms of H3K9 acetylation, H3K9 dimethylation, and H3K4 trimethylation are tightly linked to rhythmic per and tim expression.[^19]

It is now widely recognized that modulating genomic accessibility for transcription factor binding and RNA-PPI activity in a time-dependent manner to enable cycling gene expression involves the collaborative action of multiple regulatory protein complexes. Consequently, much effort has been dedicated to uncovering these histone modifiers and ATP-dependent chromatin remodelers.[^20]-[^36] For example, in mammals, multiple histone modifiers have been identified to regulate interactions between DNA and key clock transcription factors through posttranslational modifications. Positive regulators that were identified include the methyltransferase MLL1 (Mixed Lineage Leukemia protein 1), which methylates H3K4,[^25] and the histone demethylase JARID1A (Jumonji/ARID domain-containing protein 1A), which was found to prevent histone deacetylase (HDAC) activity and promote circadian transcriptional activity.[^26] The search for negative transcriptional regulators has identified SIRT1 (Sir2uin 1), an NAD[^17]-dependent HDAC that counterbalances the putative histone acetyltransferase (HAT) activity of mammalian CLK.[^27,28] More examples of histone modifiers that act as negative regulators of circadian transcription are discussed below. In contrast, less is known about the role of ATP-dependent chromatin remodelers in circadian transcription, except in the *Neurospora* clock. Four ATP-dependent chromatin remodelers have now been characterized and have been shown to modulate nucleosome density at different phases of *Neurospora* circadian transcription, with some promoting the activation phase (CLOCK ATPase (CATP) and SW1itch/Sucrose Non-Fermentable (SWI/SNF)) while others are critical to repression (CLOCK-SWITCH-1 (CSW-1)).[^29,32] The role of chromodomain helicase DNA-binding protein 1 (CHD1) is a bit more enigmatic. It is required for remodeling at the frequency (*frq*) locus, which influences DNA methylation status and consequently affects the phasing of the clock.[^32] We anticipate the same scenario in animal systems, including in *Drosophila*, in which different ATP-dependent remodelers function at different phases of circadian transcription to enable smooth transitions between permissive and repressive chromatin landscapes and vice versa to facilitate cycling transcription. So far, identification of these histone modifiers and chromatin remodelers have already led to significant progress and improved our understanding of circadian transcriptional control at the chromatin level.

The next challenge is to understand the mechanisms by which histone modifiers and ATP-dependent remodelers interact with each other as well as with circadian transcription factors to establish time-dependent changes in chromatin landscape, regulate RNAPII activity, and maintain robust cycling gene expression. For example, PER proteins have been found to be in complex with the SIN3A-HDAC1 histone deacetylase, Heterochromatin protein 1 (HP1)-Suv39h histone methyltransferase, and subunits of the Mi-2/nucleosome remodeling and deacetylase (NuRD) corepressor complexes in mouse liver extracts.[^24,33,34] Knockdown of these histone modifiers and remodeling proteins in mammalian cells expressing a bioluminescence reporter using siRNAs suggests that PER may recruit these protein complexes to circadian promoters to orchestrate rhythmic repression of CLK-BMAL1 (BMAL1 is the mammalian homolog of CYC) activity. In addition, mammalian REV-ERBα is known to interact with the nuclear receptor corepressor 1 (NCOR1)-HDAC3 complex to negatively regulate bmal1 expression.[^35] Here, we describe our recent identification of the BRAHMA (BRM) chromatin-remodeling complex as a CLK interactor in *Drosophila*, and discuss its regulation of circadian transcription by limiting CLK activity through both catalytic and non-catalytic mechanisms.[^36]

A chromatin remodeler regulates the *Drosophila* circadian clock

Using a mass spectrometry (MS) label-free quantitative proteomics approach, we observed that the core subunits of the BRM complex, including BRM, MOIRA, Bap55, BAP60, BAP111, and SNR1, interact with CLK in the nucleus of *Drosophila* Schneider (S2) cells (Table 1). Protein lysate was extracted from stable *Drosophila* S2 cell lines expressing N- or C-terminally 3XFLAG-tagged CLK. This was followed by subcellular fractionation and affinity purification using FLAG resins prior to MS analysis to identify native CLK-containing complexes in the nucleus. Raw MS data were first searched against the Uniprot database for *D. melanogaster* proteins followed by SAINT (Significance Analysis of Interactome) to statistically distinguish true interactors from background interactors. A stable cell line that expresses an empty vector was used for background deduction using a False Discovery Rate (FDR) of <0.002.[^37]

The BRM complex in *Drosophila* is part of the SWI/SNF chromatin-remodeling
family with members in both prokaryotes and eukaryotes.38 These complexes have roles in the repositioning, reconfiguring, and ejecting of nucleosomes, and have been proposed to be key regulators of the expression of many genes.38-42 Knocking down the BRM complex in mammalian cells resulted in both upregulation as well as downregulation of genes as assayed by ChIP-seq, supporting the notion that BRM functions as either transcriptional activator or repressor in a gene-specific manner.40 The BRM complex is named for its catalytic subunit, the BRM protein, which contains an ATPase domain to enable the hydrolysis of ATP for mobilization of nucleosomes. This catalytic activity can facilitate or repress access of protein regulators to the chromatin. The BRM complex has been implicated in pre-mRNA splicing regulation of developmental genes.37,42

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| Symbol + FlyBaseID | Peptide
dCount (3 reps) | AvgP | MaxP | False
dDiscovery Rate (FDR) | Peptide
count (3 reps) | AvgP | MaxP | False
dDiscovery Rate (FDR) | Control
AP-MS |
|-------------------|-------------------|-----|-----|------------------------|-------------------|-----|-----|------------------------|--------|
| brahma FBgn0000212 | 25/23 | 0.67 | 1 | 0.0395 | 10/2 | 0.71 | 0.995 | 0.0453 | 3/0 |
| bap55 FBgn0025463 | 21/23 | 1 | 1 | 0.0000 | 4/0 | 0.32 | 0.952 | 0.2590 | 2/0 |
| snr1 FBgn0011715 | 12/5 | 1 | 0.999 | 0.0004 | 24/13 | 1 | 1 | 0.0000 | 2/0 |
| bap111 FBgn0030093 | 13/4 | 0.91 | 0.999 | 0.0098 | 3/0 | 0.55 | 0.899 | 0.1582 | 0/0 |
| moira FBgn0002783 | 41/18 | 0.65 | 0.977 | 0.0772 | 33/13 | 0.27 | 0.829 | 0.3252 | 12/5 |

Table 1. Subunits of the BRM chromatin-remodeling complex interact with CLK in the nucleus of Drosophila Schneider (S2) cells as detected by FLAG affinity purification followed by mass spectrometry.

Expression via dsRNA in tim-expressing clock neurons (Fig. 1A), and transgenic flies expressing a catalytic-inactive brmK804R variant, also in tim-expressing clock neurons (Fig. 1B).36 This mutation produces BRMK804R with a lysine to arginine substitution in its ATPase site.43 We expect that both catalytic activity (i.e. ability to condense or decondense chromatin) and non-catalytic activity (i.e., ability to bind protein interactors) of BRM would be reduced in the brm RNAi mutants, whereas only the catalytic activity would be abolished in the brmK804R mutants. We therefore hypothesized that characterizing both brm RNAi and brmK804R mutants in comparison to control flies (Fig. 1C) will allow us to differentiate between the catalytic vs. non-catalytic role of the BRM complex in regulating circadian gene expression. Our results are summarized in Figure 1D.

First, we verified the functionality of the BRM complex in regulating the circadian oscillator by examining the locomotor activity rhythms of BRM complex mutants using Drosophila Activity Monitoring System (DAMS).44 Impaired clock function can be detected as changes in clock-controlled free-running rhythms, i.e. activity rhythms that are only driven by the endogenous clock when flies are held in constant darkness, as compared to wild type control. Knockdown of brm expression via RNAi resulted in the lengthening of circadian period (Fig. 1D). Interestingly, independent knockdown of other subunits of the BRM complex also result in similar period-lengthening phenotype, suggesting that the whole protein complex is required for normal circadian clock function.36 On the other hand, flies expressing brmK804R in clock neurons exhibited lengthened rhythms upon transition to free-running condition, but their phenotype quickly deteriorated to arrhythmicity (Fig. 1D). The more severe phenotype in the catalytically inactive brmK804R mutant cannot be explained easily, but it is possible that increasing the level of brm knockdown in the RNAi mutant (i.e., decrease in functional BRM protein) could lead to locomotor activity rhythm defect that is more in line with the brmK804R mutant. The observed brm RNAi knockdown in fly heads in our experiments was 40% on average.36 Nevertheless, activity rhythm phenotypes of the two brm mutants support an important role of BRM in clock regulation. This is further supported by our observation that BRM binds to per and tim promoters as assayed by ChIP-qPCR.36 Although we only investigated binding at per and tim promoters, it is likely BRM also binds to other CLK-activated target genes. The extent of the role of BRM in regulating circadian transcription will need to be confirmed by comprehensive ChIP-seq analysis.

The most surprising finding of our study was perhaps the fact that the two
classes of \textit{brm} mutants we examined exhibited opposing effects on circadian gene expression, with \textit{brm} RNAi displaying increased clock gene expression compared to control and \textit{brm} \textit{K804R} showing downregulation of clock gene expression (Fig. 1D). Since both classes of \textit{brm} mutants were predicted to show a decrease in catalytic activity, we postulate that the BRM complex normally functions to condense the chromatin at clock gene loci. Moreover, our results strongly suggested that it is likely the intact non-catalytic activity of BRM \textit{K804R} that led to the differential effects exhibited by the two classes of \textit{brm} mutants with respect to clock gene expression. We postulate that when \textit{brm} is knocked down via RNAi, the chromatin is more open due to a loss of nucleosome remodeling catalytic activity, allowing for an aberrant elevation in gene expression that results partly from an increased recruitment of general transcription machinery (Fig. 2A). While this explains the increase in clock gene expression and apparent decrease in RNAPII stalling in flies expressing \textit{brm} RNAi, the opposite effect in clock gene expression observed in flies expressing the catalytically-inactive \textit{brm} \textit{K804R} remains puzzling, especially since the chromatin is also more relaxed in \textit{brm} \textit{K804R} flies as compared to wild type (Fig. 2B). Fortunately, investigation of changes in CLK binding to the \textit{per} and \textit{tim} promoters promptly provided us with valuable clues to indicate that the non-catalytic activity retained by BRM \textit{K804R}, which is knocked down in \textit{brm} RNAi mutant, is the driving force for decreased clock gene expression in flies expressing \textit{brm} \textit{K804R}. ChIP-qPCR revealed that CLK binding to \textit{per} and \textit{tim} promoters in \textit{brm} \textit{K804R} mutants is lower compared to that of control flies, despite the more open chromatin in these mutants (Fig. 2B). This suggests that the catalytically-inactive BRM \textit{K804R} protein can influence CLK occupancy independent of ATP-dependent modifications in chromatin structure, perhaps through recruitment of repressive factors or factors that negatively impact CLK levels.

In light of this observation, along with the contrasting effects on circadian gene expression observed in \textit{brm} RNAi versus \textit{brm} \textit{K804R} mutants, we formulated a model that could explain the molecular phenotypes observed in the two classes of \textit{brm} mutants and describe the catalytic and non-catalytic function of BRM (Fig. 2C). We propose that the BRM complex recruits repressive complexes to negatively regulate CLK binding and/or restrain CLK activity during the active phase of clock gene transcription, and that over-accumulation of these repressive complexes is prevented by its ATP-dependent remodeling activity that serves to increase nucleosome density, thus counterbalancing and fine-tunes its non-catalytic function. As a result, in transgenic flies expressing BRM \textit{K804R}, the loss of catalytic activity to maintain or increase nucleosome density leads to the over-recruitment of repressive factors (Fig. 2B). The end result is a decrease in clock gene expression in \textit{brm} \textit{K804R} mutants. The initial period-lengthening phenotype of \textit{brm} \textit{K804R} mutants that quickly deteriorates into arrhythmicity in free-running conditions could potentially be explained by the fact that rhythmic circadian gene expression is highly dependent on rhythmic CLK binding to \textit{per} loci, which is significantly diminished in this mutant.36 Moreover, it is plausible to predict that the \textit{brm} \textit{K804R} mutation may impact output molecular pathways affecting activity rhythms, which could also contribute to the observed behavioral phenotype. In flies expressing \textit{brm} RNAi, knockdown of \textit{brm} expression results in the decrease of both catalytic and non-catalytic functions, leading to a twofold relief of repression when compared to the \textit{brm} \textit{K804R} mutant (Fig. 2A). The reduction in non-catalytic activity leads to the decrease in repressive complexes that normally localize to circadian loci through interactions with BRM, and the loss of catalytic activity results in a decrease in nucleosome density, which may allow for potential

*Figure 1.* The role of BRM in Drosophila circadian transcription. (A) Flies in which \textit{brm} RNAi is used to knock down \textit{brm} expression are expected to exhibit both decrease in catalytic and non-catalytic activity. Decrease in the latter is postulated to result in decreased recruitment of BRM-interacting proteins to CLK-regulated promoter regions. Interacting proteins are designated as P1, P2, and P3. Reduction in catalytic activity results in decrease in nucleosome density. (B) Flies expressing the \textit{brm} \textit{K804R} allele express a catalytically-inactive BRM protein that is unable to hydrolyze ATP to remodel chromatin (increase nucleosome density), yet retains interactions with other proteins.43 (C) Control “wild type” flies expressing native BRM protein with intact catalytic and non-catalytic activity. (D) An overview of findings from Kwok et al,36 including the phenotypes of the two \textit{brm} mutants with respect to clock locomotor activity rhythm, expression of core clock genes, nucleosome density, and CLK localization at the \textit{per} promoter.
increase of factors that have an activating effect on gene transcription. This leads to an outcome of increased circadian gene expression. Specifically, per mRNA expression was shown to be elevated in flies expressing brm RNAi (Fig. 1D). The consequent increase in PER protein levels could explain the period-lengthening effect of brm RNAi on circadian activity rhythms, as previous observations of increased or more stable PER proteins in other circadian mutants also led to period-lengthening phenotypes.

Emergent non-catalytic role of chromatin remodelers

One of the most illuminating findings in Kwok et al. was that the non-catalytic activity of BRM plays a critical role in regulating circadian transcription independently of ATP-dependent catalytic activity, possibly by recruiting repressive factors. While the role of SWI/SNF complexes and other chromatin remodelers as transcriptional regulators has come to light in recent years, many studies focused on characterizing the role of their catalytic activity in regulating gene expression by modulating nucleosome density. These studies largely utilized gene knockdown as the sole method for impairing the function of the BRM complex. Only a few studies have investigated its non-catalytic role in gene regulation. For instance, by using a dominant negative mutation in an evolutionarily conserved core BRM complex subunit SNR1, Marenda et al. showed that the interaction of SNR1 with wing-specific repressor NET and HDACs represses rhomboid expression and wing vein development. Similarly to our findings, these repressive functions are independent of ATP-dependent catalytic activity. In addition, lysine-specific demethylase 1 (LSD1) has been found to bind and function as a co-repressor of SNR1 during Drosophila wing development.

Such findings provide strong support that BRM has non-catalytic functions to repress gene expression through association with repressive factors.

Another relevant example that illustrates the importance of non-catalytic activity of chromatin remodelers is the recruitment of a repressive complex by the NuRD complex to regulate the mammalian clock. The NuRD complex is an ATP-dependent chromatin remodeler that has been shown to repress gene expression in numerous cancer cell types. Multiple subunits of the NuRD complex, including chromodomain helicase DNA binding protein 4 (CHD4), metastasis-associated protein 2 (MTA2), methyl-CpG-binding domain protein 2 (MBD2), GATAD2a, HDAC1, and RbAp48 have been observed to coimmunoprecipitate with PER2 from mouse liver extracts. The catalytic subunit CHD4 is found to bind to CLK-BMAL1 during the active phase of circadian transcription and increase transcriptional activity by recruiting RNAPII. Whereas CHD4 and MTA2 were observed to interact with CLK-BMAL1 during both the activation and repression phases of circadian transcription, other subunits of the NuRD complex that have roles in conferring repression (HDAC1, MBD2, GATAD2a, RbAp48) are exclusively localized with PER2 until the repression phase. The split NuRD complex is therefore only fully assembled when PER2
binds to CLK-BMAL1, thereby initiating full repression. The NuRD complex is also a candidate repressor that may interact with BRM to modulate CLK binding and activity. One class of histone modifying enzymes that are likely candidates are HDACs, as many HDACs have been found to be under circadian control as well as having direct interactions with clock proteins.\(^{33,35,55,56}\) The repressive Sin3-HDAC complex, which is an evolutionarily conserved protein complex that includes HDAC1 and HDAC2,\(^{55}\) has been found to coprecipitate with mammalian PER complexes and aid in the repression of circadian transcription.\(^{33}\) BRG1 (mammalian homolog of \textit{Drosophila} BRM protein) and other components of the SWI/SNF complex have also been found to interact with components of Sin3-HDAC,\(^ {57-59}\) pointing to the likelihood that BRM can directly associate with proteins involved in histone deacetylation and repression of clock genes. In addition to interacting with HDAC1- and HDAC2-containing complexes, BRG1 and other BRM complex related proteins have been observed to associate with corepressor complex NCOR1, which contains HDAC3.\(^{60}\) This particular co-repressor complex has been shown to repress gene expression in the mammalian circadian clock.\(^{35,56}\) Taken together, there is ample evidence that the BRM complex can interact with repressive factors. This supports our model in which BRM limits CLK target gene output in part through non-catalytic interactions with repressive factors (Fig. 3A). It is noteworthy that while our results suggest that BRM has a repressive role in \textit{Drosophila} clock, recent investigation into the role of a \textit{Neurospora} SWI/SNF chromatin remodeler revealed that it promotes transcriptional activation of the clock gene frequency.\(^{30}\) While the non-catalytic effects were not investigated in the \textit{Neurospora} study, varying effects of SWI/SNF complexes may be partly attributed to interaction with protein complexes that have activating and/or repressive functions through the non-catalytic activity of these remodelers. To extend this idea a little further, is it possible that the primary function of the catalytic activity of these chromatin remodelers is to tune the effects exerted by their non-catalytic activity to recruit positive or negative regulators, similar to a rheostat?

**The recruitment of BRM to CLK-activated promoters**

In addition to identifying potential repressive factors that are recruited by BRM to regulate the circadian transcriptome, studies to understand the manner in which BRM itself is recruited to clock genes will also provide insights into the mechanisms by which BRM regulates \textit{Drosophila} clock function. Although our published results did not reveal significant rhythmic BRM recruitment over the circadian cycle, the lack of rhythmic BRM binding to CLK-activated promoters could be attributed to the use of flies expressing epitope-tagged BRM under the control of the \textit{tim-GAL4} driver for ChIP analysis. We anticipate to revisit this question as ChIP analysis with a BRM antibody will likely provide a better representation of native temporal pattern of BRM recruitment. A ChIP quality BRM antibody is currently being generated.

In the mean time, the lack of a ChIP quality BRM antibody does not prevent the investigation of whether the key clock transcription factors may be involved in recruiting BRM to circadian promoters (Fig. 3B). We have already shown that both CLK and TIM can interact with BRM in \textit{Drosophila} S2 cells as well as in flies.\(^ {36}\) In addition, SWI/SNF complexes have previously been shown to be recruited to target sites through direct interactions with gene-specific transcription factors.\(^ {61-63}\) Since the role of BRM in regulating circadian gene expression appears to be repressive in nature, we investigated if BRM is recruited to clock promoters by the negative elements of the \textit{Drosophila} circadian oscillator, PER and TIM. In the \textit{Drosophila} clock, a critical event in clock progression is the heterodimerization of these two proteins.\(^ {1,3}\) Dimerization with TIM stabilizes PER in the cytoplasm and eventually leads to nuclear translocation, as TIM is responsible for shuffling PER from the cytoplasm into the nucleus upon nighttime to initiate the repression phase of CLK-activated transcription.\(^ {64}\) To investigate whether PER and/or TIM are responsible for recruiting BRM to target gene promoters, flies were subjected to constant light (LL) conditions following standard 12hr light: 12hr dark (LD) entrainment to reduce PER and TIM protein levels. Since TIM is targeted for degradation in the presence of light and PER is unstable without TIM, LL conditions should be effective in reducing the levels of both proteins. As predicted, examining PER and TIM protein levels from whole head protein extracts showed only basal levels throughout the day, with the lack of protein accumulation at ZT16 and ZT22, which we otherwise observed in LD conditions (Fig. 4A). Mobility shift of PER and TIM due to

![Figure 3. The interactions of BRM with additional factors regulate its recruitment to promoter regions and modulate circadian transcription. (A) The regulation of circadian gene expression in \textit{Drosophila} is likely orchestrated through the interaction of a chromatin remodeler such as BRM with multiple epigenetic modifiers. Examples of candidate BRM-interacting repressors are depicted. (B) The possible role of CLK as well as PER and TIM in the recruitment and localization of BRM to the \textit{per} promoter.](image-url)
progressive phosphorylation normally observed in flies housed in LD conditions is also lost in flies held in LL as assayed by western blots. As a separate verification that the LL condition was successful in disrupting the role of PER and TIM in maintaining periodicity of the clock, the behavioral phenotypes of the flies were examined (Fig. 4B). Once conditions transitioned from LD to LL, flies exhibited loss of rhythmicity, closely reflecting the loss of PER and TIM protein cycling (Fig. 4A).

To determine if reduction in PER and TIM affects BRM recruitment to the per promoter, ChIP-qPCR experiments were performed to compare flies entrained in LD and subsequently held in LL conditions and control flies that were kept in LD throughout the experiment. Interestingly, it appears that PER and TIM may be somewhat antagonistic to BRM interaction with the chromatin, as flies exposed to LL conditions exhibited higher levels of BRM recruitment to the per promoter, particularly at ZT10 and ZT16 (Fig. 4C). This suggests that PER and TIM may have roles in limiting

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**Figure 4.** Disruption in PER and TIM expression using constant light treatment (LL) affects BRM localization at circadian promoters. (A) Verification that circadian rhythms in PER (top panel) and TIM (bottom panel) accumulation and post-translational modifications are abolished in LL conditions. Protein expression patterns in control flies that were entrained for 3 d in 12 hr light: 12 hr dark conditions at 25°C are compared to flies subjected to constant light conditions (LL) for one day following 3 d of LD entrainment. Protein was extracted from fly heads as described in Kwok et al.36 and resolved by SDS-PAGE. Antibodies against PER and TIM have been previously described.36 Loading was normalized using anti-HSP70 (Sigma, St. Louis, MO). (B) Locomotor activity rhythms verify loss of rhythmicity in LL conditions. Male flies were subjected to locomotor activity assays using the Drosophila Activity Monitoring System (DAMS) (Trikinetics, Waltham, MA) as described in Chiu et al.44 Flies were entrained for 4 d in 12 hr light: 12 hr dark conditions. On the fifth day, LD conditions were shifted +6 h to confirm that flies did not exhibit entrainment defects. Starting the 8th day, flies held in LL conditions and periodicity of the clock was assessed. Shaded areas = lights-off. TUG = tim-(UAS)-Gal4 driver.70 (C) Chromatin immunoprecipitation (ChIP) assay showing BRM localization to the per promoter in LD vs. LL conditions. Transgenic flies expressing BRM-FLAG driven by TUG were harvested at the 4 indicated time points (ZT) in either LD or LL conditions. α-FLAG (Sigma, St. Louis, MO) was used in ChIP-qPCR experiments as described in Kwok et al.36 Data shown are from 3 biological replicates with technical triplicates performed during qPCR. Error bars = SEM for biological replicates (n = 3). Two-tailed t-tests were used to determine statistical differences (P < 0.05) between LD and LL treatments at each ZT. Asterisks denote significant difference observed (P < 0.05). Experimental procedures for ChIP-qPCR are detailed in Kwok et al.36
BRM accumulation at the chromatin, or serve to actively remove BRM. This presents another possible mechanism of fine-tuning the precision of CLK-activated transcription. Future experiments are necessary to further dissect the interactions between BRM and PER\(\text{T}\text{IM}^\text{}\).

Since we showed that BRM binds to CLK in *Drosophila* S2 cells and in flies,\textsuperscript{66} it is also possible that CLK itself is responsible for the recruitment of BRM to circadian promoters (Fig. 3B). There is evidence that mammalian CLK possesses activity that surpasses transcriptional activation. CLK in mammals has been shown to function as a pioneer transcription factor, with the ability to interact with nucleosome-bound DNA and promote nucleosome removal.\textsuperscript{65} CLK has also been shown to function as a histone acetyltransferase (HAT) in the mammalian clock.\textsuperscript{66} Although loss of CLK acetyltransferase activity appears to exert the most significant effect on the acetylation status of HAT activity, its ability to recruit BRM can serve to actively remove BRM. This presents the official views of the NIGMS-NIH.\textsuperscript{5}

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