Clock proteins regulate spatiotemporal organization of clock genes to control circadian rhythms

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Circadian clocks regulate ∼24-h oscillations in gene expression, behavior, and physiology. While the genetic and molecular mechanisms of circadian rhythms are well characterized, what remains poorly understood are the intracellular dynamics of circadian clock components and how they affect circadian rhythms. Here, we elucidate how spatiotemporal organization and dynamics of core clock proteins and genes affect circadian rhythms in *Drosophila* clock neurons. Using high-resolution imaging and DNA-fluorescence in situ hybridization techniques, we demonstrate that *Drosophila* clock proteins (PERIOD and CLOCK) are organized into a few discrete foci at the nuclear envelope during the circadian repression phase and play an important role in the subnuclear localization of core clock genes to control circadian rhythms. Specifically, we show that core clock genes, *period* and *timeless*, are positioned close to the nuclear periphery by the PERIOD protein specifically during the repression phase, suggesting that subnuclear localization of core clock genes might play a key role in their rhythmic gene expression. Finally, we show that loss of Lamin B receptor, a nuclear envelope protein, leads to disruption of PER foci and *per* gene peripheral localization and results in circadian rhythm defects. These results demonstrate that clock proteins play a hitherto unexpected role in the subnuclear reorganization of core clock genes to control circadian rhythms, revealing how clocks function at the subcellular level. Our results further suggest that clock protein foci might regulate dynamic clustering and spatial reorganization of clock-regulated genes over the repression phase to control circadian rhythms in behavior and physiology.

circadian rhythms | nuclear organization | live imaging

Circadian clocks are timekeepers within our cells that generate ∼24-h rhythms in gene expression and control many physiological processes ranging from sleep to metabolism to immunity (1). Circadian rhythms are self-sustained and can be entrained to environmental cues such as light (2) and temperature (3). While many of the advances in understanding the genetic and molecular mechanisms of circadian rhythms have come from either genetic screens or biochemical studies (4–7), very little is known about how intracellular dynamics of clock components affect circadian rhythms, as it has not been possible so far to study the spatial organization and dynamics of clock proteins with subcellular resolution in live cells in vivo. Furthermore, the questions of how core clock genes are organized in the three-dimensional nuclear space of individual clock cells in native tissues under physiological conditions and how that organization affects circadian rhythms remain poorly understood, as past studies were all performed using in vitro cell-culture models (8, 9).

We address these critical gaps using *Drosophila melanogaster*, which has a highly conserved yet relatively simple clock system consisting of 150 neurons (10) (Fig. 1*A*). Circadian clocks in all eukaryotes are based on highly conserved negative delayed transcription–translation feedback loops (7). Briefly, the *Drosophila* circadian clock consists of four key proteins—CLOCK (CLK), CYCLE (CYC), PERIOD (PER), and TIMELESS (TIM)—that form the feedback loop. This feedback loop drives the rhythmic activation and repression of not only core clock genes (*per* and *tim*) but also of a large number of other genes over the circadian cycle, referred to as clock-regulated genes (11). Previous studies have shown that CLK and CYC proteins act as positive transcription factors and bind to the E-boxes (enhancer box) of genes, including *per* and *tim*, to drive their transcription during the activation phase (12) (Fig. 1*B*). The repression phase is initiated when PER and TIM proteins enter the nucleus after a time delay and inhibit CLK activity, thus silencing their own expression as well as the expression of other clock-regulated genes (13) (Fig. 1*B*). PER and TIM proteins are then degraded, leading to the end of the repression phase and the start of a new cycle. In addition to this core feedback loop, there is a second interlocked feedback loop that controls the expression of the *Clk* messenger RNA (mRNA) (14, 15). CLK–CYC proteins bind to the E-boxes of the transcription factors, vrille (VRI) and PAR domain protein 1-ε (PDP1-ε), creating rhythms in their expression: VRI levels peak during the early evening, and PDP1-ε levels peaks later in the night. VRI binds to the VRI/PDP1-ε–binding boxes in the *Clk* enhancer and represses *Clk* transcription from midday to early evening, whereas PDP1-ε activates *Clk* transcription later in the night, thus resulting in rhythmic *Clk* mRNA expression. However, it has been shown that the core molecular clock is unaffected by the phase of *Clk* mRNA oscillations (16), and, moreover, recent studies suggest that PDP1-ε and VRI act downstream of the core molecular clock and regulate clock outputs, which affect rhythmic behavior (17, 18).

Significance

Almost all living organisms have evolved circadian clocks to tell time. Circadian clocks regulate ∼24-h oscillations in gene expression and control much of our behavior and physiology. Here, we reveal the surprisingly sophisticated spatiotemporal organization of core clock proteins and genes over the circadian cycle and its critical role in circadian clock function. We show that *Drosophila* clock proteins are concentrated in a few discrete foci and that they play a key role in positioning the core clock genes close to the nuclear envelope precisely during the repression phase to control circadian rhythms. These studies provide fundamental insights into cellular mechanisms of circadian rhythms and establish direct links between nuclear organization and circadian rhythms.

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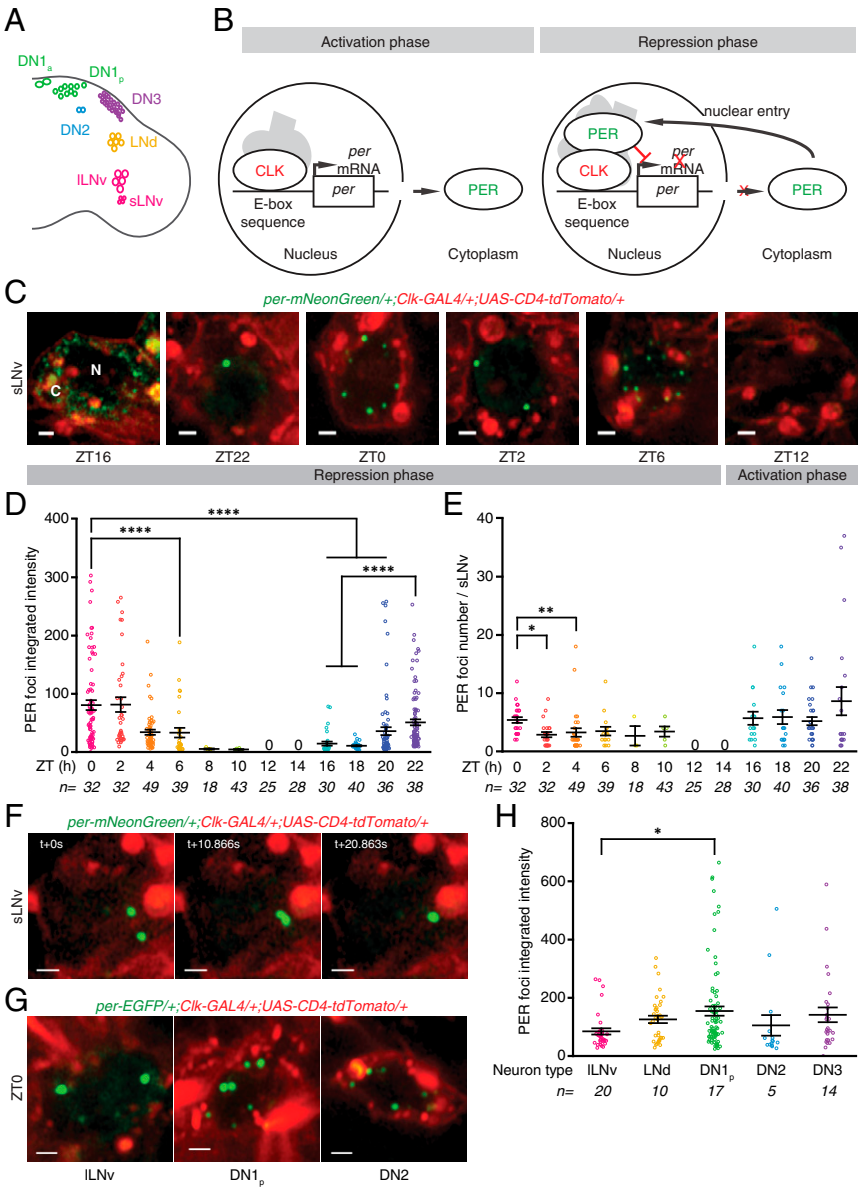

Fig. 1. PER protein is organized into a few discrete nuclear foci during the circadian repression phase. (*A*) Schematic of the *Drosophila* circadian clock network, with the major classes of clock neurons labeled. (*B*) Schematic of the core molecular clock in the *Drosophila* clock neurons. During the activation phase, the CLK protein complex binds to the E-box sequence of the *per* gene and drives its transcription. During the repression phase, the PER protein enters the nucleus and inhibits CLK transcription factor activity, silencing its own expression. (*C*) Data from *per-mNeonGreen;Clk-GAL4>UAS-CD4-tdTomato* flies entrained to LD cycles (ZT0: lights on; ZT12: lights off). Representative images of PER foci (green) in sLNvs (cell membrane labeled with tdTomato and shown in red) over the circadian cycle. N denotes the nucleus and C denotes the cytoplasm. (*D* and *E*) Quantitation of PER foci intensity (*D*) and foci number per sLNv (*E*) at specific ZTs over the LD cycle. The '0' denotes that there is no PER protein in the sLNvs. (*F*) Representative time-lapse images of sLNvs showing PER foci undergoing fusion. (*G*) Representative images of PER foci at ZT0 in other groups of clock neurons (lLNv, $DN1_p$, and DN2). (*H*) Quantitation of PER foci intensity in all classes of clock neurons at ZT0. (Scale bars, 1 μm.) The statistical test used was a Kruskal–Wallis test (*D*, *E*, and *H*). $*P < 0.05$, $**P < 0.005$, $****P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

While all oscillating gene expression in *Drosophila* is shown to be under the control of CLK protein, there are a large number of noncycling genes that are also affected by the loss of CLK protein, suggesting that *CLK* protein might be part of other gene networks in nonclock cells (19). Similar negative feedback loops function in mammals, with many of the clock proteins conserved among the two model systems (1, 7). Remarkably, these circadian feedback loops operate in virtually every cell in the human body (20), from neurons to liver cells to skin cells, in order to ensure that cellular processes occur at the right time of the day and to generate circadian rhythms in behavior, metabolism, and physiology.

While past studies have elucidated genetic and neural mechanisms of circadian rhythms (7, 21, 22), a key missing piece in our current understanding of clock regulation is how spatiotemporal organization and dynamics of core clock proteins and genes affect circadian rhythms. To address these critical gaps, we developed CRISPR-based single-cell imaging techniques that enable studies of subcellular localization and temporal dynamics of clock proteins in live clock neurons in vivo. Using these techniques, we discovered that *Drosophila* clock proteins are organized into discrete, dynamic foci and control the subnuclear localization of core clock genes to regulate circadian rhythms. Specifically, we found

that PER protein positions the core clock genes at the nuclear periphery specifically during the repression phase, when the genes are not transcriptionally active, suggesting that spatial organization of core clock genes might regulate their rhythmic gene expression. To elucidate how the nuclear envelope affects circadian rhythms, we conducted a genetic screen and found that loss of Lamin B receptor (LBR), an inner nuclear-membrane protein (23, 24), leads to disruption of *per* gene peripheral localization and circadian rhythm defects. These studies highlight the important role played by the spatiotemporal organization of clock proteins and genes and the nuclear envelope in circadian clock function, opening up questions that are fundamental to understanding core clock regulation.

Results

In order to elucidate the subcellular localization and dynamics of core clock proteins in vivo with high resolution, we generated fluorescent protein–tagged flies in which the endogenous *per* gene was labeled with mNeonGreen (25). mNeonGreen is a monomeric green/yellow fluorescent protein that is ∼3- to 5-fold brighter than EGFP, and its maturation time is ∼3-fold shorter than that of EGFP (25, 26). Specifically, we applied CRISPR/Cas9 genome editing to fuse the mNeonGreen tag to the terminal exon of the endogenous *per* gene (*SI Appendix*, Fig. S1 *A* and *B*), as previous studies have shown that the PER-LUCIFERASE carboxyl-terminal fusion protein is functional (20, 27). To determine whether the PER-mNeonGreen fusion protein is functional in vivo, we analyzed whether *per* mRNA levels oscillate with a period of ∼24 h. qPCR analysis revealed that *per* mRNA in these flies undergoes circadian oscillations, and no significant differences were detected between wild-type (WT) and *per-mNeonGreen* flies (*SI Appendix*, Fig. S1*C*). Next, we analyzed the locomotor-activity rhythms of *per-mNeonGreen* flies and found that they displayed WT-like behavior in both 12-h light-dark cycles (LD12:12) and constant darkness (DD), with ∼24-h free-running period rhythms, normal daily activity and sleep levels, and peaks of activity around dusk and dawn (*SI Appendix*, Fig. S2). Because a null mutation of the *per* locus causes loss of rhythmicity in DD (28), we tested whether a single *per*-mNeonGreen allele can rescue the rhythms of per^{01} null mutant flies. We found that *per-mNeonGreen*/per^{01} female flies, which have only one functional copy of the *per-mNeonGreen* locus and can only produce the PER-mNeonGreen fusion protein, displayed WT-like behavior in both LD and DD (94.7% rhythmicity) (*SI Appendix*, Fig. S2 *A* and *D*), demonstrating that the PER-mNeonGreen fusion protein is fully functional in vivo.

To monitor PER protein localization and dynamics, we entrained *per-mNeonGreen;Clk-GAL4>UAS-CD4-tdTomato* flies [*Clk-GAL4* is a pan-clock–neuron driver (29), and CD4 is a transmembrane protein that labels cell membranes (30)] to 12-h light-dark cycles (LD12:12; ZT0: lights on; ZT12: lights off, ZT-Zeitgeber time) and imaged PER-mNeonGreen protein in live clock neurons within intact *Drosophila* brains every 2 h over the circadian cycle. We first focused on small ventrolateral neurons (sLNvs), which express the pigment-dispersing factor (PDF) neuropeptide, as they are considered the master pacemaker neurons (31–33). Our imaging experiments performed using an Airyscan high-resolution confocal microscope revealed that the PER protein is localized and is concentrated in discrete, dynamic foci in the nuclei of sLNvs during the repression phase of the circadian cycle (ZT18 to ZT8) (Fig. 1 *C–E*, *SI Appendix*, S3*A*, and Movie S1). These results are surprising, as past studies using paraformaldehyde fixation and immunofluorescence methods suggested that the PER protein is diffusely distributed in the nucleoplasm (34, 35). However, the fixation process has been suggested to denature cellular protein-target structures and potentially lead to immunostaining artifacts (36). In fact, we also observed that a majority of the PER foci disassemble upon formaldehyde fixation (*SI Appendix*, Fig. S3*B*), explaining why these foci escaped detection in fixed samples in past studies. Another previous in vitro live-imaging study has shown that the PER protein, when overexpressed in *Drosophila* S2 (Schneider 2) cells, forms cytoplasmic foci (37). However, *Drosophila* S2 cells are regarded as "nonrhythmic" cells, as they do not express several clock genes including *Clk*, *per*, and *tim*, and, furthermore, overexpression of proteins can lead to aggregation artifacts. Our imaging experiments using tagged endogenous proteins overcome all the limitations of past approaches and enable visualization of clock protein localization and dynamics at high spatiotemporal resolution in live clock neurons within intact *Drosophila* brains.

To confirm the specificity of the PER green fluorescent signal and test whether PER foci are only observed in clock neurons when PER protein is present, we used *Clk-GAL4>UAS-per-4xsgRNA;UAS-Cas9* flies, which express four unique single-guide RNAs (sgRNAs) that specifically target the *per* gene, resulting in clock neuron–specific PER loss of function (LOF) (38, 39). As expected, we did not find any PER foci in the clock neurons of these PER LOF flies at different times over the circadian repression phase (*SI Appendix*, Fig. S3*C*). Furthermore, we tested how PER foci are affected in TIM tissue-specific LOF mutant flies (*Clk-GAL4>UAS-tim-4xsgRNA;UAS-Cas9*) (38, 39). In these flies, too, we did not detect any PER protein foci in the clock neurons during the repression phase (*SI Appendix*, Fig. S3*D*), consistent with previous observations that TIM is required for the stability of PER protein (38, 40, 41). Together, PER foci specificity is demonstrated by the absence of any foci in the clock neurons of tissue-specific PER or TIM LOF mutant flies.

In our studies, we found that PER foci first appear in the cytoplasm at ZT16 (Fig. 1*C* and *SI Appendix*, S4*A*), and they start to accumulate in the nucleus from ZT18 (Fig. 1 *C–E*) and persist until the end of the repression phase (ZT8). We did not see any significant PER accumulation in the clock neurons before ZT16. Furthermore, we found that PER cytoplasmic foci are also sensitive to formaldehyde fixation (*SI Appendix*, Fig. S3*C*). Recent studies have shown that intracellular condensate/foci assembly is often driven by weak multivalent interactions between proteins with intrinsically disordered regions (IDRs) and/or low-complexity sequences and nucleic acids (42). We found that the PER protein has large regions of disorder in its carboxyl-terminal, also shown in a past study (43) (*SI Appendix*, Fig. S4*B*). Furthermore, we found that these PER foci are highly dynamic and exhibit liquid-like properties: these foci are spherical, two foci could fuse with each other to make a bigger focus (Fig. 1*F* and Movie S2), and treatment of brains with 1,6-hexanediol, an aliphatic alcohol that disrupts weak hydrophobic interactions (42), disassembled all the PER foci in the clock neurons (*SI Appendix*, Fig. S4 *C* and *D*).

The timing of PER foci nuclear entry (ZT18) in our imaging experiments corresponds to the start of the repression phase and is consistent with previous chromatin immunoprecipitation (ChIP) studies (13). We found that PER foci continue to increase in size until ZT0 (peak repression phase) and then decrease in size until PER protein is completely degraded around ZT8 (Fig. 1*D*). PER foci are present long after (∼8 h) lights are turned on at ZT0 (Fig. 1 *D* and *E*), which has previously been shown to lead to rapid degradation of the TIM protein (44, 45), suggesting that PER proteins persist as foci even after TIM is eliminated by light. These foci are present in almost all the sLNv clock neurons especially during the peak repression phase (*SI Appendix*, Fig. S5*A*). We observed, on average, ∼5 PER foci per sLNv at ZT0 and a lesser number at later time points (Fig. 1*E*). We also observed PER foci in all other clock neuron groups in the *Drosophila* brains, suggesting that PER nuclear foci appear synchronously throughout the clock network (Fig. 1 *G* and *H* and *SI Appendix*, S5*C*). Finally, we note that we did not detect any green fluorescence (PER protein) outside the clock neurons (*SI Appendix*, Fig. S6), suggesting that the PER-mNeonGreen signal is present exclusively in the clock neurons at specific times over the circadian cycle. To

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further confirm our findings, we conducted imaging studies using a different fly line in which endogenous *per* gene was labeled with an EGFP tag (46), which has been shown to display normal circadian rhythms. We found that PER-EGFP protein also forms discrete nuclear foci in clock neurons during the repression phase (*SI Appendix*, Fig. S7).

To test whether PER-mNeonGreen foci persist in constant darkness, we entrained flies to LD12:12 cycles and released them into constant darkness (DD) and imaged PER proteins on the first day of DD. We found that PER foci persist in sLNv nuclei in constant darkness after entrainment, and the number and size of PER foci is similar in LD and DD (Fig. 2 *A*–*C* and *SI Appendix*, Fig. S5*B*). Notably, we observed PER foci in sLNv nuclei during the repression phase even after 8 d in constant darkness (*SI Appendix*, Fig. S5 *D* and *E*). As expected from past studies (34), we did not find any PER foci in lLNvs (large ventral lateral clock neurons) in DD (circadian time 24 [CT24] data were collected from 35 neurons from six brains; CT48 data were collected from 29 neurons from five brains, *SI Appendix*, Fig. S5*F*). In addition to oscillations in PER foci size and number, we found that PER foci also show a stereotypical subnuclear localization close to the nuclear envelope during the repression phase of the circadian cycle. To visualize the nuclear envelope clearly and quantify the distance between PER foci and the nuclear envelope precisely, we crossed *per-mNeonGreen* flies with *Clk-GAL4;UAS-unc84-tdTomato* flies [UNC84 is a SUN domain protein that localizes to the inner nuclear membrane (47)] and imaged PER foci during the repression phase. We observed that a majority of the PER foci are located very close (<0.5 μm, which is ∼10% of the diameter of an average clock-neuron nucleus) to the nuclear envelope during the repression phase (Fig. 2 *D*–*F* and Movies S3 and S4). Furthermore, PER foci at ZT0 (peak repression phase) exhibited significantly less mean squared displacement compared to the foci at ZT5 (*SI Appendix*, Fig. S8), suggesting that PER foci at ZT0 might be spatially constrained at the nuclear envelope. Together, these studies establish that PER protein forms dynamic nuclear foci in clock neurons that oscillate with a 24-h rhythm in their size, number, and subcellular localization.

Next, we examined PER foci dynamics and subcellular localization in clock mutants in which the 24-h period of circadian rhythms is either shortened or lengthened. Specifically, we examined mutants of a key kinase, Casein Kinase I/DOUBLETIME (DBT), which is known to phosphorylate PER protein and regulate its stability and activity (41). Flies that ectopically express dbt^L mutation (*UAS-*dbt^L) specifically in clock neurons display circadian rhythms with an average free-running period of ∼27 h (48) (*SI Appendix*, Fig. S9 *A* and *B*), while dbt^S mutation in clock neurons produces rhythms with an average free-running period of 18 h (48) (*SI Appendix*, Fig. S9 *A* and *C*). In our experiments using *Clk-GAL4>UAS-*dbt^L flies, we found that PER foci start to appear in the nucleus at ZT22 and persist until ZT12 (Fig. 3 *A* and *C* and *SI Appendix*, Fig. S9*D*), which is later compared to WT conditions, suggesting that dbt^L mutation causes delayed nuclear entry. In contrast, in *Clk-GAL4>UAS-*dbt^S flies, PER foci appear in the

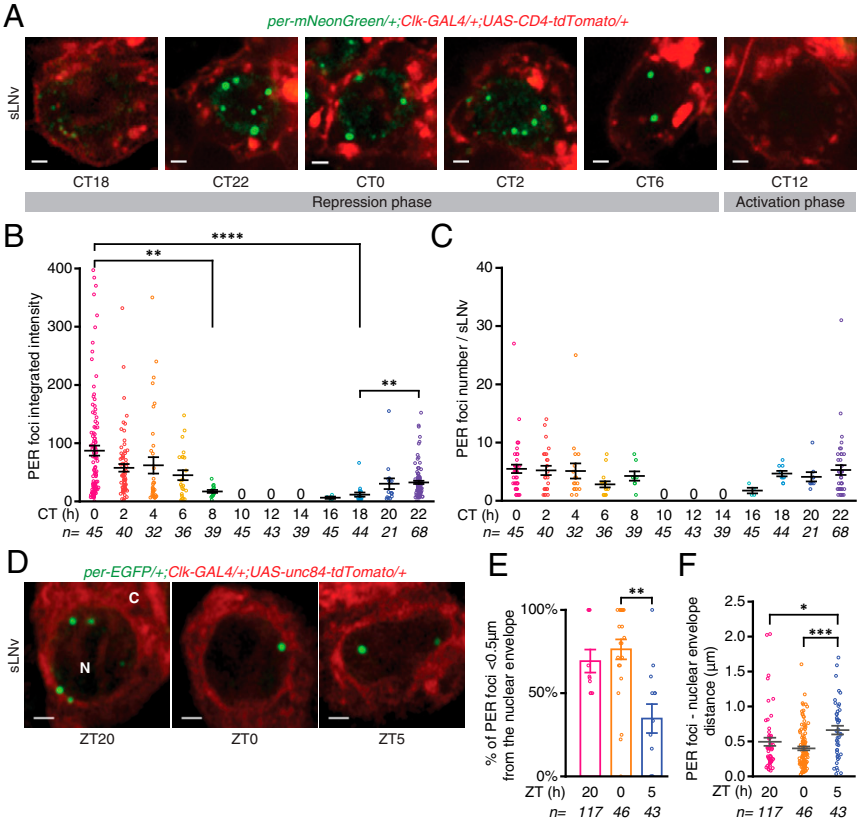

Fig. 2. PER foci persist in constant darkness and are stereotypically positioned at the nuclear envelope. (*A*) *per-mNeonGreen;Clk-GAL4>UAS-CD4-tdTomato* flies are entrained to LD cycles for 5 d and released into DD. Representative images of PER foci in sLNvs at 2-h intervals on day 1 of DD. CT refers to circadian time. (*B* and *C*) Quantitation of PER foci intensity (*B*) and foci number per sLNv (*C*) at specific CTs during day 1 of DD. (*D*) Representative images of PER foci in sLNvs from *per-EGFP;Clk-GAL4>UAS-unc84-tdTomato* flies. PER foci are located close to the nuclear envelope (marked in red) during peak repression phase. N denotes the nucleus, and C denotes the cytoplasm. (*E*) Quantitation of the percentage of PER foci that are less than 0.5 μm away from the nuclear envelope at different ZTs. (*F*) Quantitation of the distance between PER foci and the nuclear envelope at different ZTs. (Scale bars, 1 μm.) The statistical test used was a Kruskal–Wallis test (*B*, *C*, *E*, and *F*). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

nucleus at ZT16, which is slightly earlier compared to WT, and these foci disappear by ZT4 (Fig. 3 *B* and *D* and *SI Appendix*, Fig. S9*E*), in accordance with past studies that showed that PER protein is prematurely degraded in dbt^S mutant flies (48). Our studies show how PER foci dynamics and spatial distribution are regulated by DBT kinase, and our results are consistent with previous reports of different kinetics of PER protein degradation and nuclear localization in *dbt* mutants (41, 48).

Previous biochemical studies have shown that PER initiates transcriptional repression by binding to CLK protein, a positive regulator of the circadian feedback loop, which is bound to the E-boxes of the clock-regulated genes (13). To visualize CLK protein, we generated fluorescent protein–tagged flies in which *Clk* gene was labeled with mScarlet-I (49) (Fig. 4*A* and *SI Appendix*, Fig. S10*A*). We note that mScarlet-I is a bright monomeric red fluorescent protein; however, it is not as bright as mNeonGreen, the green fluorescent protein used to tag PER. We established that the tagged protein (CLK-mScarlet-I) is functional by showing that the behavior of the homozygous *Clk-mScarlet-I* flies and heterozygous flies is rhythmic (Fig. 4*B* and *SI Appendix*, Fig. S10 *B–D*), and *per* mRNA in these flies undergoes circadian oscillations similar to WT levels (*SI Appendix*, Fig. S1*C*). Furthermore, we tested if the *Clk*-mScarlet-I allele can rescue the behavioral arrhythmicity of CLK^{ar} flies, which are recessive mutant flies (50). We found that male progeny from the cross (*Clk*-mScarlet-I/Clk^{ar}) displayed rhythmic locomotor-activity behavior (83.3% rhythmic flies), suggesting that the CLK-mScarlet-I fusion protein is functional in vivo (*SI Appendix*, Fig. S10 *B* and *E*). To perform imaging experiments, we entrained *Clk-GAL4>UAS-CD8-GFP;Clk-mScarlet-I* flies [CD8 is a transmembrane protein that labels cell membranes (30)] to 12-h LD cycles (LD12:12; ZT0: lights on; ZT12: lights off) and imaged CLK protein in live clock neurons within *Drosophila* brains every 2 h. To enable visualization of endogenous CLK-mScarlet-I, we examined CLK protein in dorsal clock neurons ($DN1_p$), as they are closer to the surface of the fly brain and are therefore optically more accessible than sLNvs, which are deeper in the fly brains.

We found that CLK protein is also concentrated in discrete nuclear foci in $DN1_p$s specifically during the repression phase of the circadian cycle (Fig. 4 *C–E*). Importantly, we did not detect any distinct CLK foci in $DN1_p$ nuclei during the activation phase; instead, the CLK protein is diffusely distributed in the nucleus during these times (Fig. 4*C*). These results strongly suggest that CLK foci are formed specifically during the repression phase. CLK nuclear foci start to appear at ZT20, ~2 h after the PER protein enters the nucleus, and persist until the end of the repression phase (~ZT8) (Fig. 4 *C–E*). We also found that CLK foci colocalize with PER foci during the circadian repression phase (Fig. 4*F*). We note that, in some instances, we found red fluorescence (corresponding to the CLK protein) outside the clock-neuron network; it appears as if the red-fluorescence signal might be inside some other cells (Fig. 4*C*). These observations are consistent with past studies, which showed that the CLK protein is expressed in clock neurons and also in other cells in the brain (51) and that the CLK protein controls not only the rhythmic expression of clock-regulated genes but also the expression of many noncycling genes (19). Next, we tested whether PER is necessary for CLK foci formation in clock neurons by examining per^{01} null mutant flies. We did not find any CLK foci at ZT0 in the clock neuron nuclei of per^{01} mutant flies; instead, CLK is diffusely distributed in the nucleus in the absence of PER (Fig. 4 *G* and *H*). However, we noticed that CLK protein fluorescence level in clock neurons is lower in per^{01} mutant flies compared to control flies consistent with past studies (52), which might be a reason why no CLK foci were detected in our experiments with per^{01} mutant flies. From the above studies, we conclude that CLK protein is diffusely located in the clock neuron nuclei during the activation phase, when it drives transcription of genes, and is organized into discrete nuclear foci that colocalize with PER foci during the repression phase of the circadian cycle.

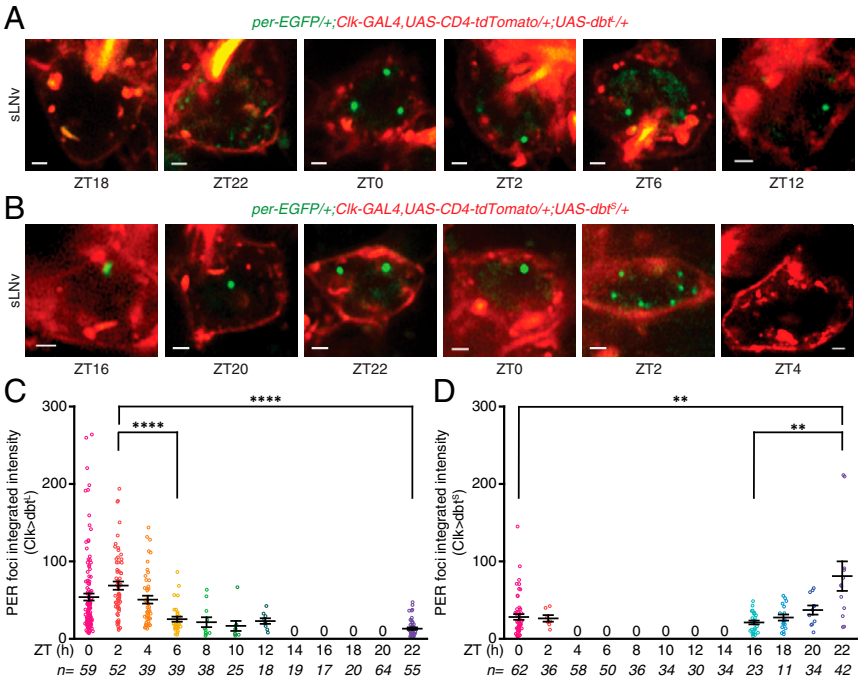

Fig. 3. PER foci dynamics and spatial organization are regulated by DBT kinase. (*A* and *B*) Representative images of sLNvs from *per-EGFP;Clk-GAL4>UAS-dbt^L,UAS-CD4-tdTomato* flies (A) and *per-EGFP;Clk-GAL4>UAS-dbt^S,UAS-CD4-tdTomato* flies (*B*) at different ZTs over the circadian cycle. (*C* and *D*) Quantitation of PER foci intensity in sLNvs from dbt^L (*C*) and dbt^S (*D*) mutants. (Scale bars, 1 μm.) The statistical test used was a Kruskal–Wallis test (*C* and *D*). $^{**}P < 0.005$, $^{****}P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

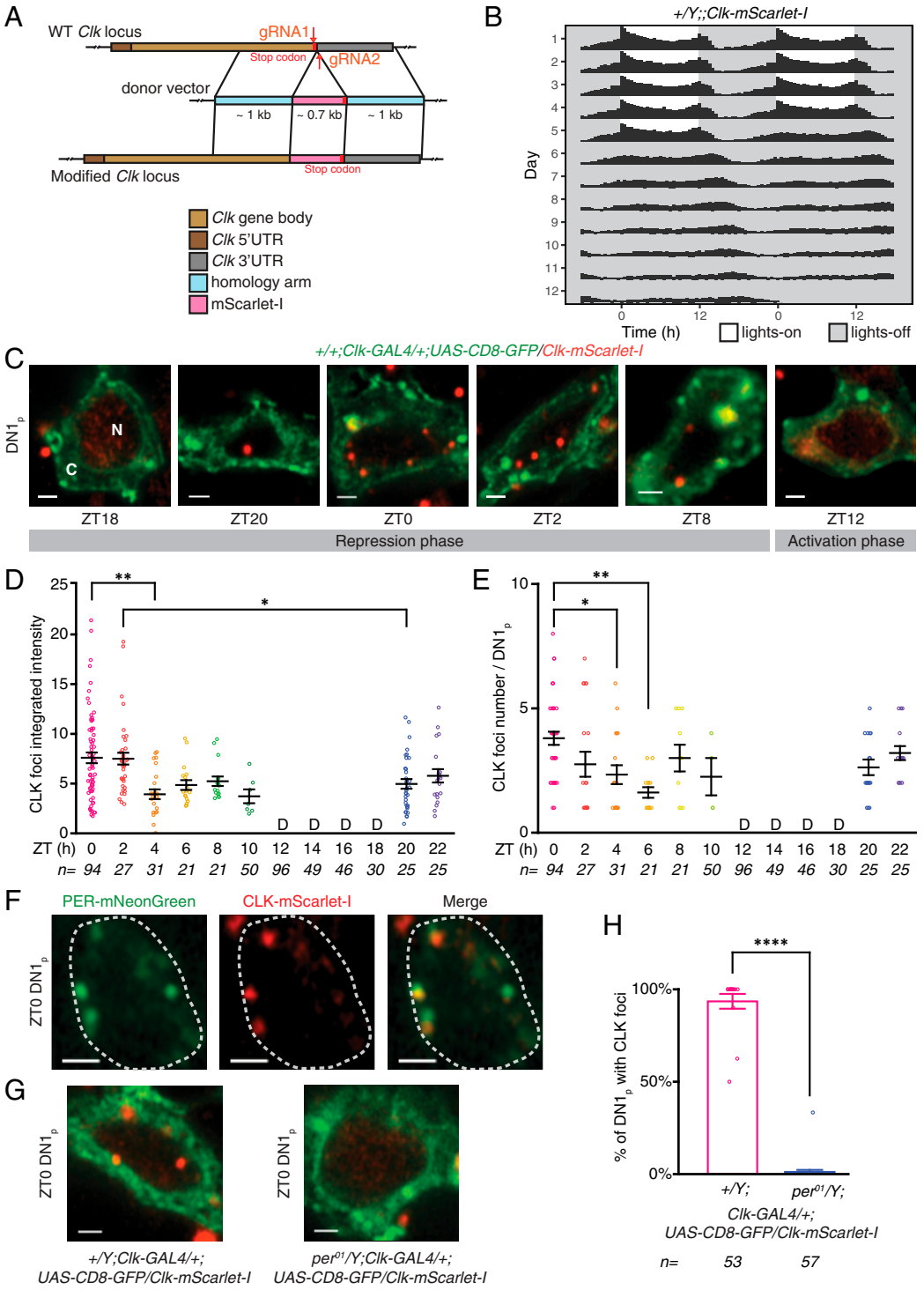

Fig. 4. CLK protein is concentrated in nuclear foci in the clock neurons during the repression phase. (*A*) Schematic of CRISPR/Cas9 genome editing to generate *Clk-mScarlet-I* flies. (*B*) *Clk-mScarlet-I* flies were entrained to LD cycles (ZT0: lights on; ZT12: lights off) for 5 d and released into DD for 7 d. Averaged population locomotor-activity profiles of *Clk-mScarlet-I* flies ($n = 62$) in LD and DD with rest–activity shown for two consecutive days in the same line. These flies display rhythmic behaviors with a period of 24.39 ± 0.10 h, with activity peaks around the time of lights on and lights off (see *SI Appendix*, Fig. S10B for details). (*C*) Representative images of CLK (red) in $DN1_p$s (cell membrane labeled with GFP and shown in green) from *Clk-GAL4>UAS-CD8-GFP;Clk-mScarlet-I* flies over the circadian cycle. N denotes the nucleus, and C denotes the cytoplasm. (*D* and *E*) Quantitation of CLK foci intensity (*D*) and foci number per $DN1_p$ (*E*) at specific ZTs over the 24-h LD cycle. 'D' denotes that CLK protein is diffusely distributed in the nucleus and is not concentrated in foci at those time points. (*F*) Representative images of CLK and PER foci colocalization at ZT0 in $DN1_p$s from *per-mNeonGreen;Clk-mScarlet-I* flies. A total of 86% of CLK foci colocalize with PER foci (from 65 CLK foci from 10 brains). (*G*) Representative images of CLK foci in $DN1_p$s at ZT0 from control (*+;Clk-GAL4>UAS-CD8-GFP;Clk-mScarlet-I*) and per^{01}*;Clk-GAL4>UAS-CD8-GFP;Clk-mScarlet-I* mutant flies. (*H*) Quantitation of the percentage of $DN1_p$s with CLK foci at ZT0 in control and per^{01} mutant flies. Each of the data points corresponds to measurements from an individual brain. (Scale bars, 1 μm.) Statistical tests used were a Kruskal–Wallis test (*D* and *E*) and a Mann–Whitney *U* test (*H*). $^*P < 0.05$, $^{**}P < 0.005$, $^{****}P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

Next, we examined how PER-CLK foci may inhibit expression of circadian genes during the repression phase. Since past work has shown that PER-CLK complex binds to the promoters of clock-regulated genes during the repression phase (13, 53) and repressed chromatin has been shown to be located close to the nuclear periphery in some cell types (54–57), we hypothesized that core clock genes might be rhythmically positioned at the nuclear envelope by the clock proteins during the repression phase. To test our hypothesis, we adapted an in situ immuno-DNA-FISH (fluorescence in situ hybridization) technique for use in adult *Drosophila* brains. We used lLNvs for these experiments, as they can be clearly identified with an antibody to the PDF neuropeptide. Our DNA-FISH experiments show that core clock genes *per* and *tim* are positioned close to the nuclear periphery in lLNvs specifically during the repression phase (ZT0) and are located in the nuclear interior during the activation phase (ZT12) (Fig. 5 *A*–*D*). Importantly, nuclear peripheral positioning of *per* and *tim* genes is disrupted in per^{01} mutants (Fig. 5 *A*–*D*), suggesting that PER protein plays a critical role in the subnuclear localization of core clock genes over the circadian cycle. We observed only one focus or two closely spaced foci per gene in the neurons (Fig. 5 *A* and *B*), consistent with past observations that homologous chromosomes are paired in *Drosophila* somatic cells (58).

We note that we were not able to detect colocalization of PER protein foci and *per*/*tim* gene dots due to technical limitations in combining DNA-FISH, which involves harsh treatments such as formamide-based DNA denaturation at high temperature, and PER foci imaging in the same set of clock neurons. However, this does not pose a challenge in interpreting our results, as past studies using ChIP and proteomics techniques have shown that PER protein binds to the promoters of the clock-regulated genes via CLK and recruits chromatin-repressive complexes to enable gene silencing during the repression phase (13, 53). Based on the above results, we conclude that core clock genes are positioned close to the nuclear periphery by PER protein during the repression phase and are repositioned to the nuclear interior during the activation phase. These results suggest that dynamic repositioning of core clock genes to different subnuclear locations over the circadian cycle might control their rhythmic transcriptional activation and repression.

To determine the molecular mechanisms underlying the localization of PER foci and core clock genes to the nuclear envelope, we conducted a behavioral screen of all known *Drosophila* lamin and nuclear envelope proteins (59, 60), *Lamin B*, *Lamin C*, *LBR*, and *fs(1)Ya*; SUN-KASH domain proteins, *koi*, *klar*, and *Msp300*; and LEM domain proteins, *Otefin*, *dMAN1*, and *Bocksbeutel*. Specifically, we knocked down the expression of these genes in the clock neuron network by crossing RNA-interference (RNAi) flies with *Clk-GAL4* flies. Through our screen, we identified that LBR, an inner nuclear membrane protein that binds to both lamin and chromatin (23, 24), is required for locomotor-activity rhythms (*SI Appendix*, Fig. S11*A*). LBR contains a nucleoplasmic N-terminal domain and a hydrophobic carboxyl-terminal consisting of eight transmembrane domains (23, 24). In our behavior studies, we observed that knockdown of LBR expression in the clock-neuron network (*Clk-GAL4>UAS-LBR-RNAi*) led to defects in rhythmic behavior (56.7% arrhythmic flies, low rhythmic strength of ~18) compared to the parental controls (*Clk-GAL4>+*, *+>UAS-LBR-RNAi*) (*SI Appendix*, Fig. S11). We note that knockdown of LBR in the clock network did not affect the total number of clock neurons or the overall morphology of the clock-neuron network (all the control [$n = 8$] and RNAi knockdown [$n = 11$] brains have four sLNvs and four lLNvs each). To quantify the knockdown efficiency of LBR double-stranded RNA (dsRNA), we knocked down the expression of LBR in all the neurons in adult flies by expressing LBR-RNAi under the control of the RU486-inducible pan-neuronal driver elav-GeneSwitch (*elavGS-GAL4*) (61) and performed qPCR experiments on fly heads. We found that LBR mRNA levels in experimental flies (*elavGS-GAL4;UAS-Dicer2>UAS-LBR-RNAi* flies fed RU486 food) are reduced by ~50% compared to the parental control flies fed RU486 food (*SI Appendix*, Fig. S12*C*). Furthermore, experimental flies fed RU486 food exhibited severe defects in locomotor-activity rhythms in LD and DD (~90% arrhythmicity in DD) (*SI Appendix*, Fig. S12 *A* and *B*). To further strengthen our findings, we used a different $LBR\text{-}RNAi^{KK110508}$ line (referred to as *LBR-RNAi2*) and drove its expression with *Tim-GAL4* to knock down the expression of LBR specifically in the clock network. Consistent with our past results, we found that a large majority of flies (~82%) displayed arrhythmic behavior in DD (*SI Appendix*, Fig. S13 *A* and *B*). Coincidentally, a past study using the $UAS\text{-}LBR\text{-}RNAi^{KK110508}$ line has also reported that LBR knockdown results in loss of behavioral rhythmicity (62).

To determine how LBR affects circadian rhythms, we tested whether disruption of LBR affects *per* gene subnuclear localization. We performed DNA-FISH experiments and found that knockdown of LBR leads to defects in the localization of *per* gene at the nuclear periphery in lLNvs during the circadian repression phase (Fig. 6 *A* and *B*). We also performed PER foci imaging experiments on flies in which LBR expression is knocked down in clock neurons. We observed that PER protein is diffusely located throughout the nucleus in a majority of sLNvs in *Clk>LBR-RNAi* flies compared to control (*Clk>+*) flies during the repression phase (Fig. 6 *C* and *D* and *SI Appendix*, S13 *C* and *D*), suggesting that LBR might be required for PER protein to be concentrated in discrete foci during the repression phase. Next, we performed qPCR experiments to quantify how *per* mRNA expression is affected when LBR is knocked down in all the neurons in adult flies, by using the RU486-inducible *elav-GS* flies. We entrained experimental (*elavGS-GAL4;UAS-Dicer2>UAS-LBR-RNAi* flies fed RU486 food) and control fly lines (*elavGS-GAL4;UAS-Dicer2>+* flies fed RU486 food, *+>UAS-LBR-RNAi* flies fed RU486 food, and *elavGS-GAL4;UAS-Dicer2>UAS-LBR-RNAi* flies fed normal food) to LD cycles and released them into constant darkness, and performed qPCR experiments on day 3 of constant darkness (DD3). We noticed that the experimental flies displayed significantly higher *per* mRNA levels during the early repression phase (CT21) compared to all the other controls, and they also did not show any significant differences in their *per* mRNA levels during the activation (CT9) and repression phases (CT21) (*SI Appendix*, Fig. S12*D* and Table S2). Together, these results suggest that LBR is required for the repression of *per* expression during the early circadian repression phase. Taken together, our results suggest that LBR plays a key role in the regulation of circadian rhythms by controlling the peripheral positioning of *per* gene and PER foci during the repression phase. These studies provide mechanistic insights into how the nuclear envelope regulates circadian gene expression and controls circadian rhythms, opening areas of inquiry related to the influence of nuclear organization on circadian rhythms.

Discussion

Our findings reveal how spatiotemporal organization and dynamics of core clock proteins and genes control circadian rhythms, providing critical insights into how clocks function at the subcellular level. Our work demonstrates that PER and CLK proteins are concentrated in dynamic nuclear foci organized at the nuclear envelope and play a crucial role in the positioning of core clock genes close to the nuclear periphery during the circadian repression phase (Fig. 6*E*).

In our live imaging studies, we found that PER protein, which acts as a negative transcription factor, is concentrated in a few discrete foci located close to the nuclear envelope during the circadian repression phase (Fig. 1*C*). CLK protein, which is a positive transcription factor, is also found to be concentrated in nuclear foci that colocalize with PER foci during the repression phase but is diffusely distributed in the nucleus during the activation phase (Fig. 4*C*). We analyzed the amino-acid sequence and found that

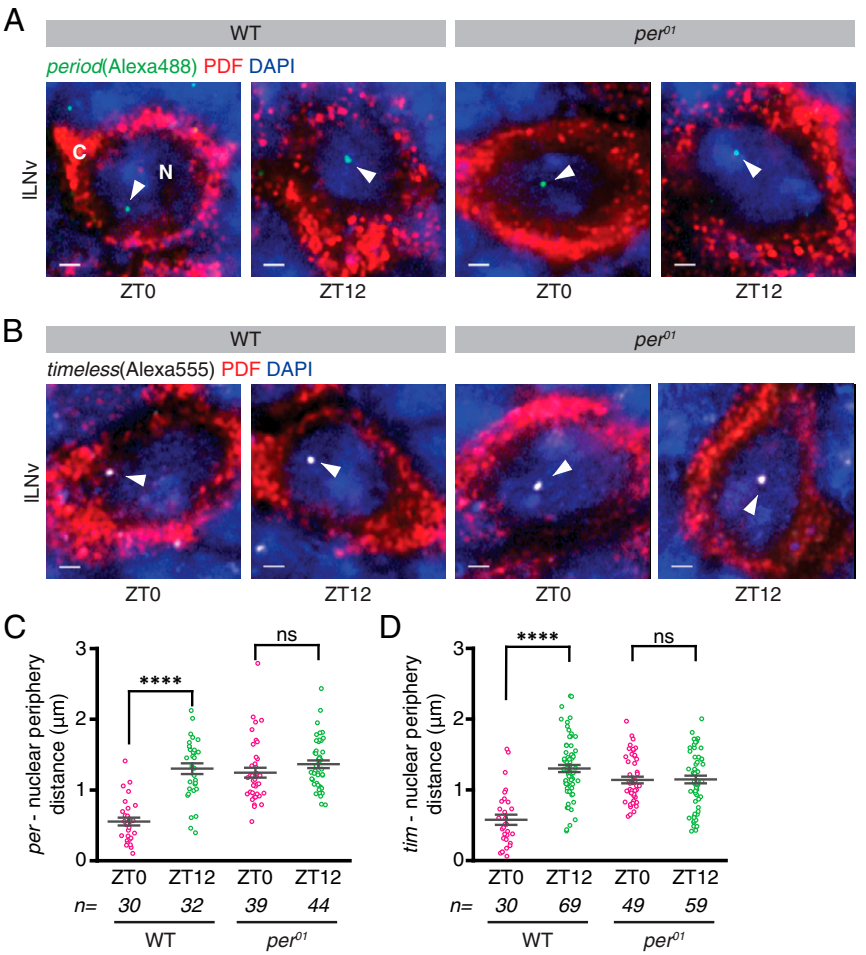

Fig. 5. Core clock genes are positioned close to the nuclear periphery by PER protein during the repression phase. (*A*) Representative images of DNA-FISH results using *per*-gene probes (green dots) during repression (ZT0) and activation (ZT12) phases in lLNvs from WT and per^{01} null mutant flies. Anti-PDF antibody (red) was used to identify lLNv clock neurons, and DAPI (blue) was used to mark the nucleus boundary. N denotes the nucleus, and C denotes the cytoplasm. White arrowheads denote the location of *per* gene in the nucleus. (*B*) Representative images of DNA-FISH results using *tim*-gene probes (white dots) at ZT0 and ZT12 in lLNvs from WT and per^{01} null mutant flies. White arrowheads denote the location of *tim* gene in the nucleus. (*C* and *D*) Quantitation of the distance between *per* gene and the nucleus boundary (*C*) and *tim* gene and the nucleus boundary (*D*) at ZT0 and ZT12 in lLNvs from WT and per^{01} mutant flies. (Scale bars, 1 μm.) The statistical test used was a Kruskal–Wallis test (*C* and *D*). $****P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

PER protein has a highly intrinsic disordered region in its carboxyl-terminus (*SI Appendix*, Fig. S4*B*). Interestingly, recent studies have shown that clock proteins from many species contain IDRs (43, 63–65); however, little is known about how these disordered regions of clock proteins affect circadian clock function. As recent studies have shown that disordered regions promote phase separation and foci formation in other biological contexts (42), our results, which reveal that PER protein is organized into discrete foci during the repression phase under physiological conditions, might provide some clues into the potential biological role of the clock protein disordered regions in circadian rhythms.

Our work highlights multiple properties of PER protein foci that enable circadian clock regulation, which we were able to uncover by studying the spatiotemporal organization and dynamics of clock proteins in their native milieu. First, we found that PER foci are dynamic and exhibit liquid-like properties (Fig. 1*F*). For example, we observed a number of fusion events, especially at time points earlier than ZT0 (peak repression phase), in accordance with our observation that foci decrease in number and increase in size during the early repression phase (Fig. 1 *D* and *E*). Second, we observed a gradual decrease in both the size and the number of PER foci at later times during the repression phase (Fig. 1 *D* and *E*), which is consistent with the fact that the PER protein is gradually degraded over the repression phase. Interestingly, these results point to a new hypothesis that PER foci might be heterogeneous and that each individual PER focus might be regulated (e.g., time of degradation) somewhat independently through yet unknown mechanisms. It remains to be determined whether PER foci might have different sets of DNA, RNA, and protein molecules and whether this heterogeneity potentially contributes to the phase differences in the expression of clock-regulated genes observed within a single cell.

Third, we observed only a few PER foci (<10) per neuron during the circadian repression phase (Fig. 1*E*). Given that clock-regulated genes number in hundreds and are spread throughout the genome, our recent results raise the intriguing possibility that clock proteins might drive clustering of clock-regulated genes into a few nuclear foci during the repression phase to enable transcriptional coregulation through common cis-acting elements (E-boxes). Past studies, which employed chromosome-conformation capture approaches, have shown that enhancer–promoter interactions of a core clock gene are under circadian control (66, 67). However, how all the clock-regulated genes are spatially organized and clustered in the three-dimensional nuclear space and whether that leads to

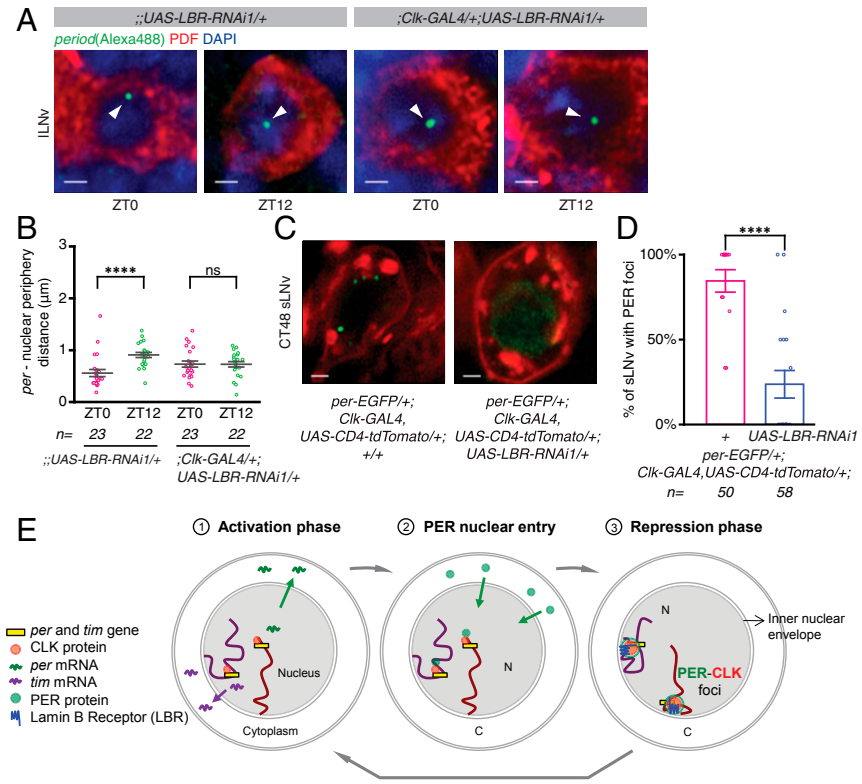

Fig. 6. LBR is required for tethering *per* gene to the nuclear envelope and for circadian rhythms. (*A*) Representative images of DNA-FISH results using *per*-gene probes (green dots) at ZT0 and ZT12 in lLNvs from control (+>*UAS-LBR-RNAi*) and *Clk-GAL4>UAS-LBR-RNAi* flies. (*B*) Quantitation of distance between *per* gene and the nucleus boundary at ZT0 and ZT12 in lLNvs from control (+>*UAS-LBR-RNAi*) and *Clk-GAL4>UAS-LBR-RNAi* flies. (*C*) Representative images of PER foci in sLNvs in control (*perEGFP;Clk-GAL4>UAS-CD4-tdTomato,+*) and experimental (*per-EGFP;Clk-GAL4>UAS-CD4-tdTomato,LBR-RNAi*) flies at CT48 on the third day of DD3. (*D*) Quantitation of percentage of sLNvs with PER foci from control and experimental flies at CT48. Each of the data points correspond to measurements from an individual brain. (*E*) A model for spatiotemporal localization of clock protein–chromatin complexes in clock neurons over the circadian cycle. (Scale bars, 1 μm.) The statistical test used was a Kruskal–Wallis test (*B* and *D*). ****$P < 0.0001$. Individual data points, mean, and SEM are shown. '*n*' refers to the number of neurons. See Dataset S2 for detailed statistical analysis.

their coregulation remains unknown. Based on our results, we propose that clock-regulated genes might be clustered via inter-/intrachromosomal interactions by the clock protein complexes specifically during the repression phase, analogous to the compaction and clustering of repressed genes into discrete nuclear foci (polycomb group bodies) by Polycomb complexes (68, 69).

Fourth, we show that PER protein is required for positioning the core clock genes to different subnuclear locations—the nuclear periphery during the repression phase and in the nuclear interior during the activation phase—over the circadian cycle (Fig. 5). To determine the underlying mechanisms linking the nuclear envelope to circadian rhythms, we leveraged the power of *Drosophila* genetics and conducted a genetic screen and identified that LBR, located in the inner nuclear membrane, is required for peripheral localization of core clock genes during the repression phase and for circadian rhythms (Fig. 6). Interestingly, previous studies have also shown that LBR is required for peripheral heterochromatin organization and silencing of one of the X chromosomes in female mammalian cells (70).

Our work demonstrates that clock proteins form discrete foci and play a key role in positioning the core clock genes close to the nuclear envelope for regulating circadian rhythms. Interestingly, past work on gene regulation has shown that spatial organization of the genome plays a crucial role in regulating gene expression in a few cell types [e.g., inactive X chromosomes in female mammalian cells (54), Hox genes during development (55), immunoglobulin genes in hematopoietic stem cells (56), and stem-cell differentiation genes in neural stem cells (57)]. While these past studies point to a cell type–specific positioning of certain gene loci that becomes established at a specific stage during development, our studies indicate that core clock genes are positioned at different subnuclear environments in clock neurons rhythmically every single day throughout the life of the organism. It will be interesting to test, in future studies, if other clock-regulated genes, and not just the core clock genes, are regulated in this manner. Our studies further suggest that circadian genome organization might be highly dynamic in space and time and that chromatin movement and positioning to specific subnuclear locations might be critical for rhythmic circadian gene expression. How clock-protein foci control positioning of clock genes to specific subnuclear locations at particular times over the circadian cycle and how chromosome dynamics affects circadian gene expression remains to be determined. Given the remarkable similarity between *Drosophila* and mammalian clock systems, we expect the cellular mechanisms uncovered here will be broadly applicable to circadian clocks in humans.

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

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