PNAS COMMENTARY

Regulation of circadian rhythms by clock protein nuclear bodies

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The natural cycles of day and night have a profound impact on the biology of nearly all living organisms on Earth. Throughout evolution, almost all organisms have evolved circadian clocks to synchronize their physiological processes with Earth's day–night rhythm (1). These clocks orchestrate ~24-h rhythms in the expression of over 40% of our genes, including those involved in sleep responses, immune responses, neural communication, and metabolic functions. While the genetic framework of circadian clocks is well understood (2, 3), the real-time dynamics and subcellular behaviors of clock proteins within both animal and cell culture models remain a focus of ongoing research (4, 5). In their PNAS publication (6), Xie et al. investigated the dynamics and subcellular localization of core clock proteins in the human osteosarcoma U2OS cells, which have been known to display circadian rhythms (7), by both stable overexpression and CRISPR tagging of the endogenous clock genes. Intriguingly, they report that while the overexpressed PER2 protein forms phase-separated condensates, the endogenous PER2, BMAL1, and CRY1 proteins assemble into highly dynamic nuclear microbodies (<100 nm in diameter) and engage in transient interactions with each other and with chromatin. This research underscores the importance of studying the dynamic interactions among clock proteins and emphasizes the need to focus on the behavior of proteins at their endogenous expression levels rather than relying on overexpressed proteins.

At the heart of circadian clocks is a transcriptional–translational negative feedback loop orchestrated by the interplay of activators and repressors—leading to 24-h rhythms in gene expression mirroring the day–night cycle of Earth. Activators induce the expression of many genes—including core clock genes which act as repressors—at specific times, while repressors gradually accumulate and eventually inhibit the action of activators (2, 3). In mammals, the CLOCK/BMAL1 complex acts as the transcriptional activator, while the PER1/PER2/CRY1/CRY2 complex serves as the transcriptional repressor (3). Previous research has shown that PER-dependent phosphorylation of CLOCK by casein kinase 1 (CK1) leads to the translocation of the CLOCK/BMAL1 complex away from chromatin, initiating the onset of the repression phase (8). Over the course of the twilight hours, repressor proteins undergo degradation, lifting their inhibitory influence on the activators, thereby resetting the circadian cycle.

In mammals, the primary circadian clock is located in the suprachiasmatic nucleus of the hypothalamus, which interprets light signals relayed from the retina and synchronizes the body's peripheral clocks (9). Interestingly, previous research has shown that cell lines, including NIH3T3 mouse fibroblasts and U2OS human fibroblasts derived from osteosarcoma, display self-sustaining and cell-autonomous circadian rhythms (7, 10). With the advent of CRISPR-based gene editing and advanced imaging techniques, these cell lines have emerged as the ideal model systems for studying protein dynamics within their native cellular contexts (5). In their PNAS publication (6), Xie and colleagues utilize super-resolution imaging in conjunction with endogenous gene tagging to probe the dynamics and spatial organization of clock proteins in synchronized U2OS cells throughout the circadian cycle.

Clock proteins across various species possess intrinsically disordered regions (4, 11, 12), which have been shown to promote the formation of membrane-less, liquid-like condensates (13). Intriguingly, recent studies in *Arabidopsis* (14) and *Drosophila* (4) have shown that their endogenous clock proteins coalesce into biomolecular condensates. Here, Xie at al. conducted experiments to determine whether mammalian clock proteins undergo liquid–liquid phase separation in synchronized U2OS cells. In their initial experiments, the authors engineered U2OS cells so that PER2 is stably overexpressed, which resulted in a 20-fold higher expression than that of the endogenous PER2 level. They demonstrated that overexpressed PER2-GFP forms liquid-like phase-separated nuclear condensates as they can fuse with each other, can rapidly recover after photobleaching, and are highly sensitive to 1,6-hexanediol, which is known to disrupt weak hydrophobic protein–protein interactions (15). Furthermore, they show that the formation of these PER2 condensates is dependent upon phosphorylation by casein kinase 1. Finally, they show that most of the endogenous nuclear BMAL1 localizes to these PER2 puncta, indicating that PER2 condensates can recruit other core clock components. Stable overexpression offers an advantage over conventional methods of transient transfection since transient transfection can lead to PER2 protein levels that are a 100-fold greater than those achieved by stable transduction (16). Nonetheless, given that constitutive stable overexpression of PER2 disrupts the circadian rhythms, the researchers questioned the physiological relevance of the nuclear condensates observed in their stable overexpression models.

To further investigate, they utilized CRISPR to engineer U2OS knock-in cells so that endogenous PER2 is tagged with GFP at

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its C terminus and conducted control experiments to confirm that the knock-in cells exhibited robust circadian rhythms. In contrast to their overexpression studies, super-resolution imaging of endogenous PER2-GFP revealed that they form hundreds of highly dynamic, smaller foci, termed "PER bodies." They estimated that PER bodies consist of ~20 PER2 proteins each and are less than 100 nm in diameter. The number of nuclear PER2 bodies exhibited a circadian rhythm, with a peak of ~650 and a trough of approximately dozens of bodies per cell. Interestingly, they reported that CK1-mediated phosphorylation is not required for PER body formation. Further, they reported that these endogenous PER bodies were not disrupted upon exposure to 1,6-hexanediol, suggesting that formation of PER bodies is not mediated by liquid–liquid phase separation. It remains to be determined whether the assembly of PER bodies is instead driven by stronger protein–protein interactions.

Xie et al. investigated the dynamics and subcellular localization of core clock proteins in the human osteosarcoma U2OS cells, which have been known to display circadian rhythms.

The authors further delved into the dynamics of other clock proteins by engineering BMAL1 and CRY1 knock-in lines. They discovered that both the endogenous BMAL1 and CRY1 proteins also cluster into smaller nuclear bodies, each comprising ~25 proteins per body. The number of BMAL1 bodies in their knock-in cell lines oscillated over the circadian cycle with a phase that is advanced relative to that of PER2 bodies, consistent with the phase of BMAL protein expression observed in mice (17). However, they did not observe any oscillations in the fluorescence intensity or the number of CRY1 bodies throughout the circadian cycle. Using their double knock-in lines, they made the striking observation that endogenous BMAL1 and CRY1 bodies rarely colocalize with PER2 bodies, indicating transient interactions between these proteins. This finding is unexpected, considering that prior biochemical research in the mouse liver (18) and cell lines (19) has demonstrated a strong affinity between mCRY1 and mPER2, indicating that they often exist within a complex. A crucial point to establish is the underlying reasons behind the observed discrepancies in protein interaction dynamics between live-cell imaging in U2OS cells and biochemical studies to deepen our understanding of circadian regulation.

Further, the researchers employed light sheet microscopy to perform time-lapse imaging and reported that these bodies exhibit rapid movement within the nucleus. PER2 and CRY1 bodies were found to be highly dynamic, typically remaining immobile for ~1 s before resuming motion. Conversely, a small percentage of BMAL1 bodies are comparably immobile for up to ~16 s, which is similar to the usual binding duration of transcription factors on chromatin (20). Based on these observations, the researchers proposed that PER bodies might transiently interact with BMAL1-CLOCK complexes, facilitating CLOCK phosphorylation, which then leads to the detachment of BMAL1-CLOCK from DNA. Investigating the behavior of BMAL1 bodies in a PER-deficient context could reveal whether the absence of PER results in reduced BMAL1 dynamics, potentially indicating increased chromatin association.

This study raises some exciting questions for the future. For example, what controls the size of the PER bodies? Given that ~20 PER2 molecules aggregate to form these entities, what mechanisms dictate the formation of such intermediate-sized complexes? The authors reported a significant difference in the number of PER bodies at 4 and 10 h postsynchronization, even though the levels of PER2 protein are similar at these time points. So, how is the assembly of PER bodies regulated? It would be interesting to measure the amount of endogenous PER2 in U2OS knock-in cells and compare it to the levels expressed in a mouse liver cell, particularly since previous research suggests that core clock genes in U2OS cells are expressed at lower levels than in the liver and far fewer cycling transcripts were identified in NIH3T3 and U2OS cells compared to the mouse liver (21). In future studies, generation of novel mouse knock-in models where endogenous clock proteins are fused with fluorescent tags would enable real-time visualization and analysis of protein dynamics in vivo. These studies could provide crucial insights into the functional implications of the varied expression levels of clock proteins on their organization and dynamics in various cells and tissues.

Recent studies employing high-resolution live imaging in various model organisms have begun to reveal a consistent pattern in the organization and behavior of circadian clock proteins (4, 14, 22): clock proteins are typically organized into highly dynamic nuclear bodies and engage in transient interactions with their protein partners. The current study has revealed that endogenous clock proteins, PER2, BMAL1, and CRY1, in human U2OS cells dynamically assemble into nuclear microbodies and engage in transient interactions among themselves and with chromatin. In Arabidopsis, the circadian clock repressor ELF3, which has a prion-like domain containing a polyQ repeat, has been previously shown to form condensates in a temperature-dependent manner via its prion-like region (14). At lower temperatures, ELF3 is dispersed within the cell and binds to DNA to inhibit transcription. In contrast, at higher temperatures, ELF3 coalesces into nuclear foci, thereby inhibiting its DNA binding and consequently alleviating transcriptional repression. Similarly, in *Drosophila* clock neurons, circadian activator CLOCK has been shown to be diffusely distributed in the nucleus during the activation phase. In the repression phase of the circadian cycle, the endogenous activator CLOCK and the repressor PERIOD proteins coalesce into ~10 dynamic nuclear foci adjacent to the inner nuclear envelope, facilitating the sequestration of CLOCK from chromatin, thereby initiating the repression phase (4). Investigating the variances in the number and size of nuclear bodies in *Drosophila* clock neurons and human U2OS cells could shed light on the divergent mechanisms of circadian clocks in insects and mammals. Advancements in real-time live-cell imaging of clock proteins are not only expanding our research toolkit but are also deepening our understanding of the complex mechanisms governing circadian rhythms.

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