Systems Chronobiology: Global Analysis of Gene Regulation in a 24-Hour Periodic World

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Mammals have evolved an internal timing system, the circadian clock, which synchronizes physiology and behavior to the daily light and dark cycles of the Earth. The master clock, located in the suprachiasmatic nucleus (SCN) of the brain, takes fluctuating light input from the retina and synchronizes other tissues to the same internal rhythm. The molecular clocks that drive these circadian rhythms are ticking in nearly all cells in the body. Efforts in systems chronobiology are now being directed at understanding, on a comprehensive scale, how the circadian clock controls different layers of gene regulation to provide robust timing cues at the cellular and tissue level. In this review, we introduce some basic concepts underlying periodicity of gene regulation, and then highlight recent genome-wide investigations on the propagation of rhythms across multiple regulatory layers in mammals, all the way from chromatin conformation to protein accumulation.

TEMPORAL GENE REGULATION THROUGH THE LENS OF THE CIRCADIAN CLOCK

Circadian rhythms in behavior and physiology are widespread across living organisms ranging from cyanobacteria to humans (Bell-Pedersen et al. 2005). Unlike other temporally ordered processes, such as embryonic development or response to acute stimuli, gene regulation considered over a normal day recurs with a well-defined period of 24 hours (Doherty and Kay 2010). These periodic patterns depend on the interactions between environmental cycles and endogenously ticking clocks (Partch et al. 2014). In mammals, these biological clocks consist of a network of hierarchical oscillators in which the master clock, located in the suprachiasmatic nucleus (SCN), controls rhythms in behavior (Mohawk and Takahashi 2011) and coordinates physiological rhythms across peripheral organs through systemic signals such as hormones and temperature rhythms (Mohawk et al. 2012). Natural light cycling in the environment constitutes the main "clock-driver," or Zeitgeber, and synchronizes the central clock in the SCN with the environment. Nevertheless, other Zeitgebers, such as temperature fluctuations and feeding rhythms, contribute to the entrainment of clocks in tissues outside of the SCN (Damiola et al. 2000; Stokkan et al. 2001). To decipher how deep the circadian rhythms are encoded within the organism, single-cell analyses have shown that circadian oscillations rely on a cell-autonomous

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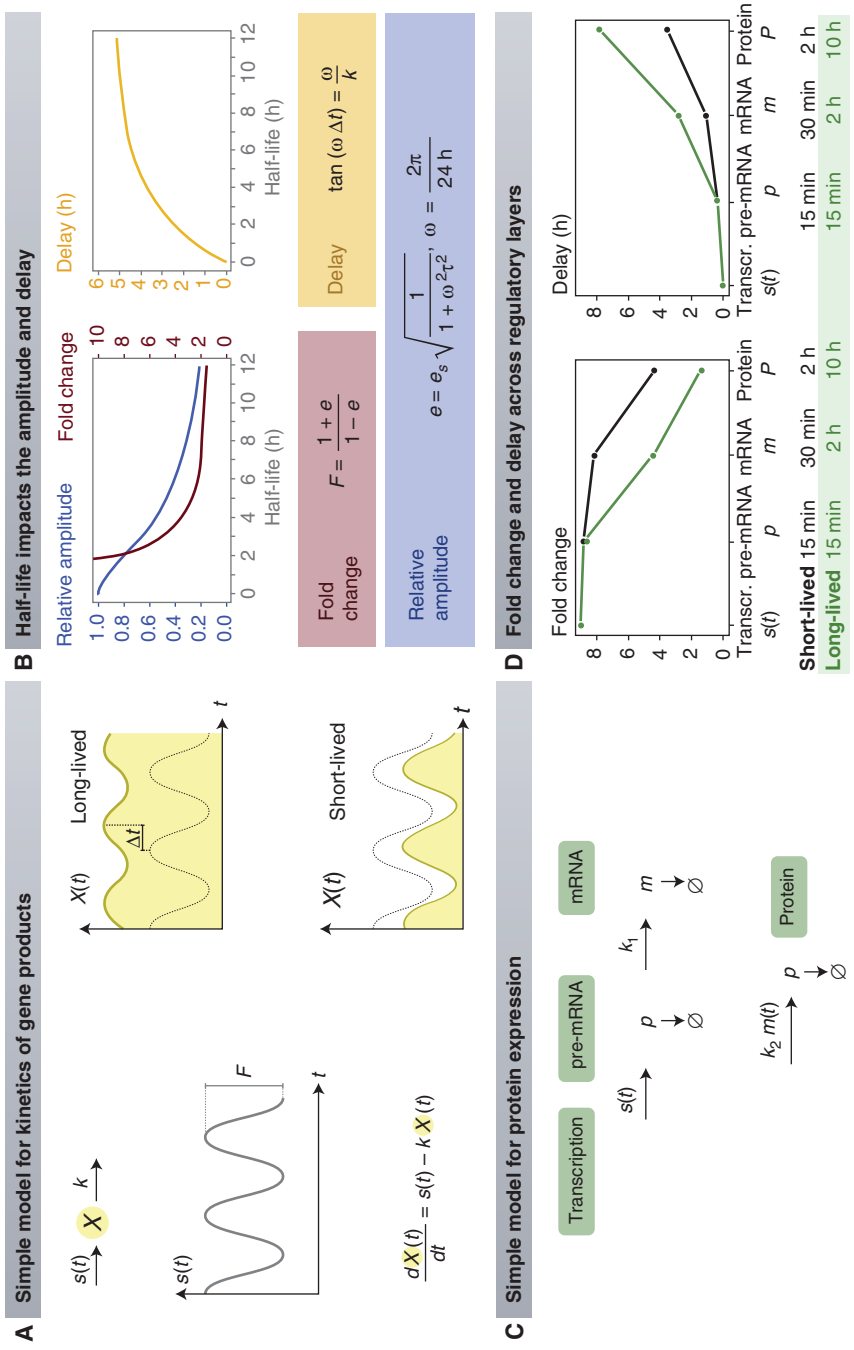

Figure 1. Constraints on transmission of rhythmic information across multiple gene regulatory layers. (*A*) Rhythmic accumulation of gene products. In the stationary state, rhythmic synthesis $s(t)$ leads to rhythmic accumulation of gene product $X(t)$. Assuming that the lifetime of X (lifetime = tau = $1/k$ = half-life/ln(2)) is independent of time, a longer lifetime of X damps amplitudes and induces longer delays (ΔT) (*top right*) compared with shorter lifetimes (*bottom right*). (*B*) Damping (*left*) and delays (*right*) of $X(t)$ as a function of half-life of $X(t)$. (*Legend continues on following page.*)

process, suggesting that potentially every cell in an organism can resonate with environmental time (Nagoshi et al. 2004; Welsh et al. 2004).

Thus, a major challenge in systems chronobiology is to unravel how the clock synchronizes physiology across distinct organs, and in particular how the clock modulates the different layers of gene regulation. In this review, we first refresh concepts underlying how rhythmic information is transmitted across each gene regulatory layer. Next, we highlight recent systems approaches that extend our understanding of circadian gene regulation, all the way from chromatin conformation to translation and protein accumulation.

CONSTRAINTS ON TRANSMISSION OF RHYTHMIC INFORMATION ACROSS MULTIPLE REGULATORY LAYERS OF GENE EXPRESSION

To start with a natural and simple scenario, let us consider a product X synthesized according to a rhythmic synthesis function $s(t)$, with a mean and relative amplitude, and is then transformed or degraded at a temporally fixed rate k (Fig. 1A). From this model, the relative amplitude of X is damped and its rhythm delayed compared with the source s (Fig. 1A). A longer lifetime (large $1/k$) relative to the circadian period increases this damping and the delay (Figs. 1A,B). For example, a half-life of 2 hours for a protein (such as DBP, a protein with robust oscillations) (Fonjallaz et al. 1996) is accompanied by a reduction in relative amplitude by a factor of 0.8 compared with the originating mRNA. Moreover, this damping is accompanied with a delay of 2.5 hours (Fig. 1B).

A basic model of protein expression that involves several of such steps sequentially, from transcription to translation, would predict damping and delays that depend on the half-lives of all gene products (Fig. 1C,D). Indeed, amplitude damping and phase delays have been observed as rhythms propagate from transcription to mRNA as well as from mRNA to protein (Le Martelot et al. 2012; Mauvoisin et al. 2014). These simple models illustrate that, without further regulation, it is in principle more difficult to transmit rhythmic information that is encoded at the transcriptional level compared with protein degradation or protein localization. However, additional active regulations relieve constraints on damping and delays. For example, half-lives that are rhythmic, rather than constant, allow phases to shift arbitrarily and boost relative amplitudes (Luck et al. 2014). Half-lives were reported to vary in circadian genes through mechanisms including mRNA-binding proteins, alternative splicing (Preussner et al. 2014), RNA methylation (Fustin et al. 2013), and phosphorylation of proteins (Vanselow et al. 2006; Mehra et al. 2009; Zhou et al. 2015). Overall, the circadian system represents an attractive model to study the dynamics of gene regulation at every layer from transcription to translation.

STATISTICAL METHODS FOR THE IDENTIFICATION OF PERIODIC GENE EXPRESSION PATTERNS

In this section, we discuss the main methods that are commonly applied to identify temporal patterns in periodic gene expression. The problem of identifying periodic signals has a long history (Fisher 1929; Hartley 1949). In the context of circadian "omics" experiments, it has been mostly tackled as a univariate problem, in which each gene in the data set is tested for

Figure 1. (*Continued*) With increasing half-life, relative amplitudes (or fold change) are damped and $X(t)$ is delayed up to a maximum of 6 hours. Tau $= 1/k$; e_s = relative amplitude of synthesis. (*C*) Simple model for protein expression. Transcription $s(t)$ produces pre-mRNA (p), which can be processed into mature mRNA (m). Proteins are synthesized at a rate proportional to the accumulation of mRNA. In the simplest model, the half-life of each gene product is assumed to be independent of time. (*D*) Damping (*left*) and delays (*right*) of rhythms in short-lived versus long-lived mRNAs and proteins. Gene products with shorter half-lives preserve rhythmic information more efficiently than those with longer half-lives. Initial fold change of $s(t)$ was chosen as 9 to illustrate damping of amplitude.

periodic expression independently. To account for testing multiple genes, this procedure is usually followed by a correction for multiple testing (Noble 2009). Because this problem has been extensively discussed (Wijnen et al. 2005; Luck and Westermark 2016), we will not be exhaustive but will only illustrate the main ideas. Methods can be broadly classified as parametric and nonparametric. A common parametric method is harmonic regression, in which a sine wave is fitted to the data assuming Gaussian noise, which leads to *F*-statistics (Fisher 1929). One way to handle periodic signals that deviate from sinusoids is to allow for higher harmonics, coupled with model selection (Costa et al. 2013). Overall, the methods based on linear transformation are simple and can be extended to handle more complex situations, such as comparisons across multiple conditions. However, caution must be taken when periodic signals deviate from sinusoids and when the noise in gene expression is far from Gaussian, such as in counts data encountered in RNA-sequencing data sets (Love et al. 2014).

Nonparametric methods may be better suited when the periodic patterns deviate from sinusoids or when there are outliers. One approach uses the ranks of the data points to analyze how the data increases and decreases over time. JTK_CYCLE compares the rank of values against an underlying sinusoidal waveform (Hughes et al. 2010). RAIN builds on the strengths of JTK_CYCLE and expands the method to detect nonsymmetrical waveforms, where the increasing and decreasing segments may be unrelated (Thaben and Westermark 2014). Nonparametric methods handle nonstandard rhythmic patterns but could be more difficult to extend to accommodate comparisons across multiple conditions.

On the other hand, the comparison of rhythmic patterns across different conditions (e.g., across tissues, genotypes, feeding conditions) has been less studied. Although many comparative studies have intersected lists of rhythmic genes based on cutoffs, this approach is often unsatisfactory because of its dependence on arbitrary cutoffs. A more refined approach is to ask whether two or more patterns are rhythmic with the same amplitude and phase. Within the framework of linear regression, the Chow test can be used for two conditions and a model selection approach can be used for three or more conditions (Chow 1960; Atger et al. 2015). Finally, we note that, with some exceptions (Leng et al. 2015; Hughey et al. 2016), the problem has been tackled gene by gene. Multivariate approaches that leverage rhythmic or mean signals across multiple genes may provide additional insights toward the regulation of gene modules across conditions.

In the remaining sections, we discuss recent advances toward obtaining a comprehensive view of circadian gene expression programs, and the mechanisms by which certain molecular machineries resonate with diurnal fluctuations.

MODEL OF THE MAMMALIAN CORE CLOCK

At the cellular level, molecular oscillators encode circadian rhythms (Bell-Pedersen et al. 2005). In mammals, although the nature of the molecular pacemaker is still not resolved (Edgar et al. 2012), it is assumed that transcriptional–translational feedback loops (TTFLs) play an important role (Partch et al. 2014; Robinson and Reddy 2014). In the current TTFL model, the CLOCK-BMAL1 transcription factor heterodimer binds E-box DNA sequence and drives the expression of *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. In turn, PER and CRY proteins negatively regulate CLOCK-BMAL1 *trans*-activation effect and repress their own transcription (Gekakis et al. 1998; Sangoram et al. 1998; Kume et al. 1999; Zheng et al. 1999). A second loop consists of the transcriptional control of the nuclear-receptors *Rev-Erbα/β* and *Rorα/β/γ* by the CLOCK-BMAL1 complex (Everett and Lazar 2014). By competitive binding to RORE-responsive-elements (RREs) on the DNA, REVERB and ROR proteins, respectively, repress or activate *Bmal1* gene transcription (Preitner et al. 2002; Akashi and Takumi 2005; Guillaumond et al. 2005; Takeda et al. 2012).

TRANSCRIPTIONAL REGULATION OF CLOCK CONTROLLED GENES

Core clock transcription factors regulate clock-controlled output genes such as the PAR bZIP transcription factors, TEF, HLF, and DBP, which bind D-boxes' DNA sequences in the genome and regulate target gene expression (Gachon et al. 2006; Stratmann et al. 2012). Interestingly, the PAR bZIP transcription factors oscillate with high amplitudes in the liver but with low amplitudes in most brain regions (Gachon et al. 2004). In mammals, a large number of transcripts show circadian accumulation, many of which are tissue specific (Panda et al. 2002; Storch et al. 2002; Zhang et al. 2014). The core clock machinery can generate tissue-specific circadian transcriptomes indirectly by activating tissue-specific transcription factors in a rhythmic manner or directly by cooperating with tissue-specific transcription factors to output rhythmic gene expression in a specific tissue (Andrews et al. 2010; Korencic et al. 2014; Zhang et al. 2015).

The mouse liver, because of its cellular homogeneity and the large amount of biological material obtainable, has been extensively studied at the genome-wide level. Around 10%–15% of genes in the liver were described as rhythmically transcribed (Hughes et al. 2009; Vollmers et al. 2009), although this figure depends entirely on the applied criteria, such as cutoffs or the set of genes considered. For example, diurnal rhythms in gene expression were shown to represent ~50% of the liver transcriptome (Mauvoisin et al. 2014). However, even in the liver, the number of cyclic mRNAs with large amplitudes (e.g., larger than twofold) remains relatively low (Kornmann et al. 2007). This fluctuating transcription is in part the consequence of temporal changes in the recruitment of core clock transcription factors themselves to the chromatin, as well as rhythmic RNA polymerase II (RNA pol II) loading (Cho et al. 2012; Koike et al. 2012; Le Martelot et al. 2012). For example, BMAL1 binds to >2000 sites in the genome including genes implicated in carbohydrate and lipid metabolism (Rey et al. 2011). Histone modifications and chromatin remodeling, which are signatures of gene activity (Bernstein et al. 2007), are also rhythmic around the clock.

Indeed, many studies reported histone modifiers and remodelers as part of the clockwork machinery, such as the histone acetyltransferase (HAT) p300 (Etchegaray et al. 2003), the histone methyltransferases MLL1 (Katada and Sassone-Corsi 2010), and MLL3 (Valekunja et al. 2013). The CLOCK protein itself was reported to show HAT activity (Doi et al. 2006). BMAL1 was recently described as a pioneer transcription factor capable of opening the chromatin (Menet et al. 2014). This is particularly interesting because rhythmic chromatin remodeling and time varying nucleosome occupancy were previously observed (Ripperger and Schibler 2006). On the other hand, biochemical analyses led to the identification of transcriptional repressors associated with core clock components such as the nucleosome remodeling and deacetylases (NuRD) complex (Kim et al. 2014), the nuclear receptor corepressor (NCoR) (Yin and Lazar 2005), Polycomb group proteins (Etchegaray et al. 2006), and histone deacetylases (Feng et al. 2011).

An interesting property of the clockwork machinery resides in its capability to sense the cellular environment through coregulators. In mouse liver, SIRT1, an NAD^+-dependent HDAC, inhibits BMAL1-CLOCK activity (Nakahata et al. 2008). Moreover, CLOCK-BMAL1 activates the transcription of the rate-limiting enzyme, NAMPT, which is involved in NAD^+ biosynthesis (Ramsey et al. 2009), ultimately resulting in SIRT1 activation and BMAL1-CLOCK inhibition. This mechanism uses metabolites to integrate energy status of the cell with the circadian clock (Berger and Sassone-Corsi 2015).

Although a more detailed understanding of circadian regulation of transcription is emerging, many aspects remain unclear. For example, how does the circadian clock associate with tissue-specific components as well as signals to transcribe the proper genetic repertoire at the right moment in the right organ and, in fine, optimize circadian physiological function? This question has been the purpose of a recent review

series (Hussain and Pan 2015). Also, the roles of histone variants (Menet et al. 2014) and distal regulatory sequences in controlling circadian transcription are still understudied and challenging. For example, a large fraction of BMAL1 binding sites (60%) is located >10 kb away from the nearest TSS (Rey et al. 2011), raising questions about whether these distal sites are enhancers. Interestingly, recent RNA-sequencing experiments revealed the presence of thousands of circadian enhancer-RNAs (eRNAs) in mouse liver (Fang et al. 2014). As is often performed in similar studies in other fields, candidate enhancers are assigned to target promoters according to genomic proximity; however, physical distance (measured in 3D) would be functionally more relevant. In the next section, we discuss recent work exploring chromatin conformation, both in general and in the context of circadian biology.

RHYTHMIC TRANSCRIPTION IN A THREE-DIMENSIONAL NUCLEUS

Our current understanding of the global spatial organization of the mammalian genome has benefitted from two main experimental approaches. First, chromosome conformation-capture assays, or "C"-based techniques, which evaluate the frequency of cross-links between genomic regions (de Wit and de Laat 2012). Among the "C" techniques, 3C reveals contact frequencies between few selected pairs of genomic fragments (Dekker et al. 2002), 4C between one locus (the bait) and the entire genome (Simonis et al. 2006), 5C between many and many loci (Dostie et al. 2006), and Hi-C monitors interactions between pairs of genomic sites genome-wide (Lieberman-Aiden et al. 2009). Moreover, ChIA-PET can identify chromatin hubs bound by a given protein (Fullwood et al. 2009). Thus, owing to the biochemical methods involved, "C" techniques usually provide information on chromatin conformation averaged over millions of cells. Second, fluorescence in-situ hybridization (FISH) uses microscopy to directly measure physical distances between genomic loci in individual cells, but with a limited throughput on the number of pairs (Fraser et al. 2015).

The emerging picture can be recapitulated as a hierarchical compartmentalization of the mammalian genome (Bickmore and van Steensel 2013; Dekker and Heard 2015). Briefly, individual chromosomes occupy discrete territories in the nucleus (Cremer and Cremer 2001) and gene rich chromosomes tend to be located near the center of the nucleus. Inside chromosome territories, different chromatin compartments segregate according to chromatin states (Lieberman-Aiden et al. 2009). Recently, Hi-C experiments revealed that mammalian genome folds into structures called topologically associating domains (TADs) (Dixon et al. 2012). TADs are 100-kb to 1-Mb scale chromatin domains that appear to be conserved between mammals (Dixon et al. 2012; Vietri Rudan et al. 2015). Moreover, CTCF and cohesin protein complexes appear to play a major role in governing the contours of chromatin folds, such as TAD boundaries and enhancer–promoter contacts (Dixon et al. 2012; Seitan et al. 2013; Rao et al. 2014; Zuin et al. 2014; Vietri Rudan et al. 2015). Importantly, functional interactions between genomic loci, such as promoter–enhancer pairs, mainly occur within a genomic scale typical of TADs, that is, dozens to hundreds of kilobases, and can be cell-type-specific (Hughes et al. 2014; Mifsud et al. 2015; Schoenfelder et al. 2015). Recently, elegant experiments using CRISPR-Cas9 genome engineering tools showed that chromatin contacts can be rearranged and transcription perturbed by removing CTCF sites or TAD boundaries (Guo et al. 2015; Tsujimura et al. 2015). So far, the best examples that explore chromatin conformation and their functional significance for processes such as transcription have come from developmental biology. The locus control region (LCR) controlling the transcription of *β-globin* genes directly contacts target promoters in specific tissues and developmental stages (Tolhuis et al. 2002; Chang et al. 2013; Deng et al. 2014; Bartman et al. 2016). Also, *Hox* genes and chromatin domains orchestrate folding rearrangements and switches in transcriptional states (Chambeyron and Bickmore 2004; Noor-

dermeer et al. 2011, 2014). Altogether, these studies illustrate the tight connections between chromatin folding and gene expression.

As previously mentioned, the circadian oscillator offers a unique model to study periodic transcriptional regulation. The circadian system is also an ideal system to explore temporal dynamics in the spatial organization of the genome and associated function on gene regulation. Does the global folding of the genome oscillate in a circadian manner? Do circadian genes cluster together? Do regulatory sequences rhythmically contact circadian gene promoters? So far, only a few studies have addressed these questions. The first one used chromosome conformation capture on chip (4C) in wild-type and clock-deficient mouse embryonic fibroblasts (MEFs) to evaluate chromatin contacts involving the *Dbp* promoter (Aguilar-Arnal et al. 2013). Interestingly, although most genomic contacts remained globally stable, there were also circadian fluctuations and clock-dependent chromatin interactions connecting *Dbp* promoter. These rhythmically contacting regions included circadian genes and genomic elements such as E-boxes, which were supported by DNA-FISH (Aguilar-Arnal et al. 2013). However, this study focused exclusively on interactions with regions on *trans*-chromosomes. A more recent study took advantage of paired-end sequencing of 4C libraries to reconstruct networks surrounding the H19 imprinting locus in human ES cells (Zhao et al. 2015). First, the investigators found the locus *Pard3* among the contacted hubs in human embryonic stem (ES) cells. Then in human HCT116 cells, in which *Pard3* was expressed in a circadian manner, the locus was rhythmically recruited to the nuclear lamina, a well-known repressive compartment of the nucleus (Kind et al. 2015). Interestingly, this recruitment was dependent on CTCF and PARP1 protein functions, the latter having been previously linked to the clockwork machinery in the mouse liver (Asher et al. 2010). Moreover, the rhythmic recruitment of *Pard3* to the lamina was corroborated with 3D DNA-FISH experiments and appeared essential for its oscillating transcription (Zhao et al. 2015). To correlate chromatin structure with gene expression, Hi-C and 3D imaging were compared with RNA-Seq around the clock, which showed correlations between chromatin folding and transcription dynamics for certain genes involved in the cell cycle and circadian rhythms (Chen et al. 2015). In this study, comparing the structure and function of the genome suggested correlation between physically interacting gene pairs. For example, 3D DNA-FISH showed that distances between *Per2* and *Clock* rhythmically fluctuate over the day; the two genes were close together when mRNA accumulation of *Per2* was low and *Clock* was high (Chen et al. 2015). Overall, these studies suggest a link between circadian transcription and dynamics of chromatin folding (Fig. 2B). Future investigations combining ChIP-sequencing for factors such as CTCF and cohesin subunits with high-resolution chromosome conformation capture assays and genome editing would help to further establish functional relationships between chromatin folding and the molecular clocks. Interestingly, recent findings suggested a role for CTCF and cohesin in insulating phases of circadianly expressed genes, probably through their function in delimiting TAD, as well as via a functional role of cohesin in connecting gene promoters to distal regulatory elements (Xu et al. 2016).

So far, we have discussed circadian fluctuations in transcription, with an emphasis on the potential role of three-dimensional conformations of the chromatin. However, only a limited overlap was found between the rhythmic accumulation of pre-mRNA and that of mature mRNA (Koike et al. 2012; Menet et al. 2012), suggesting a role of posttranscriptional mechanisms in circadian gene regulation. In the next section, we discuss recent advances in the understanding of posttranscriptional regulation in circadian biology.

CIRCADIAN POSTTRANSCRIPTIONAL REGULATION AT THE RNA LEVEL

With constant half-lives of gene products, amplitudes damp and the phase is delayed (Fig. 1). However, additional regulations, such as the rhythmic degradation of intermediate prod-

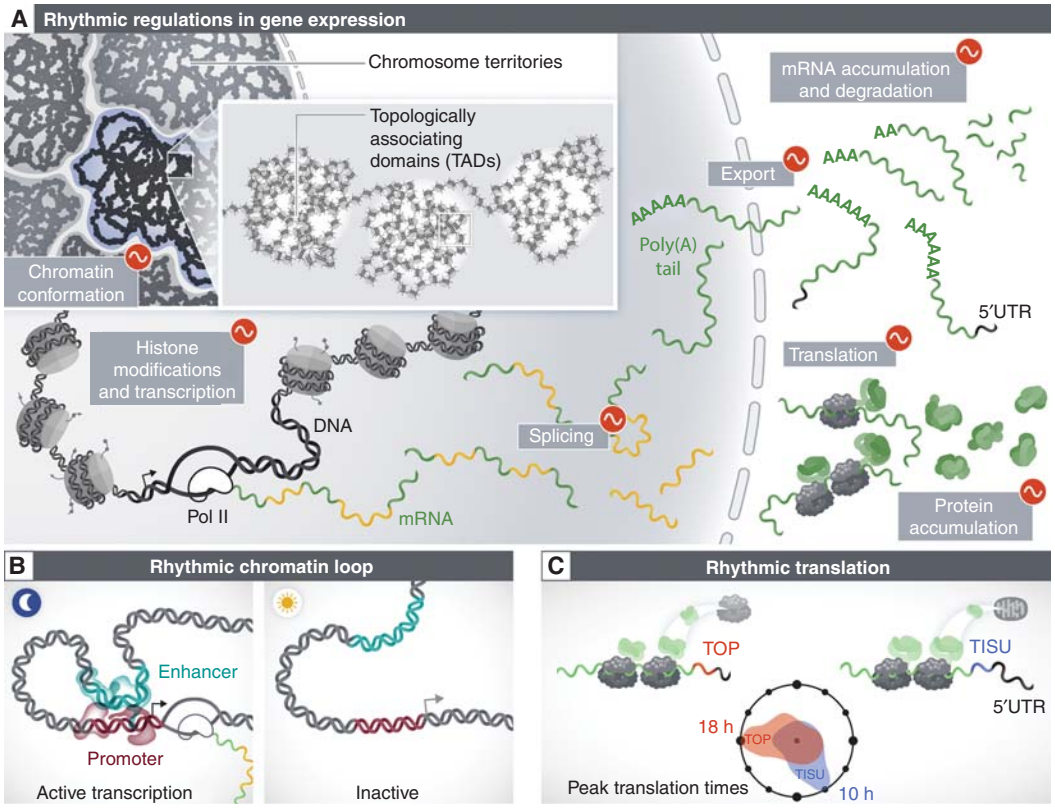

Figure 2. Circadian regulation of gene expression. (*A*) Circadian rhythms can impact gene expression at virtually any step between transcription to translation. In mammals, although many steps of gene regulation such as transcription, mRNA, and protein accumulation are known to fluctuate over the day, the role of the clock in other steps such as splicing and mRNA transport is less understood. Recent advances have highlighted other levels of regulation involving the circadian clock, such as chromatin structure and translation. (*B*) Recent advances highlighted the role of chromatin conformation in regulating circadian genes. For instance, rhythmic contacts between distal genomic regulatory sequences and gene promoters contribute to circadian gene expression. (*C*) By measuring translation rate around the clock, ribosome-profiling experiments found that translation efficiency fluctuates over the diurnal cycle. Thus, although TOP and TISU motif mRNAs accumulate constantly in mouse liver, the rhythmic translation rate of these mRNAs allows fluctuating protein synthesis encoding ribosomal (TOP) and mitochondrial (TISU) functions.

ucts, can further modulate gene expression, for example, by tuning the amplitude or shifting the phase of the driving rhythm (Le Martelot et al. 2012; Luck et al. 2014). One straightforward way to identify transcriptional and post-transcriptional regulatory events underlying circadian gene expression is to compare measures of transcription, mRNA accumulation, translation efficiency, and protein accumulation. Recently, many studies used different techniques to analyze transcriptional activity and mRNA accumulation in mouse liver around the clock. For example, the process of transcription was measured by quantifying nascent RNA chains that are associated with the chromatin (Menet et al. 2012). Others have compared RNA Pol II loading in gene bodies as a proxy for transcription with mRNA accumulation using microarrays (Le Martelot et al. 2012). Furthermore, total RNA-Seq is assumed to simultaneously estimate transcriptional activity (intronic reads) and accumulation of mature

mRNAs (exonic reads) (Koike et al. 2012; Du et al. 2014; Atger et al. 2015; Gaidatzis et al. 2015).

Although studies have identified notable genes regulated at the posttranscriptional level, such as *Tfrc* (Janich et al. 2015) and *Cirbp* (Morf et al. 2012), the genome-wide extent of posttranscriptional regulation of the circadian transcriptome varies from different studies (Koike et al. 2012; Le Martelot et al. 2012; Menet et al. 2012). This divergence is likely a result of different experimental and analysis methods used (Luck et al. 2014). A recent meta-analysis and data modeling approach considered production and degradation rates as circadian parameters, and concluded that 30% of circadian genes are regulated posttranscriptionally (Luck et al. 2014).

Altogether, genome-wide approaches measuring transcription, RNA processing, and accumulation, as well as single-gene studies, emphasized that a non-negligible amount of circadian genes are subjected to posttranscriptional regulation (Kojima et al. 2011; Beckwith and Yanovsky 2014). For example, perturbation of RNA methylation leads to a longer circadian period phenotype in both human and murine cells (Fustin et al. 2013). An important mechanism of RNA processing that greatly expands the diversity of transcripts is alternative splicing (Nilsen and Graveley 2010). Although this process could, in principle, regulate circadian accumulation of many transcripts, surprisingly few works have analyzed the contribution of alternative splicing to mammalian circadian gene expression. In fact, exon arrays have shown that relatively few circadian transcripts (0.4%) are regulated at the splicing level in mouse liver (McGlincy et al. 2012), although these transcripts included notable clock components such as *Clock* and *Npas2*. Single-gene analysis reported that rhythmic degradation of PER1 is regulated by circadian alternative splicing of the U2AF26 factor (Preussner et al. 2014). In cultured mouse cells, the temperature entrainment of the clock was shown to involve posttranscriptional mechanisms. Notably, the cold-inducible RNA-binding protein (CIRBP) is involved in temperature entrainment of the clock, possibly by binding to transcripts encoding circadian oscillator proteins, such as clock (Morf et al. 2012). Interestingly, CIRBP and RBM3, another RNA-binding protein, dictate the choice of polyadenylation sites of target transcripts in a circadian manner (Liu et al. 2013).

Circadian rhythms in poly(A) tail lengths have been known for a long time (Robinson et al. 1988). Using comprehensive methods, hundreds of transcripts showed a circadian poly(A) tail length in mouse liver, some of which correlated with circadian transcription as well as protein abundance (Kojima et al. 2012). Thus, rhythmic deadenylation of transcripts could affect circadian transcripts half-life and translation efficiency. Nocturnin (NOCT) is a cytoplasmic deadenylase rhythmically expressed and controlled by the clock. Furthermore, NOCT KO mice showed resistance to diet-induced obesity (Green et al. 2007; Li et al. 2008). Therefore, it may be tempting to speculate that NOCT could regulate the global circadian fluctuations in the length of poly(A) tails of mRNAs, but recent mouse mutant studies have not confirmed this hypothesis (Kojima et al. 2015). Interestingly, NOCT itself is posttranscriptionally regulated by the microRNA (miRNA) miR-122 (Kojima et al. 2010). miR-122 is important for hepatic circadian functions and targets genes involved in cholesterol and lipid metabolism (Gatfield et al. 2009). Using an inducible DICER knockout, an elegant study evaluated the global contribution of miRNAs to circadian gene expression in mouse liver (Du et al. 2014). Although a non-negligible fraction of circadian transcripts was affected by miRNAs, this study emphasized the resilience of the circadian clock to perturbations in miRNA biogenesis (Du et al. 2014). Taken together, these results underscore the importance of posttranscriptional regulation of molecular clocks. However, the underlying mechanisms are still not well understood and further efforts are needed to better appreciate the usage of different strategies controlling circadian gene expression at multiple steps. In the next section, we discuss recent works highlighting the role of translation in circadian biology using ribosome profiling and quantitative mass-spectrometry.

CIRCADIAN REGULATION IN TRANSLATION EFFICIENCY

The process of translating mRNA into protein is tightly regulated but its contribution to circadian biology remains poorly studied. A few reports have indicated that RNA-binding proteins such as LARK and heterogeneous nuclear ribonucleoprotein (hnRNP) complexes are implicated in circadian modulation of translation in mammals (Kojima et al. 2007; Kim et al. 2010; Lee et al. 2012). The first study to assess translation efficiency globally used microarrays of polyribosome occupancy in mouse liver around the clock (Jouffe et al. 2013). Interestingly, this work revealed that the expression of translation factors such as initiation factors, ribosomal proteins, and ribosomal RNAs underlie circadian regulation. Moreover, the rhythmic expression of ribosome biogenesis intermediates and translation initiation factors were impaired in clock-deficient mice (Jouffe et al. 2013). This link between the circadian clock, ribosome synthesis, and translation initiation raises the question of whether translation rate itself can be rhythmic. To comprehensively address this, ribosome profiling, which consists of measuring ribosomal occupancy on transcripts, is the method of choice. Translation efficiency is estimated by comparing ribosomal occupancy to transcript abundance. This approach was applied to human cell line (Jang et al. 2015) and mouse liver around the clock (Atger et al. 2015; Janich et al. 2015). The results suggested that, for most genes, when transcription fluctuates in a circadian manner, mRNA accumulation and protein synthesis follows. Nevertheless, the three studies found a few exceptions. Protein synthesis can be rhythmic from mRNAs whose abundances are constant. Conversely, mRNA abundance can be rhythmic but lead to flat ribosomal occupancy (Atger et al. 2015; Jang et al. 2015; Janich et al. 2015). For example, iron metabolism mRNAs accumulate constantly but are rhythmically translated in mouse liver (Janich et al. 2015). Because ribosome-profiling studies detect only relative effects on translation rate of individual transcripts, quantifying the absolute ribosome occupancy requires internal controls such as RNA spike-ins (Lian et al. 2016).

A remarkable observation in mouse liver and human cells is the presence of upstream open reading frame (uORFs) in core clock mRNAs (Jang et al. 2015; Janich et al. 2015). Although the functions of the short peptides translated from these uORFs are unknown, disruption of translation reinitiation perturbed the circadian period length of mouse fibroblast (Janich et al. 2015). The most complete investigation on translation efficiency included clock-deficient animals and different feeding regimen (Atger et al. 2015). Interestingly, gene sets encompassing TOP and TISU motifs in the 5′UTRs were rhythmically translated but had constant mRNA abundance (Fig. 2C). These gene sets were associated with ribosomal function, reinforcing previous observations that circadian rhythms are integrated with translation machinery (Jouffe et al. 2013). Importantly, comparing clock wild type with BMAL1 mutant suggested that feeding, rather than the clock, is the main driver of rhythmic translation in the liver (Atger et al. 2015). This is intriguing because the BMAL1 protein itself was linked to translation function (Lipton et al. 2015). Indeed, in MEFs, mass spectrometry of components precipitating with BMAL1 revealed the presence of proteins related to translation. BMAL1 was associated with translation initiation factors in the cytoplasm and promoted cap-dependent translation initiation. Furthermore, the role of BMAL1 in translation was linked to the mTOR pathway (Lipton et al. 2015).

Altogether, these findings suggest that translation is an integral part of the clockwork machinery. The dynamics of protein accumulation throughout the day is discussed in the next section.

CIRCADIAN RHYTHMS IN PROTEIN ACCUMULATION

The first studies evaluating temporal changes in protein accumulation used 2D-gel electrophoresis performed over the clock. This technique revealed that proteins in mouse liver and other

organs accumulated in a circadian manner (Reddy et al. 2006; Podobed et al. 2014). Interestingly, the identified proteins were involved in key physiological functions such as sugar metabolism in liver. More recently, stable isotope labeling of amino acids (SILAC) followed by mass spectrometry enabled quantitative analysis of the circadian proteome. This approach quantified the relative abundances of an unprecedented number of proteins in the SCN (Chiang et al. 2014) and liver (Mauvoisin et al. 2014; Robles et al. 2014) of mice. Overall, 5% to 10% of the detected proteins accumulated in a circadian manner, encoding essential biological processes such as mitochondrial oxidative phosphorylation in the SCN (Chiang et al. 2014), detoxification (Robles et al. 2014), and protein secretion in mouse liver (Mauvoisin et al. 2014). A remarkable observation in mouse liver is that virtually all secreted proteins accumulate rhythmically with a peak at Zeitgeber Time 18 but are encoded by flat mRNAs. This emphasizes the possible relevance of the circadian rhythms in controlling systemic signals. Comparing the proteomes of liver in wild-type versus clock-deficient mice suggested that feeding was the main driver of rhythmic protein accumulation (Mauvoisin et al. 2014). Notably, about half of rhythmic proteins did not come from rhythmic mRNAs, suggesting active circadian translation or rhythmic degradation. Interestingly, for the other half, the amplitudes of rhythmic proteins were damped and phases were shifted by an average of 5.5 h. Simple kinetic models incorporating protein half-life data predicted a delay of 6 h and damped of amplitudes (Fig. 1C), suggesting that the reduced protein amplitudes likely originated from long protein half-lives.

Taken together, these proteomics data, despite relatively low sensitivity compared with DNA and RNA-sequencing approaches, suggested that proteins accumulate rhythmically over the day and in a tissue-specific manner. These results were complemented by absolute quantifications of copy number for core clock-proteins over the circadian cycle in mouse liver (Narumi et al. 2016). Future studies investigating the circadian dynamics of the distribution of proteins across different cellular subcompartments might be more informative. Interestingly, recent findings suggest that mitochondria also show circadian proteome content mediated by the PERIOD complex (Neufeld-Cohen et al. 2016). Finally, critical posttranslational mechanisms such as protein folding, transport, and biochemical modifications are not discussed here.

PERSPECTIVES IN SYSTEMS CHRONOBIOLOGY

In this review, we discussed recent advances coming from large-scale molecular explorations of the mammalian circadian clock. Because the circadian system provides a unique dynamic context, it represents an exciting model for exploring functional genomics. Over the last decade, temporal analyses of gene regulation emphasized a vast and continuous regulatory landscape underlying the mammalian molecular clocks. This includes transcription, RNA-processing, and translation to mention only the most obvious.

However, important questions in circadian biology remain. The building of a comprehensive map of chromatin organization, alternative splicing, RNA transport, protein turnover, and posttranslational modifications around the clock remains exciting challenges. Furthermore, most studies on circadian biology have focused on populations of cells. Recent advances in single-cell technologies will reveal new aspects of circadian gene regulation, such as cell-to-cell variability in chromosome conformation, transcription, and mRNA accumulation (Suter et al. 2011; Nagano et al. 2013; Achim et al. 2015; Battich et al. 2015). New large-scale approaches coupled with computational modeling will be needed to tackle these critical challenges.

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