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Circadian regulation of gene expression: at the crossroads of transcriptional and post-transcriptional regulatory networks

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Gene expression programs activated in response to, or in anticipation of, environmental changes involve sequential steps, from transcription and RNA processing to nuclear export and translation. Here we review recent advances in our understanding of the multiple regulatory layers that control the oscillations in gene expression associated with daily rhythms in metabolism and physiology across eukaryotic organisms. Whereas many genes show coordinated oscillations in transcription, RNA processing and translation, others show significant temporal disconnections between these processes. Thus, circadian oscillations constitute an ideal system for examining how multiple transcriptional and post-transcriptional regulatory steps are integrated to maximize organismal adjustments to environmental conditions.

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Introduction

Living organisms adjust their functions to adapt to changes in environmental conditions. The proper control of gene expression is a crucial aspect of this adjustment and is achieved through a complex interplay of multiple processes. Transcription initiation relies on interactions between *cis*-regulatory DNA elements and *trans*-acting factors that modulate recruitment of the transcriptional machinery to the promoters of target genes. In turn, additional factors regulate transcriptional elongation and termination, and all of these steps are influenced by chromatin structure. Moreover, various cellular processes regulate mRNA levels and functionality. Splicing, 3′ end cleavage and polyadenylation, internal mRNA modifications, nuclear export, translation and mRNA decay constitute additional gene expression regulatory layers that mediate physiological, developmental and metabolic responses to internal and/or external signals.

Circadian physiology integrates most of these phenomena and is an ideal model for studying gene expression regulatory networks [1–3]. Circadian clocks are endogenous timekeeping systems that synchronize physiological events with 24-hour daily cycles. At the core of circadian systems lies a complex set of interlocked transcriptional and translational feedback loops consisting of positive elements that promote the transcription of their own repressors. These so-called core clock components are also modulated by post-translational modifications and by changes in their subcellular localization [3]. Although the identity of these components varies across organisms, the network architecture and general regulatory mechanisms of circadian systems are similar in fungi, plants, insects and mammals (Box 1).

A general picture emerging from transcriptomic studies conducted over the last decade is that physiological oscillations largely result from daily cycles in mRNA levels [4]. Although it was generally assumed that rhythmic transcription was the main source of these oscillations in gene expression, recent evidence from mammals and flies has challenged this view [5••,6,7•,8•,9•,10••,11••]. The goal of this review is to summarize recent studies that highlight the multiple regulatory layers operating at the transcriptional and post-transcriptional levels to regulate the circadian control of gene expression across eukaryotic organisms. In addition, we identify areas of research that merit increased attention, such as the role of coupling between these layers in the regulation of circadian networks. We apologize to colleagues whose work has not been included in this review due to space constraints.

Interplay between transcription factors and chromatin in the circadian control of gene expression

Transcription cycles begin with the binding of activators to gene promoters, followed by the recruitment of general transcription factors and RNA polymerase II (Pol II) to the transcription start site. A broad genome-wide correlation is observed between the binding of the master clock regulator BMAL1 and Pol II occupancy at gene promoters in mouse liver, suggesting that Poll II recruitment underlies daily rhythms in transcription in mammals [9•,11••]. Given the compact organization of DNA in the nucleus, clock-associated transcription factors must either be capable of binding directly to condensed DNA or, alternatively, their binding to DNA must be integrated with ordered changes in chromatin structure that generate

Box 1 The molecular gears

In this box, we briefly describe the main components of molecular clocks and their basic interactions in four model organisms (the mouse *Mus musculus*, the fly *Drosophila melanogaster*, the plant *Arabidopsis thaliana* and the bread mold *Neurospora crassa*).

The first mammalian core transcription-translation feedback loop (TTFL) is composed of the activators CLOCK and BMAL1 and the repressors PER and CRY. CLOCK and BMAL1 form a heterodimeric basic helix-loop-helix-PAS transcription factor, which drives the transcription of its own repressors, PER and CRY, as well as that of other clock-controlled output genes. PER and CRY proteins interact with CLOCK:BMAL1 in the nucleus to inhibit its transcription. Then, PER and CRY are degraded by ubiquitination, thereby relieving the repression of CLOCK:BMAL1 and initiating a new oscillatory cycle. The phosphorylation state of the PER:CRY complex governs its activity and subcellular distribution, and is crucial for maintaining 24-hour cycles (kinases: CKIα, CKIδ and CKIε; phosphatases: PP1 and PP5). In a second TTFL, which is out of phase with the first, retinoid-related orphan receptors (RORa, b, c) activate Bmal1 transcription and REV-ERBs (REV-ERBα/REV-ERBβ) repress it, thereby contributing to the rhythmic transcription of Bmal1 [46].

The *Drosophila* molecular clock is homologous to the one in mammals. The basic helix-loop-helix PAS transcriptional activators, dCLOCK (dCLK) and CYCLE (CYC), bind to the E-box DNA element to initiate transcription. E-boxes are present in the evening-transcribed genes *period* (*per*), *timeless* (*tim*), *vrille* (*vri*) and *Par domain protein 1ε* (*Pdp1ε*). Similar to the mammalian system, PER and TIM repress the activities of CLK and CYC, and they are also under phosphorylation control (kinases: DBT, SGG and CKII; phosphatases: PP1 and PP2A), which regulates their subcellular localization and ultimately leads to their proteasomal degradation, hence closing the loop. The second, anti-phase loop regulates *Clk* transcription. Even though the factors that initiate *Clk* transcription have not yet been described, it is clear that VRI and PDP1ε have opposite effects on *Clk* transcription; while VRI inhibits *Clk* transcription in the early night, PDP1e displaces VRI and thereby promotes transcription at a later stage [47].

In Arabidopsis, the clock is also composed of TTFLs, but the structure of this circadian oscillator is significantly more complex than the one described for other eukaryotes. In the morning, the proteins CCA1 and LHY repress the expression of *TOC1*, which in turn negatively regulates the expression of *CCA1* and *LHY*, forming a negative feedback circuit. CCA1 and LHY also activate the expression of *PRR7* and *9*, and are inhibited by these PRRs together with their homolog PRR5, forming another negative feedback loop. In the afternoon, RVE4, RVE6, RVE8/LCL5, LNK1 and LNK2, promote the expression of the clock genes *TOC1*, *PRR5*, LUX and ELF4, as well as hundreds of clock outputs genes. PRRs have been shown to repress the expression of *RVE8*, *LNK1* and *LNK2*, thereby forming another negative feedback loop. Finally, LUX, ELF3, and ELF4 form the evening complex, which represses expression of the morning-phased gene PRR9 [48]. The inter-locking nature of these loops, coupled with the defined action of each component during the day–night cycle ensures the proper functioning of the plant clock.

The TTLF in *Neurospora* is simpler, consisting of four core proteins, that is, WHITE COLLAR-1 (WC-1), WHITE COLLAR-2 (WC-2), FREQUENCY (FRQ) and FREQUENCY-INTERACTING RNA HELICASE (FRH). Transcription factors WC-1 and WC-2 bind together to form the White Collar Complex (WCC), which promotes the oscillatory expression of *frq*. FRQ interacts with FRH to form the FRQ/FRH complex (FFC), which in turns inhibits the transcriptional activity of WCC, and this complex is also phosphorylated (kinases: CKI, CKII and CamKI; phosphatases: PP1 and PP2A), leading to its proteasomal degradation and closing of the loop [49].

a more relaxed configuration. Interestingly, BMAL1 acts as a pioneering transcription factor that directly binds nucleosomes in condensed chromatin and promotes rhythmic chromatin opening at enhancer regions [8•]; this, in turn, facilitates DNA accessibility to other transcription factors that recruit additional coactivators and chromatin regulators to control rhythmic transcription [8•]. Indeed, histone modifications associated with transcriptional activation, such as H3K9ac and H3K4me3, exhibit robust circadian oscillations that broadly correlate with circadian rhythms in mRNA levels not only in mouse liver [9•,11••], but also in whole plants [12•,13], indicating that the chromatin configuration modulates the circadian transcriptome (Figure 1). In agreement with this idea, several histone-modifying enzymes have been shown to modulate circadian oscillations in gene expression through direct or indirect interactions with core clock genes in both mammalian [14] and plant circadian systems [15] and, indeed, the CLOCK protein itself has histone acetyltransferase activity [16]. In *Neurospora*, the expression of the core clock gene *frequency* is also regulated by histone methylation, chromatin remodeling factors, and rhythmic changes in nucleosomal occupancy [17–19]. Despite the importance of epigenetic control in the circadian regulatory networks in fungi, plants and mammals, no evidence of a similar regulatory layer has been described for the *Drosophila* clock.

Beyond transcription: a plethora of interconnected regulatory events

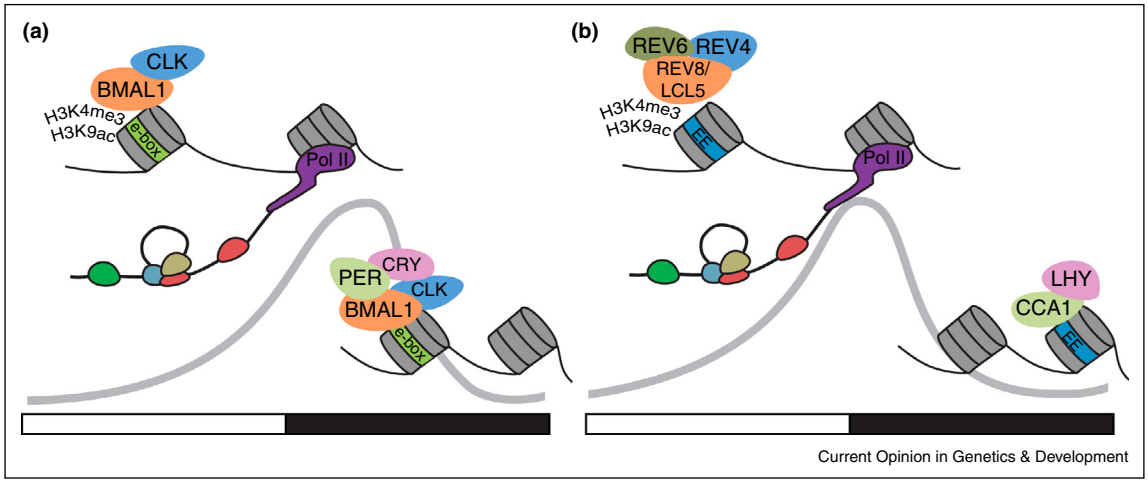
Recent studies conducted on mouse liver show that most genes cycling at the mRNA level do not show clear rhythms at the transcriptional level [8•,11••] (Figure 2). Furthermore, many genes that do not cycle at the mRNA level display significant circadian oscillations at the protein level [5••,7•] (Figure 2). These findings indicate that post-transcriptional regulatory mechanisms controlling RNA processing, stability or translation play a critical role in the control of circadian rhythms in gene expression.

Alternative splicing

Splicing events can be modulated by changes in the levels or activities of splicing factors, interactions with the transcriptional machinery and chromatin modifications [20]. As a result of these modulations, some exons are occasionally extended or skipped, or introns are retained, creating multiple mRNAs from the same gene. This process, known as alternative splicing (AS), results in a remarkable expansion of the genome coding capacity and constitutes an important mechanism controlling gene expression in response to endogenous or environmental signals [20].

AS appears to be a common mechanism co-opted in multiple organisms to couple temperature responses and *time-of-the-day* information [21]. In *Neurospora* and

Figure 1



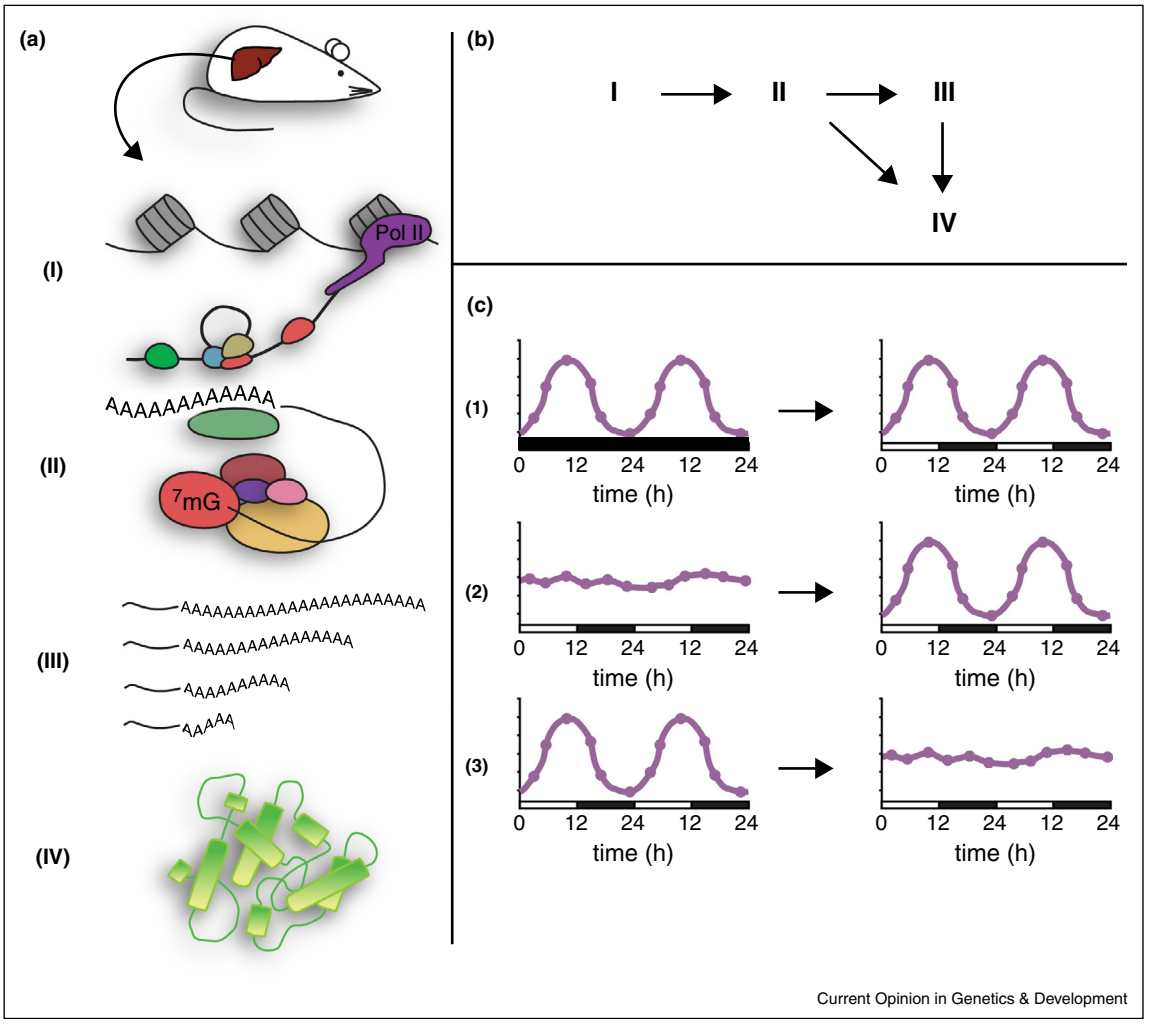
The epigenetic landscape. Mammalian **(a)** and plant **(b)** epigenetic regulation of oscillatory gene expression. (a) In the mouse liver, the histone modifications H3K4me3 and H3K9ac oscillate during the day and correlate with occupancy of the transcription factor BMAL1:CLOCK on E-boxes of oscillating genes (indicated in green, inside the nucleosomes). The transcription of these genes is delayed and only peaks at the beginning of the night. This is followed by the recruitment of CRY and PER to the E-box, which ultimately leads to histone deacetylation and transcriptional repression [14]. (b) In Arabidopsis, the same histone marks are associated with oscillations in gene expression. RVE8/LCL5, together with RVE4 and 6, activate gene expression by binding to the Evening Element (EE) of evening phased genes. In turn, CCA1 and LHY bind to the EE of these genes in the morning and repress their expression by favoring histone deacetylation [15]. White and black boxes indicate 12 hour light and 12 hour dark cycles, respectively, and gray traces depict the temporal transcription profiles. Gray barrels represent nucleosomes. Purple structures depict RNA Pol II, which is associated with the nascent RNA (dark lines) and its associated proteins (colored balloons).

Drosophila, the clock genes *frq* and *per* display temperature-associated AS events that enable proper adaptation to environmental changes [2]. Thermo-sensitive AS is also observed in core components of the Arabidopsis clock [22•,23]. In the case of the plant clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), high temperatures induce a splicing variant that produces a truncated version of the protein that inhibits its activity, constituting a self-regulatory loop that helps plants adjust to temperature changes [24]. In addition, several AS-regulated events in plant clock genes result in transcripts with premature termination codons (PTCs), which regulate mRNA levels instead of increasing proteomic diversity. This trend is common in Arabidopsis [25].

Regulation of AS by the circadian clock has been analyzed at the genome-wide level using tiling arrays in plants [26], exon arrays in mice [27] and RNA sequencing in flies [28]. Clock-regulated AS events were identified in all of these studies, although the percentage of cycling AS events was lower than that of genes oscillating at the whole transcript level [27]. Among the cycling AS events identified in mouse liver, an enrichment was found in clock genes and genes encoding drug detoxifying enzymes [27]. Interestingly, some cycling AS events also show circadian oscillations at the whole transcript mRNA level, although with different phases. In other cases, AS events and whole transcript mRNA levels exhibited different patterns throughout the day, in response to synchronizing signals such as fasting, or in different tissues [27]. This offers a unique opportunity to unravel novel mechanisms connecting AS, transcription, and other RNA-processing steps controlling steady state mRNA levels.

The mechanisms governing AS of clock-associated events and their integration with other processes, such as transcription, are just beginning to be elucidated. For example, several splicing factors show a marked oscillatory profile at the transcriptional level in mouse liver [27], and these factors are candidates for linking AS with the mammalian circadian system. In Arabidopsis, mutations in two components that affect spliceosome function, SKIP [29] and STIPL1 [30], result in long-period phenotypes and alterations in the AS ratios of several genes, including those encoding core clock components. However, it is not clear if and how circadian and/or environmental signals regulate the function of these genes. Interestingly, the first genetic link between circadian clocks and the regulation of AS was established by analyzing mutations in the Arabidopsis gene *PROTEIN ARGININE METHYL TRANSFERASE 5* (*PRMT5*), which lengthen the circadian period by modulating the AS of the clock gene *PRR9* [31,32]. *PRMT5* expression displays circadian oscillation in Arabidopsis, and thereby integrates the regulation of AS into the circadian network [31,32]. PRMT5 was also shown to be a part of the *Drosophila* clock output, since mutations in the *Drosophila* homologue disrupt rhythms in locomotor activity and

Figure 2



The oscillatory nature of gene expression can be established at several steps. **(a)** Six recent studies [5••,7•,8•,9•,10••,11••] of murine liver used high-throughput technologies to highlight the relevance of post-transcriptional modification on circadian physiology by measuring transcription levels (I), mRNA steady state levels (II), mRNA poly(A) tail lengths (III) and/or protein levels (IV). **(b)** Transcription (I) leads to circularized mRNA (II) that can undergo translation (IV) or be modified in the cytoplasm, for example, by changes in its poly(A) tail length (III). **(c)** Each of the transitions represented in (b) could: (1) sustain oscillations, (2) prevent further oscillations, or (3) be the source of an oscillatory phenomenon. Indeed, about 70% of oscillating mRNAs in the mouse liver are not rhythmically transcribed. While hundreds of genes show oscillations in their poly(A) tail length, 20% of these correspond with non-cycling mRNAs. Moreover, 20–50% of the oscillatory proteins showed no corresponding oscillations in transcript abundance. ^{7}mG, RNA cap. Each arrow in (c) represents all possible of transitions indicated in (b). Purple lines depict the temporal profile of I, II, III or IV. Symbols as in Figure 1.

affect the AS of several clock-associated genes without significantly altering the operation of the central oscillator [32]. Although the precise mechanism by which PRMT5 affects AS is unknown, PRMT5 methylates both spliceosomal proteins and histones, and represents a potential loop that can interconnect these different regulatory layers [33].

3′ end processing and polyadenylation

Changes in 3′ end processing regulate gene expression by modulating mRNA stability or translatability. Two mammalian cold-induced RNA-Binding Proteins (RBPs), CIRBP and RBM3, control polyadenylation site selection. While CIRBP regulates clock function in response to temperature fluctuation by modulating mRNA cytoplasmic accumulation [34], low temperatures induce CIRBP and RBM3 binding to the 3′ UTRs of many genes, favoring the use of distal polyadenylation sites [35]. Importantly, changes in the polyadenylation site usage of many genes regulated by CIRBP or RBM3 show strong circadian oscillations in mouse liver [35]. These changes affect the length of the 3′ UTR, and therefore the number of *cis* elements to which RBPs or small RNAs might bind, to regulate mRNA stability or translatability.

RNA methylation

m6A methylation, which is catalyzed by METTL3 and is the most common internal mRNA modification in eukaryotes, was recently found to regulate circadian behavior [36•]. Inhibition of methylation in human U2OS cells or mouse SCN slices slows the pace of the clock. In addition, many clock gene transcripts are subjected to m6A methylation, and mRNA processing and nuclear export are delayed upon *Mettl3* knockdown, providing an explanation for the increased period [36•]. An important question that remains to be addressed is to what extent m6A RNA methylation is regulated by the circadian network. RNA methylation of both non-coding RNAs and mRNAs is guided by snoRNAs. Interestingly, the level of several snoRNA host genes oscillates in the fly brain transcriptome [28], highlighting a possible mechanism by which the clock might couple transcription with this post-transcriptional mechanism.

mRNA nuclear export

mRNA export from the nucleus to the cytoplasm is a complex and energy-consuming step functionally linked to upstream and downstream events in mRNA processing. In addition to controlling polyadenylation site usage, the mammalian RNA-binding protein CIRBP was found to associate with the mRNAs of several clock genes, including CLOCK, and to regulate their cytoplasmic accumulation. CIRBP-depleted fibroblasts show a long-period phenotype that correlates with reduced *Clock* transcript levels in the cytoplasm, but normal nuclear accumulation of *Clock* mRNAs in the nucleus [34]. Temperature-dependent circadian rhythms in CIRBP expression might contribute to daily oscillations in the nuclear export of a subset of genes, including core clock genes. Interestingly, a similar long-period and delayed nuclear export phenotype is observed upon knockdown of the RNA methyltransferase *Mettl3* [36•]. The Arabidopsis clock also relies on the accurate nuclear export of mRNAs for proper functioning. Indeed, the *hos1* mutant exhibits prolonged endogenous circadian periods and low amplitude mRNA oscillations with abnormal accumulation of polyadenylated mRNA in the nucleus, a phenotype shared by four other mutants of the nuclear export machinery [37]. However, it is not clear whether changes in nuclear export are more abundant in core clock genes than in other genes, or represent a general and constitutive defect in the mutant. The emerging picture is that mRNA nuclear export is a crucial step for proper circadian timing; however, to what extent mRNA export constitutes a specific regulatory step of the circadian clock remains to be elucidated.

Polyadenylation dynamics

Once in the cytoplasm, mRNAs can be stored, translated or degraded, and poly(A) tail length is a key factor controlling these processes. Daily oscillations in poly(A) tail length were recently characterized at the genome-wide level in mouse liver, revealing that the circadian clock controls dynamic changes in the poly(A) tail length of approximately 2% of expressed mRNAs [10••]. Interestingly, rhythmic changes in poly(A) tail length were found to be associated with both cycling (43%) and non-cycling (57%) mRNA levels [10••]. This study also identified oscillations in poly(A) tail length among genes showing rhythmic transcription (evaluated measuring pre-mRNA levels) but non-rhythmic steady state mRNA levels, revealing possible coupling between transcription and polyadenylation dynamics (Figure 2). Rhythmic changes in poly(A) tail length can result from oscillations in polyadenylation in the nucleus, or deadenylation and/or re-polyadenylation in the cytoplasm. Indeed, several deadenylases and poly(A) polymerases are under circadian control and therefore likely contribute to the genome-wide oscillations in poly(A) tail length [10••].

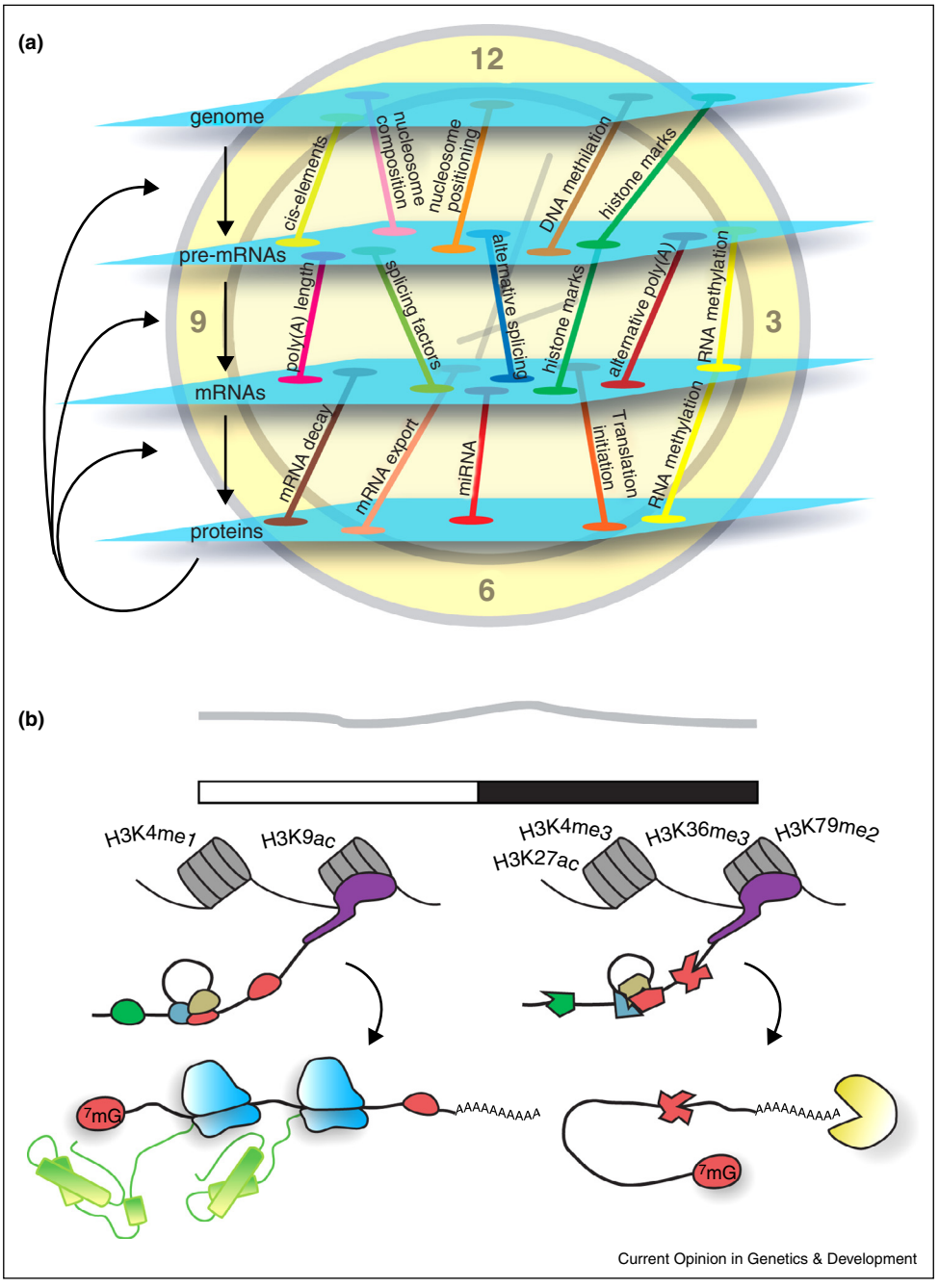
Translation

A disconnection between mRNA and protein oscillations was originally identified for a small subset of genes in mouse liver [38], and this finding was recently expanded using modern proteomic techniques [5••,7•]. This disconnection may have multiple causes, one of which is undoubtedly translational control. In this regard, the translational machinery was recently found to be under clock control in mouse liver [39]. Furthermore, a global assessment of the *Drosophila* circadian translatome confirmed the oscillatory nature of translation in flies [40•]. Interestingly, an analysis of the *Drosophila* brain transcriptome also revealed that many snoRNA host genes are under circadian control [28], and this is particularly relevant because these non-coding RNAs have central roles in ribosomal biogenesis. Additional specific steps that regulate the translation of core clock components have recently been described. For example, PER translation is controlled by TWENTY-FOUR (TYF) [41], a protein that binds both *per* mRNA and translational regulatory complexes. Another RNA-associated protein, ATAXIN-2 (ATX2), was recently shown to interact with TYF and polyadenylate-binding protein to control PER translation in *Drosophila* [42,43].

Conclusions

There is compelling evidence that circadian rhythms in gene expression are the result of regulatory events at multiple steps spanning from transcription to RNA processing and translation. While these processes have often been considered isolated steps, they can be highly interconnected (Figure 3(a)). Splicing, for example, is modulated by factors that act at the chromatin and transcriptional levels, and the splicing machinery itself can regulate chromatin and transcription [44]. If and how these processes interact to regulate rhythmic gene expression is uncertain; however, an interesting candidate to mediate the putative coupling of AS and transcription in the control of circadian rhythms in gene expression is

Figure 3



The emerging picture. **(a)** The transcriptional and post-transcriptional mechanisms of regulation represented in the scheme couple the different steps of gene expression. In the case of the circadian clock, gene expression is governed by multiple regulatory mechanisms that sustain the molecular clock through its 24-hour cycle, and ensure plasticity upon environmental inputs. Black arrows on the left indicate that gene expression constitutes a loop in which the final products affect every step of the process. 3, 6, 9 and 12 define hours on the background clock. **(b)** Genes that undergo non-cycling transcription exhibit oscillations in histone marks. This finding suggests a potential coupling between epigenetic modification and subsequent steps that regulate gene expression. The figure depicts the interesting scenario in which histone modification changes during the day shape the composition of the protein complexes associated with the nascent RNA (indicted by different shapes), ultimately regulating the fate of the mRNA. The protein complexes are shown during two extreme hypothetical situations: mRNA translation (left) and mRNA degradation (right). Light blue structures, ribosomes; yellow shape, 3′–5′ degradation of mRNAs. Other symbols as in Figure 1.

TRAP 150, which interacts with the CLOCK:BMAL1 complex and acts both as a transcriptional coactivator and as a splicing regulator. It would be interesting to identify possible mechanisms that couple other processes, such as transcription and translation. Although these processes take place in two separate compartments, there is evidence that they can be coupled through the association of nascent RNA and specific RBPs that regulate translation [45]. This could explain, for instance, the existence of several genes that oscillate at the transcriptional and translational level, without being rhythmic at the steady state mRNA level (Figure 3(b)). The existence of specific genes that show synchronized rhythms in transcription, RNA processing and translation in some tissues, but not in others, or in response to environmental perturbations, offers a unique opportunity to examine how transcriptional and post-transcriptional regulatory processes interact to control gene expression in space and time.

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