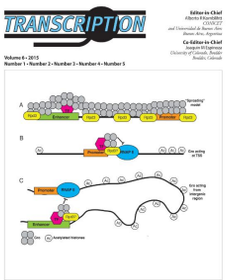

Transcription

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Clock-controlled rhythmic transcription: is the clock enough and how does it work?

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POINT-OF-VIEW

Clock-controlled rhythmic transcription: is the clock enough and how does it work?

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ABSTRACT
Circadian clocks regulate the rhythmic expression of thousands of genes underlying the daily oscillations of biological functions. Here, we discuss recent findings showing that circadian clock rhythmic transcriptional outputs rely on additional mechanisms than just clock gene DNA binding, which may ultimately contribute to the plasticity of circadian transcriptional programs.

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Circadian clock; rhythmic transcription; CLOCK:BMAL1; enhancers; transcription factors; nucleosomes; chromatin interactions

Introduction

Circadian clocks are ubiquitous timekeeping mechanisms that in eukaryotes rely on transcriptional feedback loops. In mammals, virtually every cell harbors a circadian clock that is initiated by the heterodimeric transcription factor (TF) CLOCK:BMAL1. During the day, CLOCK:BMAL1 binds DNA to activate the transcription of *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) which, when expressed, form a repressive PER/CRY complex that inhibits CLOCK:BMAL1-mediated transcription at night (Figure 1). CLOCK:BMAL1 also binds synchronously to thousands of genomic locations to initiate the rhythmic expression of genes that underlie the daily activity of most biological functions [1,2] (Figure 1). The importance of rhythmic gene expression is underscored by the many disorders developed by organisms bearing a genetically- or environmentally-disrupted clock, including obesity, diabetes, cardiovascular diseases, and cancer [3–6].

Based on the initial characterization of core clock gene expression, CLOCK:BMAL1 rhythmic DNA binding was assumed to be both necessary and sufficient for initiating 24-hour transcriptional rhythms (Figure 1). Although this remains true for the rhythmic transcription of core clock genes, analysis of rhythmic transcription at the genome-wide level revealed that the transcription of most CLOCK:BMAL1 targets is disconnected from daytime DNA binding [7–10]. More than 70% of CLOCK:BMAL1 targets are constitutively expressed or not expressed, and peak transcription of some rhythmic targets even occurs at night. Moreover, the rhythmic expression of many direct CLOCK:BMAL1 targets can be markedly altered by changes in the environment (*e.g.*, changes in feeding conditions) despite minimal effects on core clock genes oscillations [8,11]. These results challenge the prevailing idea that CLOCK:BMAL1 rhythmic DNA binding is solely responsible for the rhythmic expression of its target genes, and suggest that CLOCK:BMAL1 uses yet-to-be-characterized regulatory mechanisms to coordinate the transcription of clock-controlled genes (Figure 1). In this point-of-view, we will discuss recent work showing that CLOCK:BMAL1-mediated rhythmic transcription relies on its interaction and cooperation with additional TFs. We focus our discussion on the role of these interactions in regulating TF DNA binding dynamics within *cis*-regulatory regions, and in promoting temporal changes in enhancer-enhancer and enhancer-promoter interactions. Discussion about the role of post-transcriptional events in regulating rhythmic gene expression, and in eventually contributing to some of the discrepancies between CLOCK:BMAL1 DNA binding and mRNA cycling, can be found in some recent reviews [12–16].

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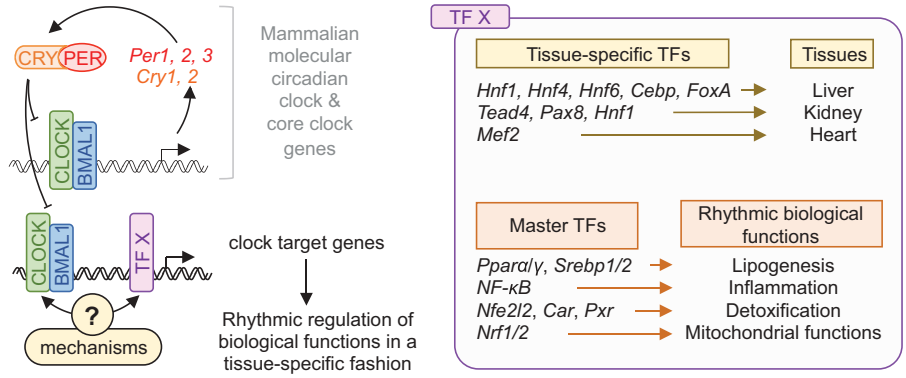

Figure 1. (Left) Schematic of the mammalian molecular circadian clock. Besides driving the rhythmic transcription of the core clock genes *Period* and *Cryptochrome*, CLOCK:BMAL1 also targets thousands of "output" genes to regulate the daily oscillations of biological functions in a tissue-specific manner. Most of these clock output genes are expressed in specific tissues and are regulated by master regulators, suggesting that CLOCK:BMAL1 interacts and cooperates with other TFs to regulate their rhythmic transcription. (Right) Examples of tissue-specific TFs and master TFs that regulate the expression of clock output genes.

2. CLOCK:BMAL1 DNA binding: more than just e-boxes?

CLOCK:BMAL1 is a basic helix-loop-helix TF that binds canonical E-boxes (CACGTG), preferentially on those organized in tandem with a 6 or 7 nucleotides spacer [2,17,18]. The genome-wide synchronous binding of CLOCK:BMAL1 during the day is primarily caused by daily rhythms in the intrinsic ability of CLOCK:BMAL1 to bind E-boxes, since rhythmic DNA binding can be recapitulated *in vitro* using nuclear protein extracts collected across the 24-hour day and DNA probes harboring E-boxes [19,20]. The diurnal oscillation of CLOCK:BMAL1 ability to bind DNA is mediated by rhythmic interaction with the PER/CRY repressor complex, and involves rhythmic PER/CRY-dependent post-translational modifications [21–23]. Like most TFs, CLOCK:BMAL1 binds almost exclusively open chromatin regions, *i.e.*, within DNase I hypersensitive sites (DHS) that comprise enhancers, promoters, and transcription start sites (TSS) [10]. These data led to the assumption that CLOCK: BMAL1 DNA binding rely primarily on the presence of an E-box within a DHS. However, recent work indicate that TFs binding near E-boxes can also modulate CLOCK:BMAL1 access to DNA.

Much of this evidence comes from the characterization of CLOCK:BMAL1 DNA binding sites at the genome-wide level in different mouse tissues by chromatin immunoprecipitation sequencing (ChIP-seq) [9,10,24,25]. Results showed that CLOCK:BMAL1 ChIP-seq peaks are mostly tissue-specific, with common sites in more than two tissues representing less than 10% of all peaks [9,10,24]. Not surprisingly, many tissue-specific peaks are located within tissue-specific DHSs, indicating that chromatin openness significantly contributes to tissue-specific CLOCK:BMAL1 DNA binding [10]. However, the majority of tissue-specific peaks are located at DHSs common to multiple tissues, indicating that factor(s) other than chromatin openness facilitate(s) tissue-specific CLOCK:BMAL1 DNA binding [10]. One such factor likely involves tissue-specific TFs (ts-TFs), whose motifs and DNase I footprints are enriched at tissue-specific peaks but not at peaks common to several tissues. A mechanism in which ts-TFs facilitate tissue-specific binding of ubiquitously expressed TFs (u-TFs) is not unprecedented [26–31], and has notably been described for the *Drosophila* CLOCK:BMAL1 ortholog CLK:CYC [32].

Facilitation of CLOCK:BMAL1 DNA binding by other TFs does not seem to be unique to ts-TFs, as activation of NF-κB by lipopolysaccharides (LPS) changes the location of ~30% of CLOCK:BMAL1 peaks genome-wide to NF-κB DNA binding sites in mouse liver [33]. The lack of repositioning in the liver of NF-κB-deficient mice treated with LPS clearly demonstrates that it is driven by NF-κB itself [33]. Remarkably, 40% of CLOCK:BMAL1 DNA binding events that are detected under standard conditions are absent in the liver of mice injected with LPS, suggesting that LPS treatment also altered the

binding of TFs that facilitate CLOCK:BMAL1 DNA binding under standard conditions [33]. Together, these data demonstrate that TFs binding near E-boxes can affect the efficiency at which CLOCK:BMAL1 binds DNA, and indicate that CLOCK:BMAL1 DNA binding is likely more plastic than initially envisioned.

While the mechanisms by which TFs facilitate CLOCK:BMAL1 DNA binding remain to be fully elucidated, they likely involve TF-mediated nucleosome displacement (Figure 2). NF-κB and many ts-TFs are pioneer TFs that can bind nucleosomal DNA to promote histone eviction and free DNA exposure [34–36]. Because most TFs bind free DNA more efficiently than DNA wrapped around histones, binding of pioneer TFs to DNA can facilitate the recruitment of TFs whose motifs are located nearby [37,38] (Figure 2). Recent work has shown that nucleosome-mediated TF cooperativity is not restricted to pioneer TFs, and actually extends to almost all TFs [39]. This suggests that virtually any TF may facilitate CLOCK:BMAL1 DNA binding. Since many TFs interact with chromatin remodelers, their binding to DNA is frequently associated with nucleosome sliding and/or histone eviction, which may then facilitate the recruitment of other TFs. Moreover, the transient conformational fluctuations inherent to nucleosomes (*i.e.*, nucleosome breathing or diffusion) can also expose a TF binding motif, and the initial binding of a TF can enhance the accessibility and the binding of a second TF [40–45].

Taken together, these data support the notion that CLOCK:BMAL1 DNA binding does not just rely on its intrinsic capacity at binding E-boxes, but also depends on how additional TFs modulate the chromatin context around E-boxes. It also opens the exciting possibility that changes in the environment reprogram circadian transcriptional programs by repositioning CLOCK:BMAL1 DNA binding through the altered recruitment of many TFs to chromatin. Testing whether CLOCK:BMAL1 DNA binding can be repositioned genome-wide despite minimal changes in its intrinsic DNA binding capacity by additional manipulations than LPS would ultimately validate this hypothesis.

3. Nucleosome and other TFs contribution to the genome-wide regulation of CLOCK:BMAL1 DHS activity

Characterization of CLOCK:BMAL1 transcriptional output revealed that CLOCK:BMAL1 DNA binding is necessary but not sufficient to rhythmically regulate the activity of its target DHSs. At the genome-wide level, the activity of CLOCK:BMAL1-bound DHSs, as measured by H3K27ac ChIP-seq signal and enhancer RNA expression, coincides with target gene transcription and not with daytime CLOCK:BMAL1 DNA binding [8]. Several reports suggest that this uncoupling between CLOCK: BMAL1 DNA binding and DHS activity is due to the nature of the TFs that bind CLOCK:BMAL1 DHSs [8,10,46].

CLOCK:BMAL1 is a pioneer-like transcription factor, as its daytime DNA binding promotes the removal of nucleosomes genome-wide [8,46] (Figure 2). While the underlying mechanisms remain unclear, they

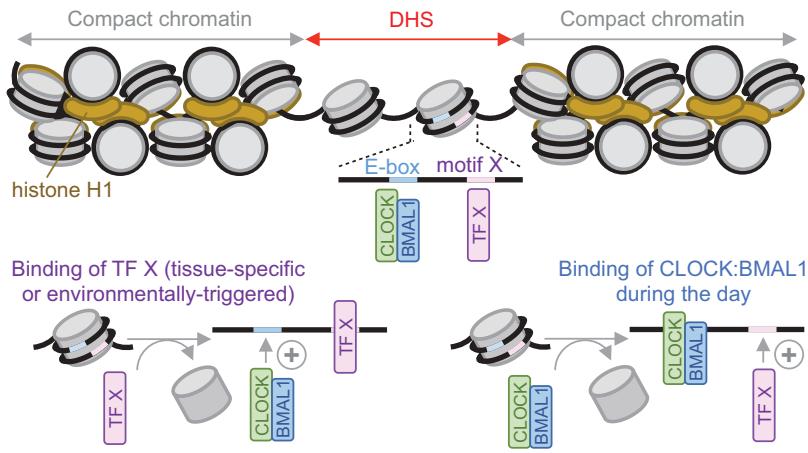

Figure 2. CLOCK:BMAL1 cooperation with other TFs and the role of nucleosomes. Binding of a tissue-specific or master TF to DNA competes with nucleosome compaction, thereby increasing exposure of naked DNA and potential E-boxes and facilitating CLOCK:BMAL1 DNA binding. Conversely, CLOCK:BMAL1 DNA binding during the day, which promotes nucleosome eviction, increases the exposure of naked DNA and motifs for other TFs, and thereby facilitates the binding of other TFs.

likely involve CLOCK:BMAL1 capacity at binding nucleosomal DNA [46,47], the co-recruitment of chromatin remodelers like CHD4 to E-boxes [48], and/or nucleosome diffusion on DNA [42,43,45]. Evidence that CLOCK:BMAL1 facilitates daytime H2A.Z incorporation at its DHSs suggests that the contribution of nucleosome diffusion could be more particularly relevant. H2A.Z ChIP-Seq signal in mouse liver is rhythmic and peak during the day at CLOCK:BMAL1 DHSs, whereas it is arrhythmic and low at all time points in *Bmal1*$^{-/-}$ mice [46]. Interestingly, nucleosomes containing the histone variant H2A.Z exhibit increased diffusion on DNA, which consequently facilitates TF DNA binding and promotes further histone displacement [45]. Thus, by mediating histone eviction, H2A.Z incorporation, and exposing free DNA, CLOCK:BMAL1 likely generates a chromatin landscape that is favorable for the binding of TFs during the day (Figure 2). Consistent with this idea, DNase I footprints for the ts-TFs HNF6 and CEBPA are decreased at CLOCK:BMAL1 DHSs in *Bmal1*$^{-/-}$ mouse liver, but not at DHSs untargeted by CLOCK:BMAL1 [10]. Binding of HNF6 is rhythmic and peaks during the day at several CLOCK:BMAL1 DHSs but not at DHSs only bound by HNF6 [46]. Binding of the circadian repressors REV-ERBα and REV-ERBβ is enriched at CLOCK:BMAL1 DHSs targeting rhythmic genes peaking at night, *i.e.*, within genes with a peak of transcription that is in antiphase with CLOCK:BMAL1 DNA binding [8,49]. More generally, a meta-analysis of TF ChIP-Seq datasets in mouse liver indicates that u-TFs, but not ts-TFs, recruited near CLOCK:BMAL1 binding sites likely contribute to the heterogeneity of CLOCK:BMAL1 DHS activity and target gene transcription [8]. Additional evidence is now required to experimentally validate these findings and test the extent to which u-TFs binding underlies CLOCK:BMAL1 DHS transcriptional activity.

Findings from other systems strongly suggest that DHS activity and target gene transcription relies on cooperative binding of several TFs, and that this cooperative TF binding itself likely involves nucleosome displacement and/or removal. Indeed, findings from the ENCODE project and others demonstrated that TF ChIP-Seq peaks are almost exclusively concentrated in open chromatin regions, and that DHSs are highly enriched in TF DNA binding motifs [50–53]. Many experiments have shown that the combinatorial arrangement of TF DNA binding motifs, along with TF cooperation when co-bound to DNA, are critical for regulating DHS transcriptional activity [53,54]. A particularly compelling example is the comparative analysis of TF ChIP-Seq peaks in the liver of six closely related rodents, which revealed that the loss of one TF motif in a species often results in the loss of binding of other TFs, and this even if their motifs are preserved [55]. Consistent with this result, and reminiscent to what has been observed at CLOCK:BMAL1 DHSs, knocking out one TF also decreased other TF ChIP-Seq signal at co-bound DHSs [55]. This combinatorial role of TF binding sites in regulating DHS activity is further exemplified by results showing that the enhancer activity of PPARγ binding sites does not rely on just PPARγ, but rather depends on varying contributions from dozens of TFs binding in the immediate vicinity, including interactions between combinations of these TFs [56].

While cooperative TF binding can involve various mechanisms including direct protein-protein interactions, the role of nucleosomes and especially of TF-mediated cooperative nucleosome displacement has emerged as a ubiquitous feature for how TFs regulate DHS activity and target gene transcription [39,53,57–59]. Recent studies have shown that gene expression not only correlate with the reduction in nucleosome occupancy at promoters and enhancers but that this correlation can be predicted based on TF expression levels and on the chromatin-remodeling capabilities of the different TFs [60]. Nucleosomes have also been shown to decrease the dwell time of TFs on DNA and, consequently, to directly reduce bursts of transcription [61].

In summary, data from various systems indicate that DHS transcriptional activity is regulated through the cooperative binding of multiple TFs, and that nucleosome hindrance of TF binding motifs provide a mechanistic underpinning for TF cooperation. Similar mechanisms could explain how CLOCK:BMAL1 regulates DHS activity and subsequent rhythmic gene expression.

4. CLOCK:BMAL1, rhythmic DHS-DHS interactions, and control of transcription

Comparison of BMAL1 cistromes between tissues not only revealed that ts-TFs facilitate CLOCK:

BMAL1 DNA binding, but also uncovered that interactions between CLOCK:BMAL1 DHS and other DHSs likely contribute to initiating rhythmic transcription [10]. Most genes exhibiting similar CLOCK:BMAL1 ChIP-Seq signal between three different tissues were surprisingly rhythmic in only one or two tissue [10]. While TFs binding at CLOCK:BMAL1 DHSs could explain the differences in rhythmic transcription for some targets (through the regulation of CLOCK:BMAL1 DHS activity by u-TFs, Figure 3), the presence of other DHSs located in CLOCK:BMAL1 target loci was often associated, and positively correlated, with rhythmic transcription for many target genes [10]. This suggests that other DHSs contribute, and may even cooperate, with CLOCK:BMAL1 DHSs to drive rhythmic transcription. Consistent with this hypothesis, day/night comparison of mouse liver DHS-DHS interactions revealed that CLOCK:BMAL1 target rhythmic transcription coincides with rhythmic interactions between CLOCK:BMAL1 DHSs and other DHSs, with more interactions occurring during the day when CLOCK:BMAL1 binds DNA [10]. These results are consistent with recent findings showing that rhythmic transcription in mammals is associated with rhythmic long-range interactions between *cis*-regulatory elements [62–65], and suggest that CLOCK:BMAL1 control of rhythmic transcription is associated with the presence of rhythmic interactions between its DHSs and other DHSs (Figure 3).

Whether the formation of rhythmic chromatin interactions is causal or consequential of rhythmic transcription is still unknown, and this topic remains somewhat controversial in other fields. While many reports showed that long-distance contacts between enhancers and TSS activate transcription [66–70], some conflicting results have been reported [71]. In particular, DHS-DHS interactions have been proposed to be necessary but not sufficient for transcription activation, as interactions can be found in a poised state for later activation [72–74]. The mechanisms bringing different DHSs into physical contact remain unclear as well. The mediator complex has long been thought to stabilize contacts between *cis*-regulatory regions. This notion is however challenged by a recent study showing that destabilization of the mediator complex decreases the transcriptional activity of interacting DHSs without affecting DHS-DHS interactions [75]. A phase separation model where transcription factors, chromatin regulators, and co-activators would form liquid phase transcriptional condensates has emerged as an exciting model to not only explain how different DHSs may physically interact, but also how TFs may cooperate at the

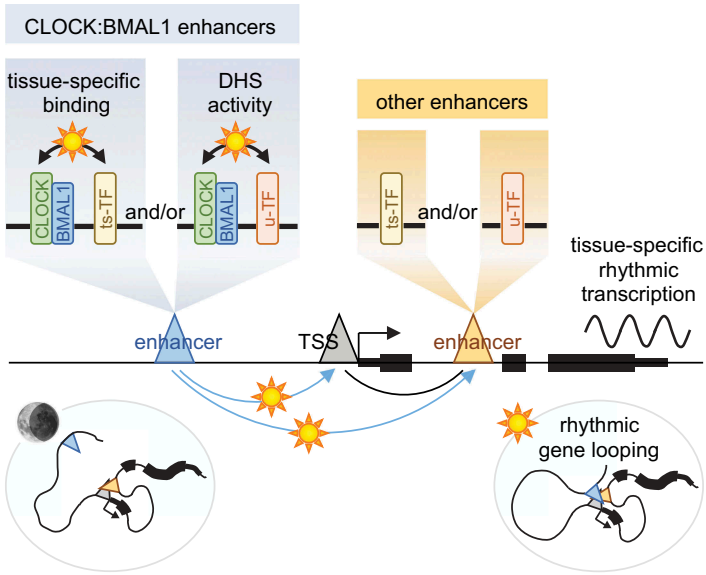

Figure 3. Rhythmic transcription of CLOCK:BMAL1 target genes at the genome-wide level relies on TF cooperation at CLOCK:BMAL1 DHSs as well as rhythmic physical interactions between CLOCK:BMAL1 DHSs and other DHSs. See text for details.

single DHSs level [76,77]. These "gel-like" condensates exhibit properties of liquid droplets in that their formation is mediated by liquid-liquid phase separation and they can undergo fission and fusion [76]. They are formed by cooperative interactions between multivalent molecules, are enriched in proteins containing intrinsically disordered regions, and can be modulated by post-translational modifications [76,77]. Recent findings showed that the number of TF binding sites, along with their density across a short DNA region, is positively associated with the formation of transcriptional condensates [78]. Interestingly, transcriptional condensates were also shown to contribute to long-range communication between enhancer-promoter pairs, and thus facilitate higher-order organization of the three-dimension genome [78]. Given that core clock proteins harbor extensive intrinsically disordered regions, this phase separation model may represent an attractive mechanism by which the circadian clock components regulate transcriptional activation as well as repression [77,79]. The recent finding that phosphorylation of Pol II C-terminal domain switches its localization from a functional type of condensate to another [80] may also provide a conceptual framework to understand the reasons behind the dramatic changes in phosphorylation profiles over the course of the day exhibited by most core clock proteins [22,81,82]. Specifically, the nighttime recruitment of hyperphosphorylated PERs to CLOCK:BMAL1 DHSs may drive a switch from an active daytime type of transcriptional condensate to a repressive nighttime type of condensate.

5. Reprogramming of circadian transcriptional programs, and the role of other TFs

Rhythmic gene expression is ubiquitous, and more than half of the mammalian genome is rhythmic in at least one tissue [83–85]. Yet, rhythmicity is tissue-specific, and rhythms in gene expression faithfully mirror the biochemical, biological, and physiological functions that each tissue subserves [83–85]. Reports over the last few years also revealed that rhythms in gene expression are surprisingly plastic. Changes in the environment like feeding conditions, aging, and antibiotics treatment, can reprogram rhythmic transcriptional programs without dramatically affecting the oscillations of clock gene expression [11,86–91]. Although rhythmically expressed genes are not all directly targeted by CLOCK:BMAL1, this reprogramming of rhythmic gene expression also applies to CLOCK:BMAL1 direct targets [e.g., 8]. The mechanisms mentioned above that involve TFs binding at CLOCK:BMAL1 DHSs, and interactions between CLOCK:BMAL1 DHSs with other DHSs, could contribute to the alteration of CLOCK:BMAL1 target gene expression by environmental changes despite minimal changes in core clock gene oscillation (Figure 3).

Because many rhythmically expressed genes regulate defined biological processes (*e.g.*, lipogenesis in the liver, immune response in myeloid cells), their expression is also regulated by specific master transcriptional regulators (*e.g.*,*Srebp1/2* and *Pparα/γ* for lipogenesis, *NF-κB* for immune response) (Figure 1). Thus, the expression of most CLOCK:BMAL1 targets is regulated, and likely coordinated, not only by core clock genes but also by master regulators. As discussed above, integration of transcriptional signals from CLOCK:BMAL1 and master TFs can occur at the level of a single DHS, with master regulators regulating CLOCK:BMAL1 DNA binding and inversely. The genome-wide repositioning CLOCK:BMAL1 DNA binding after NF-κB activation exemplifies this possibility [33; see above]. In addition, master regulators can also bind to the DHSs that synergistically interact with CLOCK:BMAL1 DHSs (Figure 3). Environmental changes that affect the transcriptional activity of master TFs are likely to affect the dynamics of DHS-DHS interactions and, consequently, of target gene transcription. It is tempting to speculate that some synergistic DHS-DHS interactions involve dominant and subordinate DHSs, and that a dominant DHS can regulate the activity of a subordinate DHS via direct chromatin interaction. Such a model could explain why rhythmic interactions between CLOCK:BMAL1 DHSs and other DHSs are detected for rhythmically expressed target genes. A dominant CLOCK:BMAL1 DHS would make rhythmic contacts with a subordinate DHS to regulate its activity, ultimately leading to rhythmic transcription. Conversely, a dominant (arrhythmic) DHS would regulate the activity of a subordinate CLOCK:BMAL1 DHS across the 24-hour day and lead to arrhythmic gene expression. By altering the transcriptional activity of

master TFs, environmental changes may affect the dominant-subordinate relationship between a CLOCK:BMAL1 DHS and another DHS, and consequently lead to the reprogramming of rhythmic transcriptional programs genome-wide. Consistent with this idea, genes whose rhythmic expression is more resilient to changes in environment, such as core clock genes, harbor multiple CLOCK:BMAL1 DHSs, and each DHS contains multiple E-Boxes [8]. The expression of such genes would be less likely regulated by a master TF within a single DHS and less prone to changes in the balance between dominant-subordinate DHS-DHS interactions. Future experiments aiming at testing this model will shed light on the possible mechanisms underlying the plasticity of circadian transcriptional programs.

6. Conclusions

The genome-wide characterization of rhythmic gene expression has revealed that the circadian clock imprints 24-hour rhythms on transcriptional programs that are already under the regulation of specific master TFs. The circadian clock adds a layer of transcriptional regulation (a temporal one) to genes being regulated by other TFs, and this may explain why knocking out core clock genes is not developmentally lethal in any organisms. While not immediately lethal, clock-controlled rhythmic transcription is critical for organismal health, as exemplified by the many diseases developed by organisms bearing a genetically- or environmentally-disrupted clock [3–6]. In our view, an emerging picture is that every gene is virtually capable of being expressed rhythmically under a given condition or another, which has relevance to understand the negative impact of clock disruption in specific environmental and/or pathologic conditions. Perhaps as importantly, the plasticity of rhythmic gene expression between conditions could be leveraged in comparative approaches to define the mechanisms by which circadian clock effects on the regulation of transcription at the level of a single CLOCK:BMAL1 DHS, as well as the level of DHS-DHS interactions.

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Disclosure statement

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