HHS Public Access

Author manuscript

Nat Rev Genet. Author manuscript; available in PMC 2018 March 01.

Published in final edited form as:

Nat Rev Genet. 2017 March ; 18(3): 164–179. doi:10.1038/nrg.2016.150.

Transcriptional architecture of the mammalian circadian clock

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Abstract

Circadian clocks are endogenous oscillators that control 24-hour physiological and behavioral processes in organisms. These cell-autonomous clocks are composed of a transcription–translation-based autoregulatory feedback loop. With the development of next-generation sequencing approaches, biochemical and genomic insights into circadian function have recently come into focus. Genome-wide analyses of the clock transcriptional feedback loop have revealed a global circadian regulation of transcription factor occupancy, RNA polymerase II recruitment and initiation, nascent transcription and chromatin remodelling. The genomic targets of the circadian clock are pervasive and are intimately linked to the regulation of metabolism, cell growth and physiology.

Introduction

Life on earth evolved in the presence of a 24-hour energetic cycle driven by the solar day. To adapt and respond optimally to the daily environmental cycles, living systems utilize internal timing systems that resonate with the 24-hour day[1] (Figure 1a,b). Across life forms, the internal timing system can be seen at the transcriptional level, giving rise to divergent gene networks in different organisms that oscillate on a 24-hour basis[2]. 'Circadian clocks', as these endogenous timers are known, seem to be the product of convergent evolution, as the design principles of circadian transcriptional circuits share a common network motif[3] (that is, negative feedback with delay (Figure 1c)), although the specific components are divergent across the kingdoms of life[2, 4–7]. The link to the daily solar energetic cycle is most obvious in photosynthetic organisms[8] but is also a conserved motif in the nutrient cycles of higher organisms[9–11]. Whether circadian clocks evolved to adapt to the energetic demands of the

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Competing interests

The author declares competing interests. See the article online for details.

FURTHER INFORMATION

Link 1: http://www.genecards.org/Search/Keyword?queryString=circadian%20clock

Link 2: CircaDB, doi: 10.1093/nar/gks1161, http://circadb.hogeneschlab.org

Link 3: BioGPS Circadian Genomics Screen, doi: 10.1016/j.cell.2009.08.031, http://biogps.org/plugin/406/circadian-genomics-screen/

Competing interests

The author is a co-founder and scientific advisory board member of Reset Therapeutics, Inc.

Subject categories

Biological sciences / Cell biology / Circadian rhythms [URI /631/80/105]

Biological sciences / Genetics / Gene expression [URI /631/208/199]

environment or to avoid the detrimental consequences of solar radiation remains a topic of debate[1, 6, 11].

Irrespective of why clocks evolved, it is evident today that clock genes and cell-autonomous clocks are ubiquitous. In mammals, for example, we no longer have a neuro-centric view of a master clock located only in the brain. Instead, the discovery of "clock genes"[12] in the 1990s showed that virtually all cells express these genes and have the capacity to generate circadian oscillations[13, 14]. The circadian clock in mammals is now conceptualized as a hierarchical system in which a brain clock located in the hypothalamic suprachiasmatic nucleus (SCN) acts as a master pacemaker to synchronize or entrain peripheral clocks distributed throughout the body[15–19] (Figure 1d). This system-wide architecture begs the questions: how is this hierarchy coupled and integrated, and what are the functions of clocks in tissue- and cell-specific contexts?

The molecular mechanism of circadian clocks in mammals is generated by a cell-autonomous transcriptional autoregulatory feedback loop. The 'core' clock genes include *CLOCK and BMAL1,* which encode activators, and *PER1, PER2, CRY1* and *CRY2*, which encode repressors. Current topics of active investigation include the components of this core circadian clock gene network, how environmental and systemic inputs impinge upon the core clock mechanism[20, 21], and how the core clock network regulates downstream output pathways. Recent work has provided insight into the genomic targets of the core clock pathway and has led to some surprises concerning the pervasiveness of circadian regulation in gene expression. A substantial fraction (~5–20%) of genes expressed in any particular cell or tissue have been found to undergo circadian oscillations at the mRNA level[22]; however, this level of regulation (that is, steady-state mRNA rhythms) is only one of a multitude of layers of circadian regulation in gene expression. Virtually every regulatory stage in gene expression that has been examined carefully — from transcription, splicing, termination, polyadenylation, nuclear export, microRNA regulation, translation to RNA degradation — has revealed additional layers of circadian control[23, 24]. Thus, both transcriptional and post-transcriptional regulation of circadian gene expression are key nodal points of control.

Our understanding of the mechanism of the circadian clock in mammals has been informed by progress on two fronts: first, the biochemical definition of components of the circadian activator and repressors complexes, and second, the determination of the structure of key domains of the clock proteins (Box 1). This Review will focus on molecular, biochemical and genomic advances in our understanding of circadian regulation in mammals. I will emphasize work on the genomic targets of the core circadian transcriptional pathway and will highlight studies revealing global circadian oscillations in RNA polymerase II (RNAPII)-mediated transcription and their relation to the dynamic circadian regulation of chromatin state and chromatin modifiers. For non-mammalian model systems and aspects of circadian biology focused on photic regulation, metabolism and immunity, there are other excellent reviews[8, 25–37].

Box 1

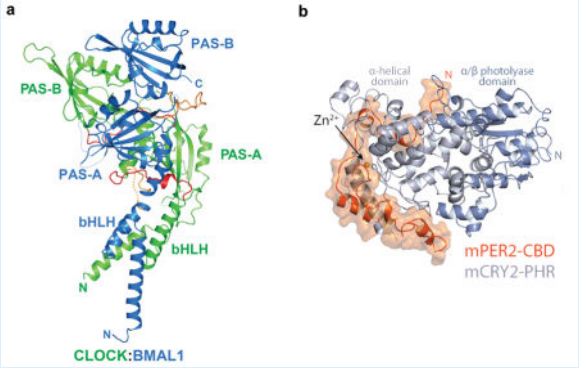
Structural biology of clock proteins

To achieve a mechanistic understanding of clock function, structural biology will be essential[154, 155]. The three-dimensional structures of a number of circadian proteins and their complexes have been solved using X-ray crystallography. These include the bHLH, PAS-A and PAS-B domains of the CLOCK:BMAL1 heterodimeric transcriptional activator complex (PDB code 4H10) (see figure a)[156]; the PAS-A/PAS-B domains of the mammalian PERIOD proteins[157, 158], the photolyase homology region (PHR) of mammalian CRY1[159], the PHR of CRY2 in complex with FBXL3[160], and the PER2-CBD (CRY-binding domain) in complex with CRY1[161] or CRY2 (PDB code 4U8H) (see figure)[162]. The structure of the CLOCK:BMAL1 heterodimeric complex is asymmetric with CLOCK wrapping around BMAL1. The PAS-A and PAS-B domains of the two subunits interact with their complementary domains but with two different interfaces: the PAS-A domains have symmetrical interactions involving the β-sheet surfaces; while the PAS-B domains have a head-to-tail interaction in which the β-sheet surface of BMAL1 interacts with the α-helical surface of CLOCK. The PAS-A/PAS-B domain configuration of BMAL1[156] (but not CLOCK) is identical to that seen in the PAS-A/PAS-B domains of the mammalian PERIOD proteins[157, 158] suggesting that PER could interact with CLOCK and replace BMAL1[163]. Interestingly, a crystal structure of a another member of the bHLH-PAS heterodimeric protein family, HIF-2α-ARNT, reveals a completely different overall domain arrangement when compared to CLOCK:BMAL1, but specific domain interface interactions are preserved between the respective bHLH, PAS-A and PAS-B domains of the two subunits[164] (see REF.[165] for a comparison of these bHLH-PAS structures). The recognition of the CACGTG E-box DNA binding motif of the CLOCK:BMAL1 bHLH region is similar to that seen for other bHLH proteins[166].

The crystal structures of mammalian CRY2 have been determined in five functional states: 1) the photolyase homology region (PHR) by itself (apo); 2) the FAD-bound CRY2 PHR; 3) the CRY2-FBXL3-SKP1 complex; 4) the KL001-bound CRY2 PHR; and 5) CRY2 in complex with the PER2-CRY-binding domain (CBD)[160, 162, 167]. Both the CRY1 and CRY2 apo structures adopt the canonical photolyase fold and can be aligned most closely to the Drosophila (6–4) photolyase[159, 160]. However unlike (6–4) photolyases and cryptochromes from plants and *Drosophila melanogaster*, the mammalian CRY1 and CRY2 PHRs do not have the FAD cofactor bound. In CRY2, FAD can bind with low affinity, and the FAD-binding pocket has a more open conformation perhaps explaining the lower affinity of FAD[160]. CRY2 in complex with FBXL3 reveals an extensive interface with the FBXL3 leucine rich repeat (LRR) domain, but most interestingly is the C-terminal tail of FBXL3 inserting into the FAD-binding pocket revealing a novel substrate recognition motif by the SCF^{Fbxl3} ubiquitin ligase[160]. The small molecule stabilizer of CRY, KL001[168], binds the FAD pocket, but also extends beyond the pocket where the FBXL3 C-terminal tail enters, so as to be in a position to block the FBXL3-CRY2 interaction to stabilize CRY[167]. Finally, the known interaction between CRY and PER proteins, which mutually stabilize one another, has been illuminated by the recent crystal structures of the CRY1/PER2-CBD[161] and CRY2/PER2-

CBD[162] complexes. The PER2-CBD forms an extended conformation on CRY2 that begins at a secondary pocket critical for CLOCK:BMAL1 binding and winds around the CRY2 C-terminal helix to sterically hinder the recognition of CRY2 by the SCF^{Fbxl3} ubiquitin ligase (see figure b). Thus, the overlapping interfaces on CRY2 for PER2-CBD and FBXL3 provide a structural basis for the competition of PERs and FBXL3 for controlling the stability of CRY[162]

The existing crystal structures beg the question of how PER and CRY interact with CLOCK:BMAL1 as a quaternary complex. In different contexts, CRY1 can interact directly with CLOCK:BMAL1 without PER in vitro[169] and while in other experiments PER has been shown to be essential for interaction with CLOCK:BMAL1 in vivo[163]. Likely PER is involved in reducing the occupancy of CLOCK:BMAL1 on DNA[170] as seen in *D. melanogaster*[171], while CRY1 may be able to repress CLOCK:BMAL1 on DNA[170, 172]. Because the native CLOCK:BMAL1/PER:CRY quaternary complexes are megadalton in size and involve other interacting proteins, and important domains of these proteins are intrinsically disordered, the solution of these complexes likely will require a combination of crystallography, NMR, and cryo electron microscopy methods in future work.



The core circadian clock gene network

Core components of the mammalian circadian clock

In mammals, the mechanism of the circadian clock consists of the activators, CLOCK (and its paralog NPAS2) and their heterodimeric partner BMAL1 (or ARNTL), which are basic-helix-loop-helix (bHLH) – PER-ARNT-SIM (PAS) transcription factors[38, 39]. The CLOCK:BMAL1 complex binds to regulatory elements containing E-boxes in a set of rhythmic genes encoding the repressor proteins PERIOD (*Per1, Per2, Per3*) and CRYPTOCHROME (*Cry1, Cry2*)[39–41] (Figure 2a). In mouse, CLOCK:BMAL1 activation occurs in the daytime, leading to transcription of the *Per* and *Cry* genes in the afternoon and the accumulation of the PER and CRY proteins in the late afternoon or evening[42]. The PER and CRY proteins interact with each other as well as with the serine-threonine kinases Casein kinase 1δ (CK1δ) and CK1ε[43], and translocate into the nucleus at night, where they interact with CLOCK:BMAL1 to repress their own transcription[42, 44]. As repression progresses, *Per* and *Cry* transcription decline, and the PER and CRY protein levels decrease

since their half-lives are relatively short and specific E3 ligase complexes target the PER and CRY proteins for ubiquitination and subsequent degradation by the proteasome[43, 45]. Once negative feedback repression is relieved by turnover of the repressor complex, transcription by CLOCK:BMAL1 can begin anew to start a new cycle of transcription the next morning. Importantly, core genes such as *PER2* and its regulatory kinase, *CSNK1D,* have been found to underlie human sleep timing disorders[46, 47], demonstrating a clear role for clock genes in humans (Box 2).

Box 2

Human genetics of clock genes

Validation of the core circadian transcriptional mechanism elucidated in rodents (Figure 2) has come from the Mendelian disorder, familial advanced sleep phase disorder (FASPD), in which the timing of sleep-wake cycles in affected individuals are shifted several hours earlier than normal[173]. Dominant mutations in both *PER2* and *CSNK1D* have been shown to be linked to FASPD and to be causative in humans[46, 47]. In large-scale human genome-wide association studies (GWAS) for fasting glucose levels and increased risk of type 2 diabetes, common variants in the *MTNR1B* locus, which encodes melatonin receptor 1B, and in *CRY2* have been documented[174–177]. Melatonin signalling is a major output pathway of the suprachiasmatic nucleus (SCN) in mammals, and levels of melatonin are circadian, with high levels at night and low levels in the day[178] reflecting a physiological signal of darkness. The *MTNR1B* mutations have been proposed to act in pancreatic islets to impair insulin secretion but this remains subject to debate[179–181]. Recently, large-scale GWAS for ‘morningness’ and sleep timing preference (chronotype) have identified a number of genes known to be associated with circadian rhythms in animals[182–184]. These include *RGS16*, a regulator of G-protein signaling that is enriched in the SCN and has a long period phenotype with a loss-of-function mutation[185]; the core clock gene *PER2*; *VIP* (*Vasoactive intestinal polypeptide*), a neuropeptide in the SCN involved in oscillator coupling[186]; *HCRTR2*, the receptor for hypocretin or orexin that is linked to narcolepsy in humans and dogs[187]; and *FBXL3*, an F-box protein that is the recognition domain for an E3 ubiquitin ligase complex that degrades the CRY proteins[85]. GWAS using sleep duration as a phenotype have identified a few loci (*PAX8, VRK2* and *UFL1*)[183, 188, 189], but their relation to sleep mechanisms remains obscure. Overall, it is reassuring that a number of GWAS hits are associated with genes with known circadian and sleep functions, thus fortifying the notion that circadian transcriptional mechanisms will likely apply to human biology as well.

In addition to the *Per* and *Cry* target genes, CLOCK:BMAL1 also activate the nuclear receptors REV-ERBα and REV-ERBβ (encoded by *Nr1d1* and *Nr1d2*, respectively), which compete at REV-ERB/retinoic acid-related orphan receptor (ROR) binding elements (ROREs) with RORα, RORβ and RORγ (encoded by *Rora, Rorb, Rorc*, respectively)[48–50] (Figure 2a). The rhythmic expression of REV-ERBα/β leads to the repression of *Bmal1* and *Clock*, which induces a rhythm in these genes that is in anti-phase with the *Per* gene expression rhythms[48]. These interlocked feedback loops of activators and repressors form a canonical positive- and negative-feedback gene network motif[51]. A third CLOCK:BMAL1-

driven transcriptional loop involves the PAR-bZip (proline and acidic amino acid-rich basic leucine zipper) factors DBP (D-box binding protein), TEF (thyrotroph embryonic factor) and HLF (hepatic leukemia factor). These proteins interact at sites containing D-boxes with the repressor NFIL3 (Nuclear Factor, Interleukin 3 Regulated; or E4BP4), which is driven by the REV-ERB/ROR loop (Figure 2a) [52, 53]. Together, these three interlocking transcriptional feedback loops can generate cycles of transcription with various phases of expression depending on the combination of *cis*-elements (E-box, RORE, D-box) in the promoters and enhancers of specific target genes (Figure 2b) [54].

Core clock protein interactions with chromatin and chromatin-modifying complexes

At the beginning of the transcription cycle, the activators, CLOCK and BMAL1, interact with the histone acetyltransferases (HAT) p300 and CREB binding protein (CBP), respectively, to acetylate histones and provide an accessible chromatin state for transcription[55–58]. CLOCK has been reported to have intrinsic HAT activity and to acetylate histone H3K9 and H3K14[59, 60]. The NAD^+-dependent histone deacetylase (HDAC) sirtuin 1 (SIRT1) associates with CLOCK, BMAL1 and PER2[61, 62], and a circadian rhythm in NAD^+ levels driven by the expression of the CLOCK:BMAL1 target gene *Nampt* leads to a rhythm in SIRT1 activity which feeds back to inhibit the CLOCK:BMAL1 complex[63, 64].

CLOCK and BMAL1 have also been shown to be associated with the methyltransferase MLL1 (mixed lineage leukemia 1)[65]. Interestingly, MLL1 interacts with the δ19 activation domain of CLOCK and is thought to be important for the recruitment of CLOCK to DNA[65]. Moreover, MLL1 is associated with rhythmic H3K4 trimethylation (H3K4me3)[65], a common histone marker associated with active gene promoters. Also, MLL1 is acetylated, and the metabolic regulator SIRT1 rhythmically deacetylates MLL1, thereby affecting its methyltransferase activity[66]. The related methyltransferase MLL3 exhibits circadian occupancy and is associated with promoters carrying rhythmic H3K4me3 marks; however, MLL3 recruitment to DNA does not depend on BMAL1 or CLOCK[67].

CLOCK and BMAL1 also interact with the histone lysine demethylases JARID1a and LSD1, which are both thought to promote the recruitment of CLOCK:BMAL1 to *Per* gene promoters to enhance transcription[68, 69]. BMAL1 interacts with TRAP150 (thyroid hormone receptor-associated protein-150), a co-activator that promotes CLOCK:BMAL1 binding to circadian target genes and links CLOCK:BMAL1 to the Mediator complex subunit MED1 to recruit the general transcriptional machinery[70]. RNAPII is both recruited and initiated on a circadian basis as discussed in more detail below[71, 72].

During the transition from activation to repression, the CLOCK:BMAL1 complex interacts with the PER-CRY repressor complex at the beginning of the night as the levels of PER and CRY accumulate and translocate into the nucleus[42]. The PER complex is large and contains at least 25 proteins[73–75]. Initially CLOCK:BMAL1 are associated with two components of the Mi-2/nucleosome remodelling and deacetylase (NuRD) transcriptional corepressor complex, CHD4 and MTA2. Interestingly, PER complexes without CLOCK:BMAL1 lack CHD4 and MTA2 but contain other members of the NuRD complex such as MBD2, GATAD2a, HDAC1 and RbAp48[75], suggesting that during this transition phase there is a split NuRD complex which then comes together to form a reconstituted NuRD corepressor

complex to inhibit CLOCK:BMAL1 transcription. The CLOCK:BMAL1 complex also recruits the polycomb repressor complex (PRC2), leading to the trimethylation of H3K27 at target genes and thereby inhibiting gene expression[76, 77]. In addition, CLOCK:BMAL1 recruits the Ddb1-Cullin-4 ubiquitin ligase complex to circadian target genes to promote H2B monoubiquitination at E-box sites to covalently mark genes for subsequent recruitment of the PER complex[78]. At the peak of repression in the middle of the night, the PER complex is also associated with the transcriptional repressor complexes, SIN3-HDAC[79] and Hp1γ–Suv39h histone methyltransferase[80], which contribute to CLOCK:BMAL1 repression by deacetylation of histone H3K9 and H4K5 as well as di- and trimethylation of H3K9. The PER repressor complex also includes NONO, a protein involved in RNA processing and WDR5, a component of a histone methyltransferase complex[73]. At later times during the repression phase, PER complexes include the RNA helicases DDX5 and DHX9, as well as the large subunit of RNAPII and SETX, a helicase that promotes transcriptional termination[74]. Thus, recruitment of a number of chromatin-modifying complexes as co-repressors is necessary for PER- and CRY-mediated repression of CLOCK:BMAL1. During the circadian cycle, transcriptional activation and repression are accompanied by dynamic epigenetic transitions in chromatin state that poise the genome for rhythmic gene expression.

Regulation of circadian period length by repressor stability

The stability of the core repressor proteins is an important determining factor in the dynamics of the transcriptional feedback loop, which in turn regulates the period length of the oscillation. The stability of PER and CRY is regulated by their phosphorylation state and by their ubiquitination by E3 ligase complexes (Figure 2a). For example, the *tau* mutation in CK1ε shortens circadian period length *in vivo* and destabilizes the PER proteins by phosphorylation of a regulatory site that marks the PER proteins for ubiquitination by β-TrCP and proteasomal degradation[43, 81–83]. By contrast, mutations in the F-box protein FBXL3, which is one of four subunits of the SCF (Skp1/Cullin/F-box protein) E3 ligase complex, lengthen circadian period and stabilize the CRY proteins by decreasing ubiquitination and subsequent proteosomal degradation[84–86]. In addition, CRY ubiquitination by SCF^{Fbxl3} is triggered by AMPK-mediated phosphorylation, which provides a link to metabolism via nutrient-responsive AMPK activity[87].

Stability and turnover of PER proteins is primarily regulated by CK1δ and CK1ε[43, 83, 88, 89], by the balance between these two protein kinases and phosphatase 1[90], and by the stoichiometry of PER and the kinases[91, 92]. Recent work has shown that a dual-kinase, multisite phosphoswitch regulates PER stability and its ubiquitination by the β-TrCP E3 ubiquitin ligase complex[93].

In the case of CRY, stability and turnover are regulated by the balance of the two paralogues FBXL3 and FBXL21 (Figure 2a) [94, 95]. Contrary to expectation, FBXL21 antagonizes FBXL3 to reduce the E3 ligase activity of SCF^{Fbxl3} on CRY1 and CRY2 to stabilize them[95]. Although SCF^{Fbxl21} does possess E3 ligase activity, it is less efficient than SCF^{Fbxl3} in the ubiquitination of CRY[95]. However, FBXL21 interacts more strongly with CRY than FBXL3, which protects CRY from SCF^{Fbxl3} activity[95]. The subcellular localization of FBXL3 and FBXL21 also differ, with FBXL3 acting as the primary E3 ligase for CRY in the nucleus,

whereas FBXL21 acts as the primary E3 ligase for CRY in the cytoplasm[95]. Finally, in genetic experiments exploring the interaction of the CK1ε^{tau} mutant with the *Fbxl3*^{Afh} mutant, a broad range of periodicities were seen depending on the combination of alleles[96], suggesting that the PER and CRY pathways independently affect the ultimate period length *in vivo*.

Genome-wide clock architecture

While previous work has examined the transcriptional regulation of CLOCK:BMAL1 target genes such as *Dbp, Per1* and *Nr1d1* in detail, identity of the circadian cistrome has only been recently defined. Using chromatin immunoprecipitation followed by sequencing (ChIP-seq), genome-wide DNA binding sites for BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1 and CRY2 have been identified in mouse liver during the circadian cycle by a number of groups[71, 97–100].

Genomic targets of the core circadian activators

Occupancy of CLOCK:BMAL1 at the *Dbp* locus, which encodes a transcription factor that modulates expression of clock output genes, is circadian, with peak binding occurring in the middle of the day[101]. At the single-cell level, CLOCK and BMAL1 recruitment to E-boxes in the *Dbp* gene are interdependent and circadian[102]. BMAL1 binding was observed to be dynamic and stochastic, and fluctuations in binding depend on the proteasome[102, 103], which is consistent with a 'kamikaze' model of transcriptional activation[99]. On a genome-wide level, CLOCK and BMAL1 occupancy also peak within a narrow window of time during the middle of the day, and the phasing of CLOCK:BMAL1 occupancy is not tightly phase-locked with the RNA expression of their target genes[71, 97, 98]. This flexible phase relationship can be explained at least in part by the combinatorial action of other *cis*-acting elements (RORE and D-box) within the tripartite clock gene network[54] (Figure 2a,b). For example, the *Cry1* gene, which peaks much later in phase than the *Per* genes, is regulated by both E-box and RORE elements[104].

To view the dynamics of the occupancy of the core circadian activator complexes genomewide, all core clock proteins have been assessed together in the mouse liver[71]. At the *Per1* gene, the activators BMAL1 and CLOCK bind in a cyclic manner at the promoter, between circadian time (CT) CT0 and CT12, with maximal binding observed at CT8. In genome-wide analyses, CLOCK and BMAL1 bind to over 4,600 and 5,900 sites, respectively, corresponding to ~3,000 unique genes in the liver[71] (Figure 3b). At highly expressed target genes, CLOCK:BMAL1 DNA-binding motifs are enriched for a tandem E-box motif[97]. Native liver extracts show that CLOCK:BMAL1 bind tandem E-box sites cooperatively with higher affinity to more effectively compete with other E-box binding factors such as USF1[105].

The hepatic steroid receptor coactivator 2 (SRC-2) is recruited to DNA in a diurnal cycle and overlaps extensively with CLOCK:BMAL1 sites[106]. SRC-2 acts as a potent transcriptional coactivator of CLOCK:BMAL1, and null mutations in *Src-2* mice lead to an abnormally early phase of the activity rhythm[106], suggesting that SRC-2 has a role within the circadian clock system.

In addition to the liver, the circadian cistrome has recently been described in mouse pancreatic islets[107]. Comparing the occupancy of BMAL1 between the liver and pancreas, about 40% of target genes overlapped, with disparate binding sites clustering at distal regulatory regions of tissue-specific genes. Interestingly, even within the overlapping set of target genes, BMAL1 was found to bind to distinct enhancers in liver and pancreas[107], consistent with the existence of tissue-specific circadian *cis*-regulatory modules[108].

Genomic targets of the core circadian repressors

In contrast to the activators, the repressors, PER1, PER2 and CRY2, bind genomewide at night between CT12 and CT20, peaking between CT15 and CT17)[71] (Figure 3b). CRY1 has a bimodal pattern, with a major peak at CT0 (Figure 3b). The repressors CRY1 and CRY2 bind to significantly more sites in the genome than PER1 and PER2, with many thousands of these sites being independent of CLOCK:BMAL1 and containing DNA-binding motifs for nuclear receptors[71] (Figure 3c). Consistent with this finding, CRY1 interacts with a number of nuclear receptors and is a corepressor for the glucocorticoid receptor[109]. The nuclear receptors REV-ERBα and REV-ERBβ also occupy DNA rhythmically, with a peak in the daytime[110, 111]. REV-ERBα competes with RORα for DNA binding at RORE and RevDR2 sites to regulate circadian gene target genes such as *Bmal1* and *Cry1*, where it recruits N-CoR and HDAC3 to repress gene expression[50, 111]. Interestingly, REV-ERBα also interacts at other genomic sites that are enriched for tissue-specific transcription factors such as HNF6 in the liver[50]. At these sites, the action of REV-ERBα is independent of its DNA-binding domain and defines a different set of target genes that are directly involved in metabolism. Finally, SRC-2 has been proposed to modulate an oscillation in chromatin accessibility — via recruitment of PBAF (Polybromo-associated Brg/Brahma-associated factors) members of the SWI/SNF (SWItch/Sucrose Non-Fermentable) complex — to promote REV-ERB loading and enhance the amplitude of circadian gene expression[112]. Thus, the circadian cistrome is highly dynamic, and involves not only the core circadian E-box transcriptional regulators, but is interlinked via REV-ERBα/β as well as DBP and NFIL3 to secondary and tertiary gene targets with a wide range of phases of gene expression.

Genome-wide circadian RNA expression

To examine the functional consequences of the circadian cistrome, RNA sequencing (RNA-seq) has been used to profile cycling genes in the mouse liver[71, 98, 99]. For example at the *Dbp* and *Per2* genes, strand-specific whole-transcriptome RNA-seq revealed a robust circadian oscillation using both the intron RNA signal as a proxy for pre-mRNA and the exon RNA signal as a proxy for mRNA[71, 113, 114]. As was previously observed in microarray experiments, thousands of genes cycle at the steady-state mRNA level in the liver as well as in many other tissues[22, 115]. As more regions in the mouse have been profiled, more than half of all genes have been found to cycle at the mRNA level in at least one tissue, and importantly, cycling genes are enriched for drug targets – more than half of the top selling 100 drugs on the market in the United States have a circadian target gene[22]. This emphasizes the importance of the optimizing the timing of drug administration for circadian drug targets, which can both enhance efficacy while at the same time reduce side effects.

A wide range of cycling RNA species has been found using RNA-seq. In addition to cycling protein-coding transcripts, oscillating long intergenic non-coding RNA (lincRNA), microRNA and antisense RNA transcripts have been found in mouse liver[98, 99, 116]. Using Global Run-On sequencing (GRO-seq)[117] to identify enhancer RNA (eRNA) transcripts[118], thousands of oscillating enhancers have been found, and the phase of these cycling eRNAs corresponds with the phases of transcription of nearby genes[119]. Motif analysis of cycling enhancers at different circadian phases revealed that there was phase-specific enrichment of transcription factor motifs and that four sets of motifs were enriched at different times[119]. E-box motifs were enriched between Zeitgeber time (ZT) ZT6 and ZT9, corresponding to the peak of CLOCK:BMAL1 occupancy. D-box motifs were enriched between ZT9 and ZT15, corresponding to the expression of DBP and the low point of NFIL3 repression. RORE and RevDR2 motifs were enriched between ZT18 and ZT24, corresponding with the low point of REV-ERB repression of the RORs. ETS (E26 transformation-specific) binding motifs were enriched between ZT0 and ZT3[119], which may correspond to GM129 or CHRONO repression[120–122]. Overall, the circadian cistromic results agree well with their transcriptional readouts as assessed by eRNA and gene expression analysis: the tripartite core circadian transcriptional feedback loop (E-box, D-box, RORE) drives the majority of circadian gene expression on a genome-wide level.

Using whole-transcriptome RNA-seq or nascent-seq combined with mRNA-seq, two divergent sets of cycling genes have been found (cycling nascent transcripts and cycling mRNA levels)[71, 98] (Figure 4a). Only ~20% of cycling mRNAs also had a corresponding cycling nascent transcript[71, 98], suggesting that other RNA processes likely contribute to steady-state circadian mRNA expression (Figure 4b). A number of different classes of cycling transcripts have been observed: 1) cycling transcription and cycling steady-state mRNA; 2) cycling transcription and no cycling mRNA, and 3) no detectable cycling transcription, but cycling mRNA levels. The first class is typical for the core circadian genes and their high-amplitude direct targets. The second class is typically seen in highly expressed genes in the liver that have mRNAs with long half-lives such as albumin. The third class represents transcripts that could become rhythmic via circadian variation in mRNA turnover, RNA splicing[123], polyadenylation[124], miRNA regulation[116] and other post-transcriptional steps. Whether rhythmic translation or protein levels ultimately occur has also been revealing. For example, rhythmic diurnal polyadenylation occurs in 2.3% of expressed genes in the liver, and one-fifth of these genes (class III) lead to rhythmic protein expression correlated with polyA tail length in the absence of rhythmic mRNA levels[124]. Ribosomal profiling has also revealed substantial numbers of examples of circadian translation in U2OS cells and liver[125, 126]. Interestingly there are at least 150 examples of translationally driven oscillations in the liver for which no mRNA rhythms are present[125]. Thus, both transcriptional and post-transcriptional regulation are critical for circadian gene expression.

Genome-wide circadian regulation of transcription, RNAPII and chromatin states

In the mouse liver, cycling pre-mRNA levels (using the intron RNA signal) were coordinately expressed in time with a peak at CT15 (Figure 4c). Thus, there is a peak of globally coordinated circadian transcription that occurs at the beginning of the night during

the active or awake portion of the mouse circadian rhythm, which is not seen at the mRNA level (which has a bimodal pattern). To analyse the global rhythms in nascent transcription further, RNAPII occupancy has been measured during the circadian cycle[71, 72]. The large subunit of RNAPII (POLR2A) contains a C-terminal domain (CTD) that is post-translationally modified at different stages of transcription[127–129]. RNAPII is recruited into the pre-initiation complex in a hypophosphorylated state (recognized by the 8WG16 antibody)[130]. Surprisingly, RNAPII-8WG16 occupancy is highly circadian in the mouse liver, with a peak that is correlated with the intron RNA peak (Figure 4d). The first step in the initiation of RNAPII involves phosphorylation on serine 5 (Ser5P) of the CTD of RNAPII[131]. RNAPII-Ser5P occupancy is also circadian[71], suggesting that both recruitment and initiation of RNAPII are under circadian control. Using an antibody against the RPB2 subunit of RNAPII (POLR2B), RNAPII occupancy for promoters and gene bodies is rhythmic and co-varies over time[72]. Rhythmic loading of RNAPII to promoters rather than a rhythmic transition from a paused state to productive elongation accounts for the majority of rhythmic transcription in the mouse liver[72].

Given these circadian rhythms of RNAPII, chromatin states associated with RNAPII transcription have been examined during the circadian cycle[71, 72, 99]. Figure 5 illustrates genome views of histone modifications that are characteristic of promoter, enhancer and elongation states[132–138]. Histone H3K4me3, H3K9ac and H3K27ac are enriched at promoters and undergo circadian rhythms in histone modifications at the *Per1* gene (Figure 5a). On a genome level, circadian rhythms in RNAPII occupancy as well as histone H3K4me3, H3K9ac and H3K27ac modifications are present in the majority of expressed genes irrespective of whether RNA cycling occurs or not[71]. Both RNAPII occupancy and H3K4me3 marks are highly dynamic and show circadian oscillations that peak in the early night (CT12) for all expressed genes, cycling intron RNA genes and non-cycling genes (Figure 5b).

In summary, in the mouse liver there is a highly stereotyped, time-dependent pattern of core transcription factor binding, RNAPII occupancy, transcription and chromatin states (Figure 6). There are six distinctive phases of the circadian cycle: 1) a poised state at the beginning of the cycle (at CT0) in which CLOCK:BMAL1 and CRY1 bind to E-box sites in a transcriptionally silent state; 2) a derepression phase at CT4 as the levels of CRY1 decline; 3) a major activation phase at CT8 during which CLOCK:BMAL1 occupancy peak along with p300 and CBP recruitment and are accompanied by histone H3K4me1 and H3K9ac activation marks; 4) a temporally coordinated transcriptional activation phase at CT12–15 in which RNAPII recruitment and pre-mRNA transcript expression peak, followed by histone H3K4me3 and H3K27ac marks; 5) a classic repression phase at CT15–18 in which PER1, PER2 and CRY2 occupancy peaks and transcription declines; and 6) a transition between the end of repression and the beginning of the next cycle. Interestingly, the co-activator CBP interacts with the repressor PER2 at the end of the repression phase and CBP occupancy has a second peak at this time[71], perhaps maintaining an open chromatin state during the transition from repression to activation. CRY1 occupancy also bridges this transition period and interacts with CLOCK:BMAL1 to begin the next cycle.

Unlike embryonic stem cells, where 30% of genes are paused and carry bivalent histone marks[139, 140], genes that are unexpressed in mouse liver are not enriched for paused RNAPII (Figure 5b)[71]. Interestingly, the histone H3K4me3 promoter mark lags RNAPII occupancy by about 1 hour in cycling genes, and the H3K36me3 elongation mark lags about 3 hours[72], consistent with the idea that these marks arise as a consequence of RNAPII transcription. Thus, circadian transcriptional regulators are clearly involved in RNAPII recruitment and initiation, as are the histone modifications associated with these events, but additional steps such as promoter proximal pausing, pause release and productive elongation probably have additional regulatory roles (Figure 7). At enhancer sites, CLOCK:BMAL1 is a pioneer transcription factor and can bind to nucleosomes to promote rhythmic chromatin remodelling and incorporation of the histone variant H2A.Z[141] (Figure 7). Cycling enhancers also display circadian rhythms in eRNA expression in conjunction with tissue-specific transcription factors[107, 119]. In the case of cycling genes, RNAPII recruitment, initiation and productive elongation are all rhythmic and occur in phase, leading to associated rhythms in histone modifications[72]. In the case of expressed but non-cycling genes, RNAPII recruitment and initiation are also rhythmic, leading to rhythmic histone modifications at the promoter. However, subsequent steps in pausing, pause release and productive elongation may no longer be under circadian regulation[71]. Because RNAPII pausing at promoter-proximal regions and pause release are major regulatory steps in transcription, it will be important in the future to determine whether pausing factors such as negative elongation factor (NELF) and DRB-sensitivity-inducing factor (DSIF), as well as the positive transcription elongation factor-b (P-TEFb) complex, are under circadian control[129, 142] (Figure 7).

Conclusions

Circadian modulation of transcription occurs on a genome-wide scale far greater than that seen previously by gene expression profiling. The circadian clock in the liver regulates RNAPII occupancy on a genome-wide basis and drives genome-wide circadian modulation of chromatin states. These circadian rhythms in chromatin state poise the genome for transcription on a daily basis to coincide with the daily metabolic demands of the organism and to optimize the energy utilization of gene expression[143]. In addition to the circadian modulation of chromatin states, the three-dimensional architecture of the nucleus and the interaction of active and repressive chromosomal domains undergo circadian oscillations (Box 3) [144–148]. Thus, the four-dimensional regulation of the genome and its nuclear architecture are ripe for study. In addition to the pervasive circadian regulation of metabolism[11, 149], strong links of the circadian gene network to cell growth and cancer[150–152], immune function[29, 30] and signalling[153] are apparent and will be important therapeutic targets in the future.

Box 3

Four-dimensional regulation of the genome

It is well established that chromatin interactions occur in three dimensions (3D)[190–192]. However, temporal changes in 3D genomic interactions or in both space and time (the fourth dimension) are an emerging area (“the 4D nucleome”[193]). Circadian cycles in

chromosome organization have been observed using circular chromosome conformation capture (4C) methods at the *Dbp* locus, a well-known CLOCK:BMAL1 target gene[144]. Although chromosome interactions surrounding the *Dbp* locus were not reported to change during the circadian cycle, the frequency of long-range inter-chromosomal interactions did vary. Inter-chromosomal interactions were highest at the peak of *Dbp* gene expression and were lowest at the opposite phase, when *Dbp* gene expression was low. The *Dbp* circadian interactome was dependent on *Bmal1* since *Bmal1* −/− knockout cells were similar to that seen at the trough of *Dbp* rhythms[144]. 4C analysis on a BMAL1-bound enhancer upstream of the circadian gene, *Nr1d1* (which encodes REV-ERBα) also found that interactions were largely stable during the circadian cycle[148]. However, global analysis of cohesin and CTCF revealed that cohesin–CTCF co-binding sites insulated cycling genes with different phases of expression and that cohesin–non-CTCF sites were associated with high amplitude circadian cycling[148]. In addition, genome-wide chromosome conformation capture (Hi-C) combined with RNA-seq expression has been used in human fibroblasts to search for circadian interactions, and a Laplacian analysis framework has been implemented to quantify dynamical structure–function relationships in the genome[145]. Using multicolor 3D-FISH, *CLOCK* and *PER2*, which have rhythmic and antiphasic structure–function dynamics, were shown to also have a correlated rhythm in spatial distance between them[145]. Finally, using 4C to probe the *H19* imprinting control region in human embryonic stem cells, a large inter-chromosomal interactome was identified[146]. The interactome was organized by PARP1 and CTCF, and interestingly active loci enriched for circadian genes were found to be recruited to repressed lamina-associated domains (LADs) in the nuclear periphery. In serum-synchronized HCT116 cells, there was a circadian rhythm of recruitment of circadian loci to the nuclear envelope that was dependent on PARP1 and CTCF. This was associated with the acquisition of H3K9me2 repressive marks and attenuation of transcription. Thus, circadian regulation of transcription also involves the recruitment and repression of circadian loci to LADs in the nuclear periphery[146].

Acknowledgments

The author thanks Linda Koch and three anonymous reviewers for their constructive comments. Apologies to those whose work was not cited owing to content and length constraints. The author thanks Nobuya Koike and Tae-Kyung Kim for critical contributions to the research presented here. This work was supported by the Howard Hughes Medical Institute and NIH grants R01AG045795 (J.S.T.) and R21MH107672 (Genevieve Konopka and J.S.T.). J.S.T. is an Investigator in the Howard Hughes Medical Institute.

Glossary

convergent evolution
process in which unrelated organisms independently evolve similar traits as a result of having to adapt to similar environments or selective pressures

network motif
As defined by Uri Alon, a small set of recurring regulatory patterns in a network

oscillation

a repetitive variation of a variable in time with a stable frequency or period

E-box
for CLOCK:BMAL1, the consensus cis-regulatory element is CACGTG

REV-ERB/retinoic acid-related orphan receptor (ROR) binding elements (ROREs)
the cis-regulatory element for the nuclear receptors REV-ERB/ROR

D-box
the cis-regulatory element for the PAR-zip transcription factors, DBP, HLF and TEF

phases of expression
the time of the day when peak gene expression occurs

period
the length of the circadian rhythm measured from specific phase points in each cycle such as the peak-to-peak interval

phosphoswitch
in the case of the PER2 protein, a phosphoswitch model is proposed where two competing phosphorylation sites (one for the FASPD site (Box 2) and one for the β-TrCP binding site) determine whether PER2 has a fast or slow degradation rate.

‘kamikaze’ model of transcriptional activation
Ubiquitin-dependent proteolysis of transcriptional activators suggesting a role for activator degradation in RNAPII elongation and requirement for reloading of newly synthesized activators

cistrome
the *in vivo* genome-wide location of transcription factor binding-sites

Global Run-On sequencing (GRO-seq)
a nuclear run-on assay method that captures nascent transcripts from initiated RNAPII followed by next-gen sequencing

Circadian time (CT)
A standard of time based on the free-running period of a rhythm (oscillation). By convention, CT0 is the beginning of the subjective day, and CT12 is the beginning of the subjective night.

nascent-seq
next-gen sequencing of nascent RNA transcripts based on chromatin-associated RNA transcripts, nuclear run-on global transcripts such as GRO-seq or PRO-seq, or RNA sequencing of transcripts immunoprecipitated with RNA polymerase II

Zeitgeber time (ZT)
A standard of time based on the period of an environmental synchronizer or zeitgeber, such as the 24-hour diurnal cycle of light and darkness. Under standard light-dark cycles, the time

of ‘lights on’ usually defines zeitgeber time zero (ZT 0) for diurnal organisms and the time of lights off defines zeitgeber time twelve (ZT 12) for nocturnal animals.

Laplacian analysis
a framework used in many disciplines to quantify topologies in which autonomous entities reach a consensus without a central direction. In the 4D nucleome, it is used to describe the underlying topology of Hi-C interactions in the genome.

lamina-associated domains (LADs)
regions of condensed chromatin that are bound by the nuclear lamina. LADs are enriched for repressive histone marks such as H3K9me2.

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Biography

Joseph S. Takahashi received his Ph.D. in Neuroscience from the University of Oregon, Eugene, in the laboratory of Michael Menaker. He is currently the Loyd B. Sands Distinguished Chair in Neuroscience, an Investigator in the Howard Hughes Medical Institute, and Chair of the Department of Neuroscience at the University of Texas Southwestern Medical Center in Dallas. His laboratory is known for the use of forward genetics and positional cloning in the mouse as a tool for discovery of genes underlying behavior and for the discovery of the mouse and human clock genes that led to a description of a conserved circadian clock mechanism in animals. He is a member of the US National Academy of Sciences, the American Academy of Arts and Sciences and the National Academy of Medicine.

Key points

- The mammalian circadian clock mechanism is cell autonomous and composed of a transcription-translation negative feedback loop. These clocks are distributed throughout the body and regulate tissue-specific rhythmic functions.
- The core circadian transcriptional regulators drive gene expression rhythms in thousands of genes. CLOCK:BMAL1 target genes in the mouse liver regulate genes in all fundamental metabolic pathways suggesting that the clock system in intimately imbedded in cellular metabolism.
- Circadian activators and repressors recruit a wide array of chromatin modifiers leading to dynamic changes in the poise of the genome with time of day.
- RNAPII is recruited and initiated genome-wide in a circadian manner in the mouse liver leading to genome-wide circadian changes in histone modifications.
- Circadian CLOCK:BMAL1 gene targets are directly linked to metabolism, immune function, cell proliferation, cancer and signaling.

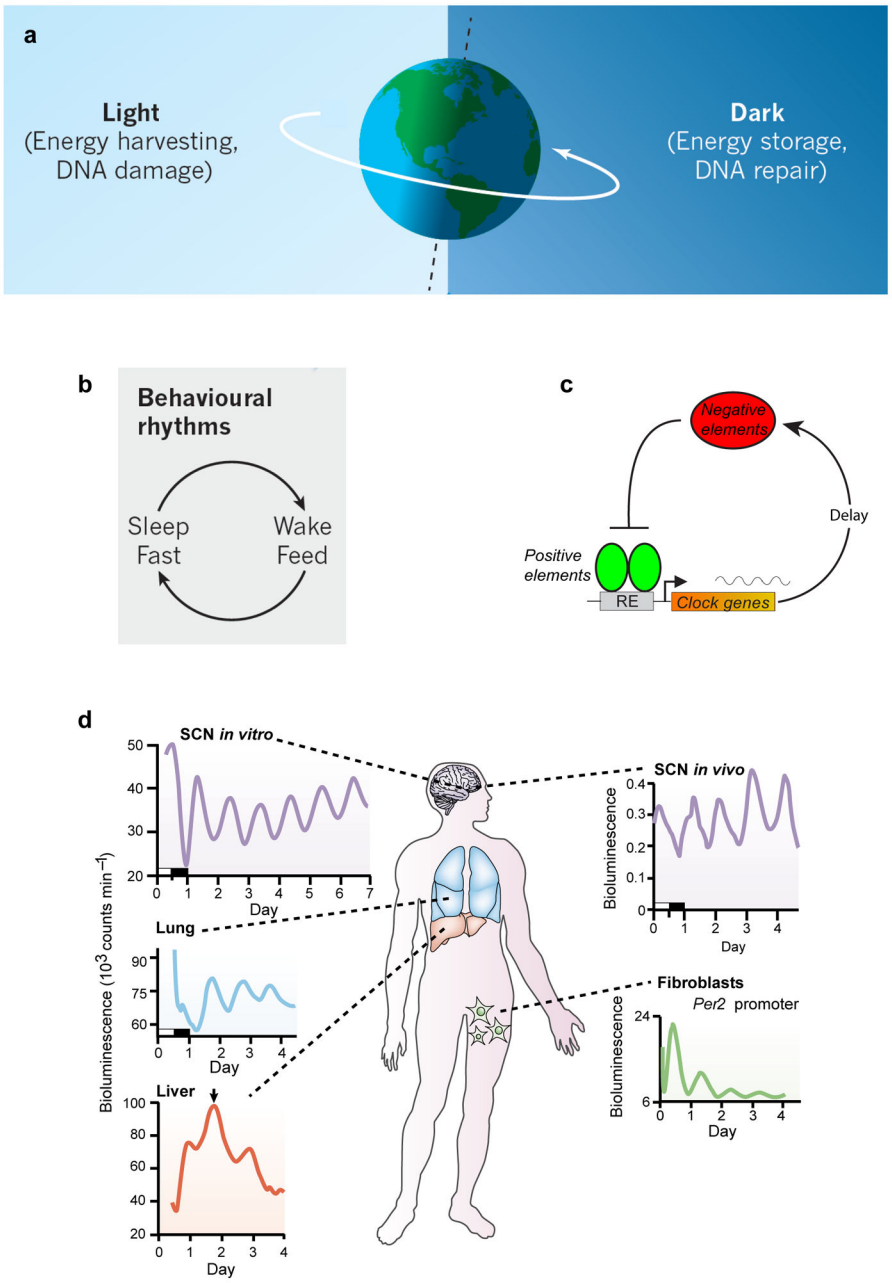

Figure 1.

Circadian rhythms are adaptations of organismal physiology to resonate with the 24-hour solar energetic cycle on earth. **a**) The earth's 24-hour rotation leads to a diurnal cycle of light and darkness that drive an energy harvesting and energy storage daily rhythm. Solar irradiation also imposes a cycle of DNA damage and recovery. Reproduced with permission from REF.[11]. **b**) Animals have behavioral rhythms of sleep-wake and feeding and fasting occur on a 24-hour basis in synchrony with the solar day. Reproduced with permission from REF.[11]. **c**) A conserved network motif of circadian clocks involves a transcription-translation negative feedback loop with delay. **d**) In mammals, circadian clocks are cell autonomous and are found in all major organ systems and tissues of the body. A hierarchical organization

exists in which the hypothalamic suprachiasmatic nucleus (SCN) acts as a master pacemaker to synchronize behavioral and physiological rhythms throughout the body. Modified with permission from REF.[15].

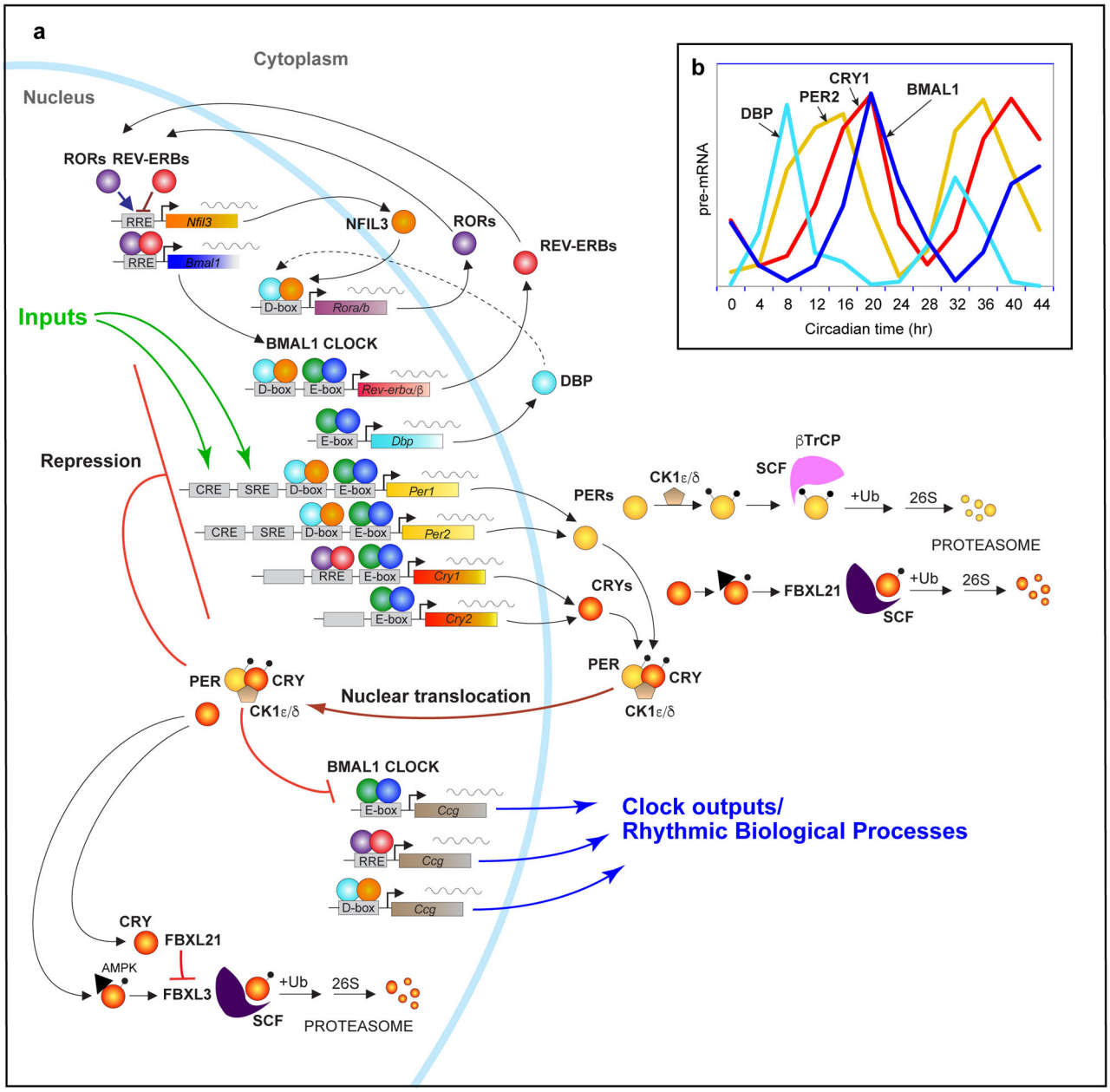

Figure 2.

The circadian gene network in mammals. **a**) At the core, CLOCK and BMAL1 activate the *Per1, Per2, Cry1* and *Cry2* genes, whose protein products interact and repress their own transcription. The stability of the PER and CRY proteins are regulated by parallel E3 ubiquitin ligase pathways. CLOCK and BMAL1 also regulate the nuclear receptors, Rev-erbα/β, which rhythmically repress the transcription of *Bmal1* and *Nfil3* that is driven by the activators, RORa/b. NFIL3 in turn represses the PAR-bZip factor, DBP, to regulate a rhythm in the ROR nuclear receptors. These three interlocked transcriptional feedback loops represent the three major transcriptional regulators of the majority of cycling genes. Different combinations of these factors generate different phases of transcriptional rhythms

as exemplified by the RNA profiles of *Dbp, Per2, Cry1* and *Bmal1* in the mouse liver (**b**). Additional rhythmic output genes (so call “Clock-controlled genes or Ccg’s) are transcriptionally regulated by the three loops acting on E-box, RRE and D-box elements in the regulatory regions of target genes.

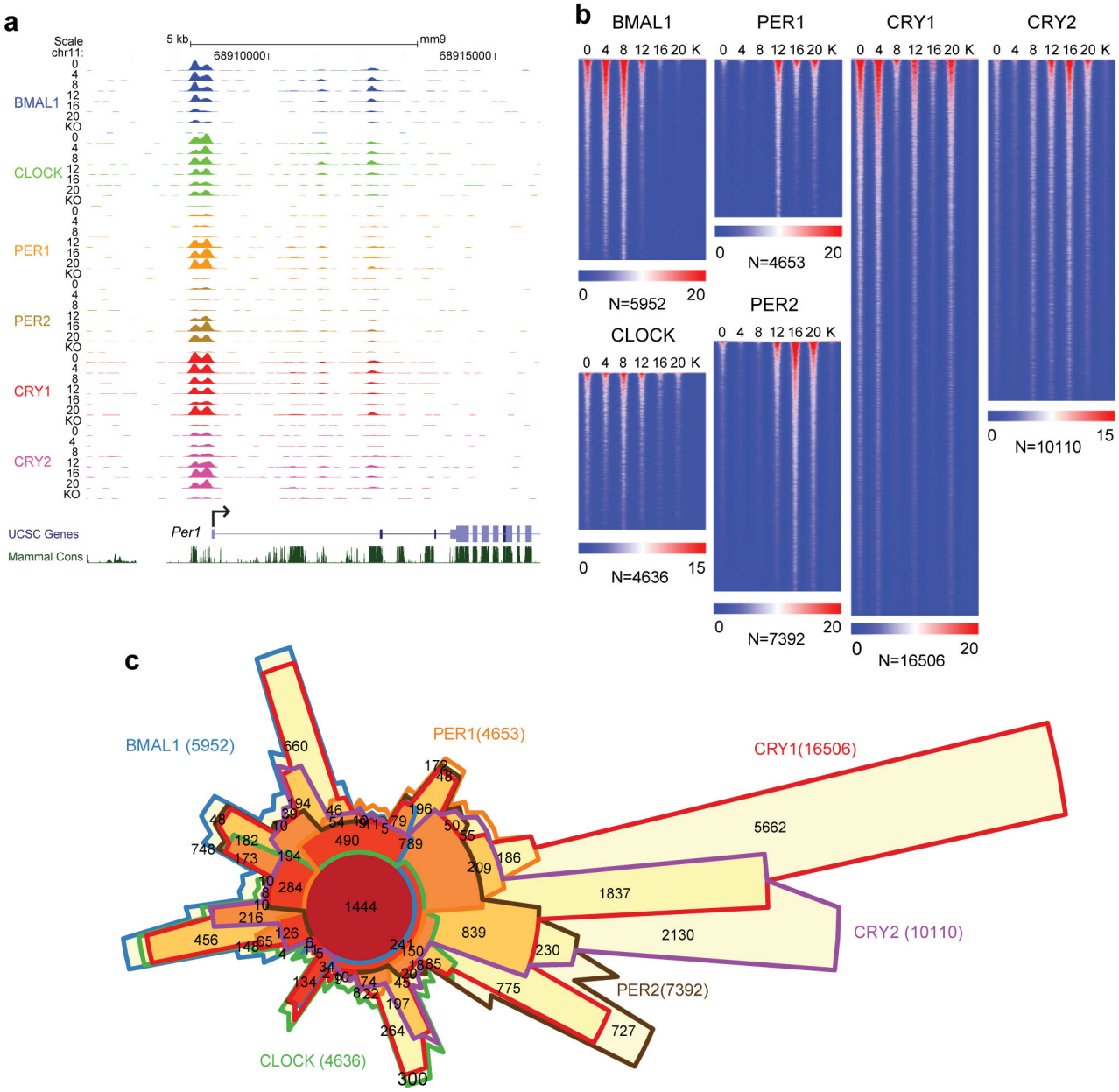

Figure 3.

The circadian cistrome in the mouse liver. **a**) UCSC genome browser view of ChIP-seq profiles of circadian transcription factors at the *Per1* gene at 6 circadian times of day [0, 4, 8, 12, 16, 20 CT (hr)]. The colors of the wiggle plots of ChIP-seq occupancy indicate the following transcriptional regulators: BMAL1 (blue), CLOCK (green), PER1 (orange), PER2 (gold), CRY1 (red), CRY2 (pink). KO indicates a ChIP-seq sample from a knockout mouse for each transcriptional regulator. **b**) Heat map views of genome-wide DNA binding for BMAL1, CLOCK, PER1, PER2, CRY1 and CRY2 measured over 500 bp fragments encompassing the binding sites. Each peak in the genome is represented as a horizontal line, ordered vertically by signal strength. Six time points are shown beginning at CT0 and ending

at CT20 from left to right. Knockout (K) mouse control is shown on the far right of each panel. The number of peaks in the genome is indicated at the bottom of each panel. The blue-red gradient indicates the coverage for all binding sites in the genome. The number of binding sites (N) in the genome are indicated at the bottom of each heat map. **c**) Chow-Ruskey diagram showing the 6-way overlap of BMAL1, CLOCK, PER1, PER2, CRY1 and CRY2 peaks in the genome. The red circle in the middle represents the overlap of all six factors. Lighter shades of red, orange and yellow represent fewer overlaps of subsets. The boundaries for each protein are color coded: BMAL1 (blue), CLOCK (green), PER1 (orange), PER2 (brown), CRY1 (red) and CRY2 (purple), and the areas of each domain are proportional to the number of binding sites. Modified/Reproduced with permission from REF.[71].

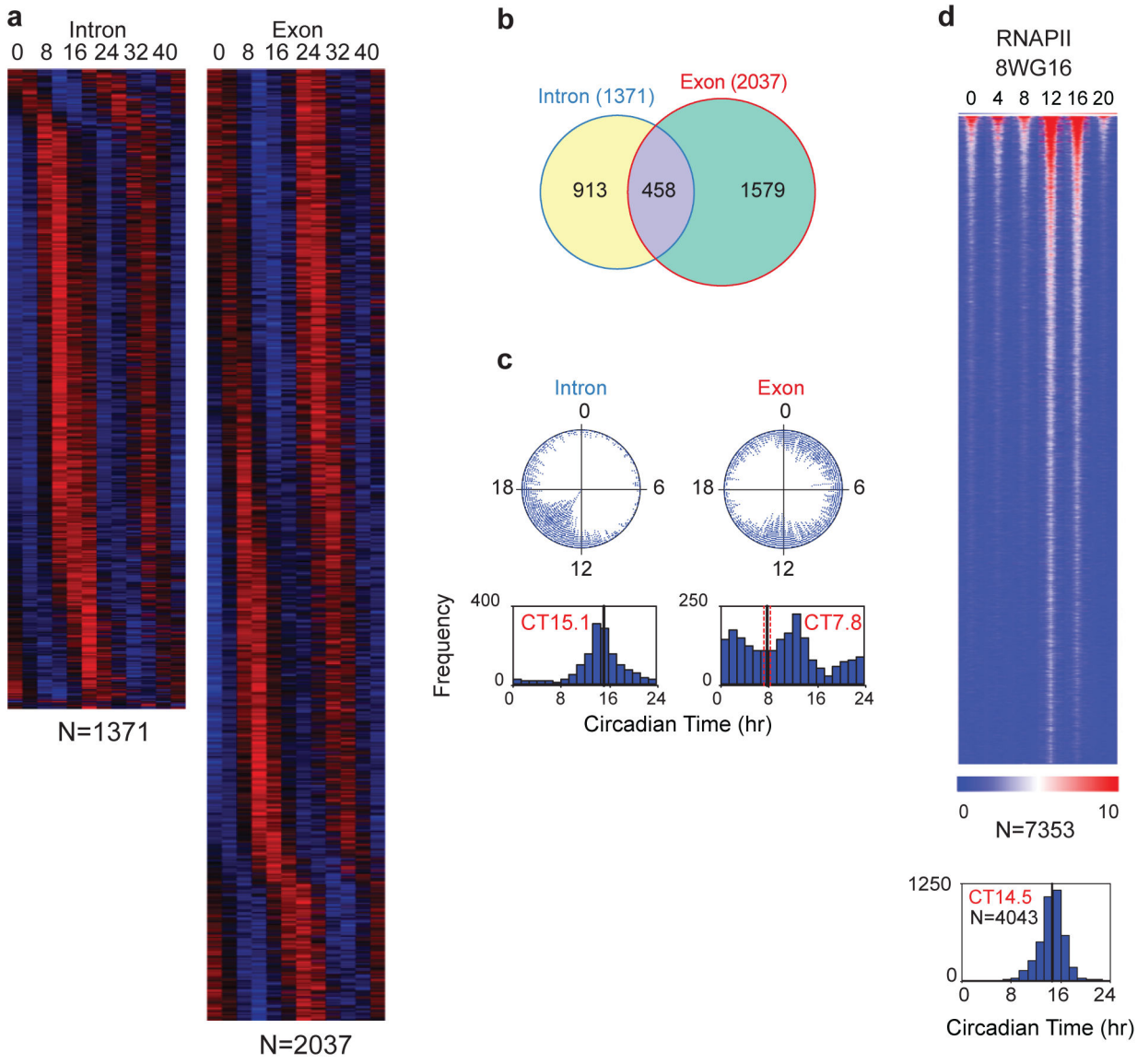

Figure 4.

Whole-transcriptome RNA-seq analysis of circadian gene expression in the mouse liver. **a**) Heat map view of intron (left) and exon (right) RNA cycling genes. Each gene is represented as a horizontal line, ordered vertically by phase in hours indicated at the top. Twelve time points are shown beginning at CT0 and ending at CT44 from left to right. “N” indicates the number of cycling genes in each category. b) Venn diagram of intron and exon cycling genes showing only 22% overlap. **c**) The phase distribution of cycling genes. The phase of each transcript rhythm is represented in a dot plot (top) and histogram plot (bottom). **d**) Heat map view of cycling RNAPII-8WG16 occupancy across the genome of the liver with a peak occurring at night. Modified/Reproduced with permission from REF.[71].

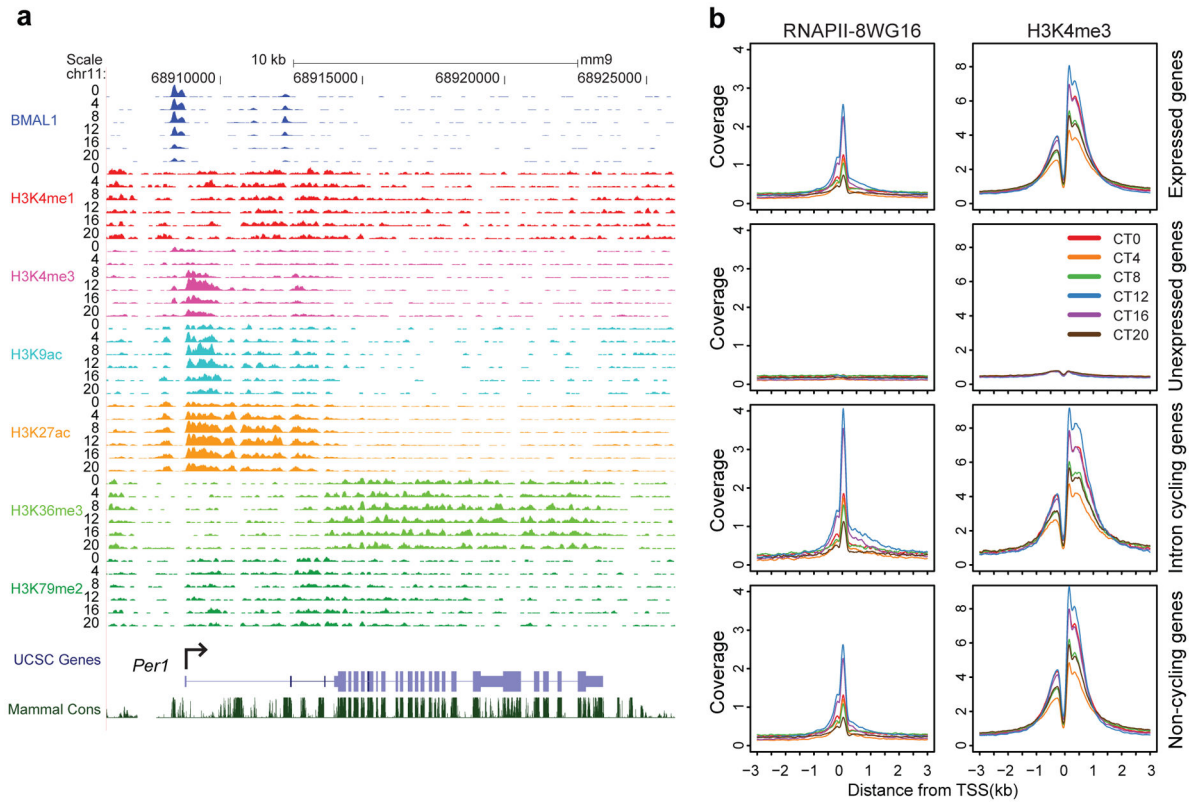

Figure 5.

Circadian chromatin states in the mouse liver. **a**) UCSC genome browser view of histone methylation and acetylation at the *Per1* gene at 6 circadian times of day [0, 4, 8, 12, 16, 20 CT (hr)]. The colors of the wiggle plots of ChIP-seq signal indicate the following: BMAL1 (blue), H3K4me1 (red), H3K4me3 (pink), H3K9ac (aqua), H3K27ac (orange), H3K36me3 (light green), H3K79me2 (dark green). **b**) Binding profiles of RNAPII-8WG16 and H3K4me3 at the transcription start site (TSS) +/− 3kb in 12,680 expressed genes (top row), 8,945 unexpressed genes (second row), 1371 intron cycling genes (third row) and 5,839 non-cycling genes (bottom row). Modified/Reproduced with permission from REF.[71].

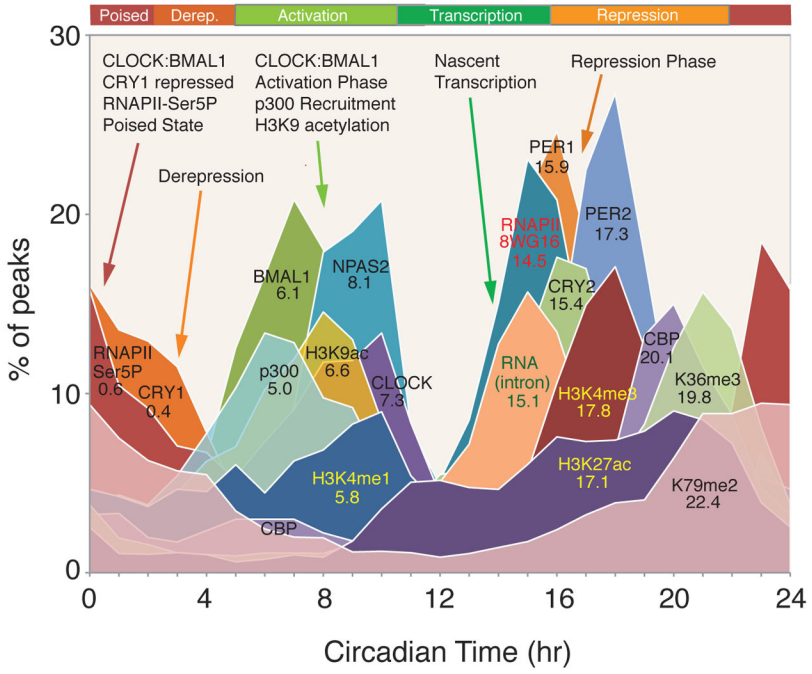

Figure 6.

Circadian transcriptional landscape in the mouse liver. Histograms show the phase distributions of each factor as a function of time of day. Reproduced with permission from REF.[71]

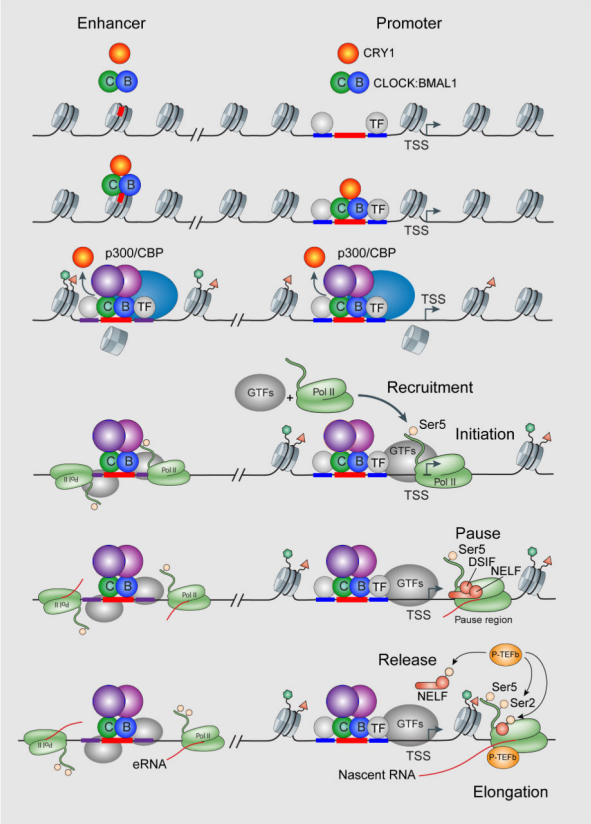

Figure 7.

Circadian transcriptional regulation at enhancer and promoter sites during the CLOCK:BMAL1 activation phase in the daytime. CLOCK:BMAL1 act as pioneer transcription factors at enhancer sites. Circadian recruitment and initiation (as seen by RNAPII-Ser5 marks) of RNA polymerase II occurs at both distal enhancers and proximal promoter sites. However, the circadian regulation of pausing factors (NELF, DSIF) and pause release factors (P-TEFb) remain to be determined. Modified with permission from REF.[129].