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Review article

Spatiotemporal chromatin dynamics - A telltale of circadian epigenetic gene regulation

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ABSTRACT

Over the course of evolution, nature has forced organisms under selection pressure to hardwire an internal time keeping device that defines 24 h of a daily cycle of physiological and behavioral rhythms, known as circadian rhythms. At the cellular level, the cycle is governed by significant fractions of transcriptomes, which are under the control of transcriptional and translational feedback loop of clock genes. Intriguingly, this feedback loop is regulated at multiple stratums such as at the transcriptional and translational levels, which direct a cell towards producing a robust rhythm by sustaining the repeated stoichiometry of protein products. Moreover, with the advent of state of the art paradigms, epigenetic regulation of circadian rhythms has been becoming more evident at present time. Light-induced recurring fluctuations in chromatin acetylation concurrent with the binding of RNA Pol II and integration of miRNAs monitor the chromatin modifiers or clock genes expression to drive temporal rhythmicity. Furthermore, CLOCK protein intrinsic histone acetyl transferase activity, the interaction of CLOCK-BMAL-1 with HAT enzymes, and the involvement of many histone deacetylases also maintain the rhythmic protein profile. Additionally, the critical role of the rhythmic methylation pattern of clock genes in battery of cancer and metabolic disorders also defines its importance. Therefore, in this review, we focused on accumulating all the present data available on epigenetics and circadian rhythms. Interestingly, we also gathered evidence from the available literature pinpointing towards the dynamic nature of chromatin architecture governed by long and short-range regulatory elements DNA contacts arising daily, that was thought to be steady otherwise.

1. Introduction

As the Sun rises, diurnal (day-time active) species start to forage, while nocturnal (night-time active) species sleep due to various genomic, physiological, and behavioral changes, which is referred as circadian rhythms. Nearly all organisms on the earth, from archaebacteria to higher eukaryotes show cyclic changes in biological processes, which enable them to adapt in diverse environmental changes to attain better fitness and survival [1]. Circadian rhythms define many aspects of physiology and behaviors ranging from sleep-wake cycle, hormonal levels, daily changes in body temperature, blood pressure, food intake to impairment in cellular metabolism, that occur throughout 24 h even in the absence of external cues [2,3]. Circadian rhythms are generated and controlled by time measuring system known as biological clocks, which enable adaption synchrony with earth's environmental period [4]. Interestingly, such coordination and integrated output of organisms generated by every cell of the body are known as oscillators. Within every cell, there exists a clock composed of many transcription factors (TFs); activators and repressors known as clock genes that drive the individual cell to show rhythmic oscillations of homeostasis and metabolism. Mounting evidence gathered from studies has indicated that about 3–30% of the transcripts expressed in a rhythmic manner are controlled by clocks within each cell depending on the tissue and specific cell type [5–8]. These significant fractions of the transcriptome are termed as clock-controlled genes (CCGs). Across all the life forms, intricate central and peripheral oscillators maintain biological clocks to maintain the daily cyclic rhythm. In mammals, the suprachiasmatic nucleus (SCN) located at the anterior hypothalamus is the master clock or central oscillator for the entire body. One of the most obvious rhythms in mammals is sleep-wake cycle, which is entrained by light when SCN receives light signal via retina through retino-hypothalamic tract. SCN is composed of > 10,000 densely packed neurons, firing in synchrony upon light perception and transmitting chemical and hormonal signals to other parts of the body, thereby

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causing synchronization between every cell. Apart from light, food availability and temperature also act as signals in order to optimize synchronizations of the biological clock. However, one of the intriguing properties of circadian rhythm is that it can operate freely even in the absence of external signals. The molecular mechanism that governs the rhythmicity in every cell is structured as intricate and hierarchical networks of an interconnected transcriptional-translational feedback loop (TTFL) of clock genes [9]. There are eight major clock genes, namely Brain muscle ARNT like Protein-1 (BMAL-1), Circadian locomotor output cycles kaput (CLOCK), Period (PER1, PER2, and PER3), Cryptochrome (CRY1 and CRY2) and Casein Kinase (CKIδ/ε) have been reported so far. The heart of TTFL is driven by four proteins, including two activators BMAL-1 and CLOCK and two repressors PER and CRY. It is the temporal transcript level of positive components (CLOCK and BMAL-1) and negative components (PER and CRY) and their interconnected transcriptional and translational feedback loop, which is hardwired in every cell that determines rhythmicity. To impinge such rhythmic clock controlled transcriptome in every cell, the core component of the TTFL undergoes rhythmic multilayered regulation at both transcriptional and translational levels. Moreover, together with TTFL dynamics, chromatin remodeling also imparts robust characteristic to the core molecular regulatory mechanism of clock genes that brings in epigenetic regulation into the array of circadian timing. Genome-wide ChIP-Seq studies on mice liver demonstrate that circadian chromatin remodelers introduce rhythmic histone methylation at H3K4 and K27 and acetylation at H3K9 and K14 on several clock and CCGs promoters, in concert with RNA Pol II recruitment [10]. Accumulating evidence in chronobiology indicates that the CpG sites undergo rhythmic methylation to oscillate in synchrony with the cell cycle [11] - a phenomenon which is often gated by the biological clock [12–14]. Intriguingly, misregulation of circadian rhythm may converge into a number of pathological outcomes such as diabetes, inflammation, sleep disorders, metabolic syndrome, cardiovascular diseases, and cancer. These pathological outcomes signify that circadian clocks are tightly engaged in a wide variety of metabolic and homeostatic programs [15]. In addition to this, light-inducible expression of miR-132 modulates the expression of chromatin remodelers and translational regulators such as Methyl CpG-binding Protein 2 (MeCP2), E1A-associated protein p300 (EP300), Jumonji, AT-rich interactive domain 1(JARID1A), and B-cell translocation gene 2 (BTG2A) ultimately influencing *Period* gene expression and light entrainment, which is vital to the clock gene rhythmicity [16]. Since epigenetic regulation of clock genes and CCGs holds a quintessential key for dynamic expression of genes imparting coordinated output for biological processes, Hence, this review focuses onto the key epigenetic mechanisms fundamental to the regulation of core clock TTFL enginery.

2. TTFL machinery: a connection between circadian rhythms and cellular homeostasis

TTFL is the principal regulator which regulates endogenous rhythm within every cell to control rhythmic gene expression, protein modification and secretion [17]. During start of the day, at the core of the TTFL network of every cell, the activator component BMAL-1 and NPAS2 (Neuronal PAS domain 2, a paralogue of CLOCK) or BMAL-1 and CLOCK heterodimerizes and precisely interacts with chromatin and alters domains such as E-boxes of clock genes, thereby, driving the expression of the negative components *Period* (*Per*) and *Cryptochrome* (*Cry*) genes [18]. In the span of 6–8 h when PER and CRY proteins accumulate and reach a stoichiometric threshold, they form complexes and directly bind to BMAL-1-CLOCK/NPAS2 heterodimers in the nucleus, hence inhibiting transcription of their own as well as other cognate clock genes. In essence, it is the building up of negative component gene products to a critical concentration, which represses its own expression straightaway or implicitly via other regulators. Subsequently, the protein product is diminishing and a succeeding run begins in the next 24 h window [19]. Apart from transcriptional and translational control over TTFL, post-translational modification (PTM) also plays a critical role in the maintenance of PER and CRY protein levels in the cytoplasm. Research in comprehending the mechanism of TTFL have indicated that the site of phosphorylation on PER plays a decisive role in the nuclear entry of PER/CRY complex and degradation of PER2 [20,152]. Post-translational control involving many CK1δ and CK1ε [21–23] by competition between two kinases and phosphatases [21], and through the relative amount of PER and the kinases [24,25], which are accountable for regulating PER proteins stability and turnover. Recently work on PER stability has also shown dual-kinase, multiple PTM phospho-switch sites-mediated regulation and its ubiquitination by β-TrCP E3 ubiquitin ligase complex [26]. The decreased concentration of PER and CRY protein products relieves transcriptional repression on CLOCK-BMAL-1 complex and thus permits CLOCK-BMAL1-driven transcription again to progress, subsequently beginning an oscillatory rhythm in circadian gene expression. The time to finish the TTFL mechanism and the time it takes for PER and CRY to degrade and de-repress transcription is approximately about a day and thus ensues rhythm [27]. Yet other clock genes, the nuclear receptor retinoic acid receptor (RAR)-related orphan receptor (ROR) boosts *Bmal-1* and Clock genes expressions; instead, nuclear receptor subfamily 1, group D, member 1 (NR1D1, also known as *Rev-erbα*) is known to repress their expressions. This auxiliary feedback mechanism for controlling *Bmal-1*, Clock genes expression is also defined as the “sub-loop of clock genes” (Fig. 1) [8,28]. The core clock mechanism TTFL remains consistent within every cell in an organism whereas the intricate function of different organs or tissue such as heart, lungs, brain, kidney, and liver in humans are mainly controlled by the downstream target genes of core clock genes. These downstream targets include 1st order CCGs such as D site of albumin promoter (albumin D box) binding protein (*Dbp*), *Rev-erb*, *Rors*, E4 Promoter binding protein 4 (*E4bp4*) and Deleted in esophageal cancer1/2 (*Dec1/2*) and 2nd, 3rd order CCGs (genes involved in a wide array of physiological pathways, cell cycle, and metabolism *etc.*) [76]. Noticeably, degree of expressivity of 1st, 2nd and 3rd order of CCGs and control over other downstream genes (at transcriptional and translational level) by them ultimately regulate the physiological and behavioral function of a particular tissue.

3. Circadian dynamics of chromatin fiber

DNA compaction is one of the crucial mechanism by which most of the eukaryotic cells downsize their megabase-sized genome in order to accommodate it into a very tiniest place nucleus in the cell. In essence, chromatin is an intricate mesh of DNA wounded to histones that exist in diverse conformational states which appear as constellation made up of DNA, proteins, and RNA [29]. Chromosomal DNA wound around histone octamers and is known as nucleosomes. These nucleosomes can undergo various states depending on the stages of cell-like beads on a string to solenoid structure. Based on the higher order of compaction, chromatin is further divided into loosely packed gene rich regions euchromatin and tightly packed gene poor regions heterochromatin. However, it is reasonably very obvious that at the time of transcription to make the gene regulatory elements accessible to TFs, RNA Pol II and activators, euchromatin must undergo into reversible open chromatin state. Research into chromatin dynamics has demonstrated that mostly the amino terminus of histones are protruded as tails that can be biochemically altered and confers different affinity states towards DNA in a spatiotemporal manner. Therefore it is seemingly evident that a number of amino acids present at the tails are disposed to many PTM such as acetylation of lysine (H3K14ac and H4K12ac) and phosphorylation of serine (H3S10ph). These are in general connected to loosening up of chromatin and hence initiate gene expression [19]. In addition to that methylation on different residues of lysine (H3K4me1, H3K4me2, H3K4me3, H3K36me1, and H3K79me2) also contributes to that activation of transcription. On the other hand hypoacetylation,

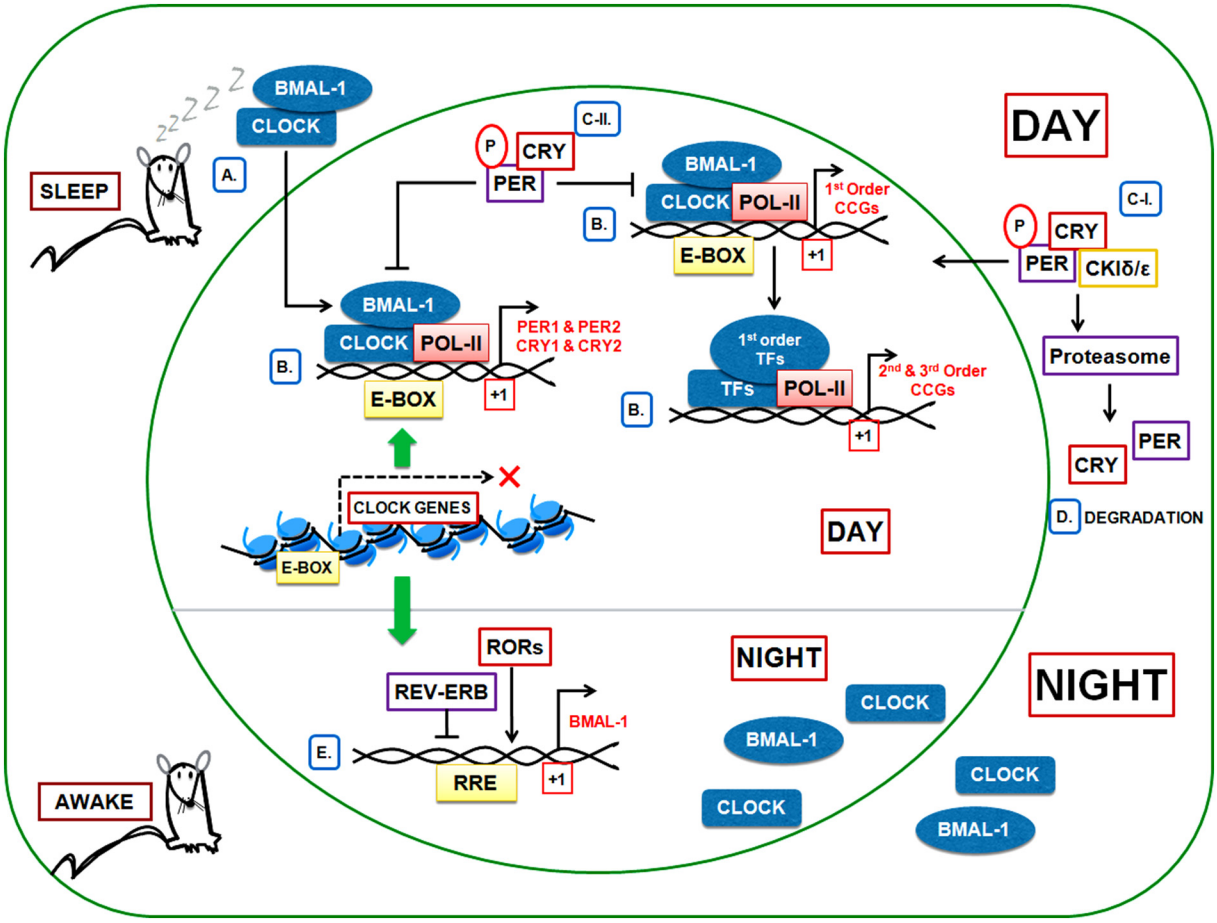

Fig. 1. The transcriptional and translational feedback loop (TTFL) machinery. (A) At subjective day BMAL-1 heterodimerizes to constitutively expressed CLOCK; (B) CLOCK-BMAL-1 binds to E-BOX of clock genes and 1st order CCGs promoter, simultaneously driving their expression with *Per* and *Cry* gene peaking at mid circadian day; (C-I) By the evening time PER/CRY reaches threshold and phosphorylated by CKIδ/ε, later on, forms heterodimer and reverts back to nucleus; (C-II) Inhibits CLOCK-BMAL-1 complexes; (D) PER/CRY levels are maintained and degraded by CKIδ/ε through proteasome system; (E) Following subjective night 1st order protein products such as RORs and REV-ERB binds to RRE of BMAL-1 promoter to drive circadian rhythmicity of BMAL-1 and follow next cycle through subloop. Mice being a nocturnal animal prefers to sleep at day time while remains active at night.

BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles kaput; CKI, casein kinase I; CRY, cryptochrome; PER, period; P, protein phosphorylation.

hypophosphorylation and addition of mono, di or tri-methylation groups on lysine depending on the residue number (H3K9me3 and H3K27me3) also hint to chromatin repressive mode [19]. Additionally DNA itself can undergo reversible methylation at CpG sites by means of engagement of loads of chromatin remodeling proteins. Hence, chromatin provides an interactive hub to chromatin modifying proteins, TFs, and nucleic acid polymerase [30] so as to drive a gene expression or DNA replication. Furthermore, nucleosomes alter the affinity of TF for the underlying DNA through stearic interactions [31]. For the entrainment of robust endogenous circadian rhythm, most of the species utilize light as the principal environmental signal. Central to this vital property, light triggers changes in the gene expression involved in the core clock feedback mechanism. It has already been reported that there is a light regulated component known as a light responsive module (LRM) within the promoter region of *Period* genes [32,33]. LRM consists of E-Box, non-canonical E′-Box and D-Boxes, which show the temporal binding of chromatin activators at pre-transcriptional levels. Most of these studies involved Zebrafish PAC-2 reporter cell line since many tissue and cell lines of Zebrafish both *in-vivo* and *in-vitro* have shown remarkable entrainment of the clock because of the presence of photoreceptive cells which perceive light directly. Stably transfected PAC-2 cell lines with −1.8 Kb promoter region of *Per2* fused with EGFP revealed the presence of LRM in the promoter region by an increase in relative luminescence upon light exposure. Remarkably series of deletion and site-directed mutagenic studies within this promoter region indicate the light inducible E-box and its interaction with D-Box for driving *Per2* expression. E-box exhibited light driven expression of *Per2* in particular by light triggered cAMP pathway and CLOCK-BMAL-1 recruitment onto E-Box, which indicated the involvement of clock after light exposure. However, D-box mediated enhanced *Per2* expression relied on direct stimulation of thyrotroph embryonic factor (TEF, a bZIP transcriptional activator binds to D-box) and PTM by light exposure [33]. Interestingly, follow up studies involving genes relevance to light induction and all showed downregulation upon treatment with cycloheximide (an inhibitor of *de novo* protein synthesis) except *Per2* [32]. Thus these studies indicated the temporal binding of activators such as CREB (CRE binding protein) through cAMP, CLOCK-BMAL-1 and other TFs at LRM during pre-transcriptional levels and speculated a change in chromatin state required for driving *Per2* expression upon light induction. With the advancement in classic immunoprecipitation techniques and involvement of ChIP and DNase I hypersensitive experiments, it became easier to identify the changes in the chromatin state and involvement of chromatin modifiers. In 2003, Etchegaray *et al.* reported the first instance of chromatin modulation within the promoter of mouse *Per1*, *Per2*, and *Cry1* gene. Mouse liver tissue nuclear extracts at different circadian time points revealed rhythmic H3 acetylation and

RNA Pol II binding at promoter regions by ChIP and DNase I assay. Interestingly, these dynamic chromatin changes are in synchrony with that of the steady mRNA levels confirming the significance of the modulation. Investigations dedicated to understanding the chromatin changes around circadian CCG *Dbp* showed dynamic epigenetic modification around the regulatory elements coinciding at ZT7 and ZT19. Remarkably *Dbp* shows maximum mRNA expression at ZT7 and minimum at ZT19. It undergoes daily acetylation of H3K9, methylation of H3K4me3 and diminution in chromatin density paralleled by CLOCK-BMAL-1 occupancy to many E-Boxes, thereby, decreasing in overall chromatin density and binding of CLOCK-BMAL-1 recruits RNA Pol II leading to increased mRNA levels at ZT7. Whereas antiphasic cycles are accompanied by dimethylation of H3K9 (H3K9me2), enrichment of heterochromatin protein 1 (HP1α) and elevated histone density throughout the 24 h except at ZT6-ZT9 [34–36]. The core function of HP1α is to silence the gene expression by interacting with other chromatin modifiers in addition to binding with H3K9me2. However, the exact mode of action behind HP1α is yet to be known. Subsequently, interaction between CLOCK/NPAS2 and histone acetyltransferases (HAT) such as p300, CREB binding protein (CREBBP), p300/CBP associated factor (PCAF) and nuclear receptor coactivator 3 (NCOA3) have also been reported which are ultimately required for the addition of acetyl group specifically to lysine 9 of H3 to positively regulate clock genes appearance [133]. In addition to acetylating histones, HATs activity is also regulated by associated factors known as co-activators like PCAF and NCOA3 that are required for the HATs activity. These studies indicated that *in-vitro* relative luciferase read out under *mPer1* promoter was also found to be enhanced in the presence of p300 confirming its role. Moreover, mice liver nuclei revealed enhanced precipitation of CLOCK with p300 at CT6 as compared to CT18. Consequently, this precipitation data is overlapped with higher *mPer1* and *mPer2* levels at that time point. Nevertheless, studies including the mutation in mCLOCK HAT activity domain also did not alter its binding capacity with transcriptional co-activators [34,37,38]. Parallel to this study in *Drosophila* also reported that daily cycles of *per* and *tim* occurs in concert with CLOCK (CLK)-CYCLE (CYC) (Homologs of mouse CLOCK-BMAL-1) binding to upstream/intronic E-boxes, acetylation of histone H3K9 and trimethylation of histone H3K4 [39]. ChIP assay at different time points explored histone signatures at regulatory sequences indicated open chromatin codes for *per* and *tim* concerted with mRNA peak at ZT10. Remarkably these epigenetic dynamic changes in *Drosophila* show a conserved method of regulating the circadian gene expression throughout 24 h indulging epigenetics into it. In last decade pinpointing mutagenic studies in core clock genes regulatory elements with *in-vitro* reporter assay have discovered many crucial regions in the promoter. This detailed comprehensive study of a promoter of *mPer1* and *mPer2* involved deletion reporter constructs of the promoter and measuring relative bioluminescence *in-vitro* in cell lines. The promoter of clock genes includes multiple E′-box, D-box and canonical E-box. Rat-1 cells transfected with a promoter containing only E-box and stimulated with dibutyryl cAMP (dbcAMP) showed a phase of 24 h similar to wild type construct. This reveals that the E-box is a main oscillator driver region for *mPer1*. Similarly, the conserved regions of *Per2* of human and mice containing noncanonical E″ boxes are involved in the phase delaying of approx. 1.3 h as compared to the wild type construct. However, mutant D-box deletion reporter construct showed a decline in expression level upon dbcAMP induction, thereby, involved in high amplitude generation [40]. These studies, therefore, unveiling the role of functional clock controlled elements involved in the phase delaying, high amplitude generation and main oscillation driver regions in the promoter regions makes it prone to have more layers of regulation.

Remarkably, CLOCK itself has been revealed to perform HAT activity alone [37] and together with its companion BMAL-1 display increased activity on E-boxes of the clock genes [37,41]. Compelling evidence also advocates the role of acetyl-CoA binding motif and high C-terminal sequence similarity with ACTR (Src family member, HAT) in CLOCK intrinsic activity. Myc-tagged CLOCK transiently expressed in mammalian cells exhibited significant HAT activity in cells as well as in gel HAT assays. They also determined the HAT specificity of CLOCK on H3 lys9 and lys14. However, mutation in a homologous sequence containing motif A revealed reduced HAT activity. Also the ectopic expression of mCLOCK-mutA in clock homozygous mutant MEF did not rescue the circadian expression of *mPer1* and *Dbp* gene thereby, explaining the importance of CLOCK epigenetic activity and transactivation at specific times. Additionally, CLOCK also acetylates non-histone proteins including BMAL-1 which in turn entices with CRY1 towards CLOCK-BMAL-1, allowing the negative limb of TTFL to enunciate its function of repression [41,42]. Further, the discovery of CLOCK HAT activity and circadian acetylation of BMAL-1 lead to probe whether CLOCK-BMAL-1 heterodimerization required acetylation of BMAL-1. Studies involving the overexpression of truncated amino terminal of CLOCK (CLOCKΔn) lacking PAS domain was unable to precipitate BMAL-1. Importantly the acetylation of BMAL-1 did not occur though the CLOCKΔn mutant still had a HAT activity domain. Site-directed mutagenesis in HAT domain of CLOCK lead to reduced BMAL-1 acetylation at K537 indicates that CLOCK also acetylates non-histone protein as well. Surprisingly ectopic expression of mutant BMAL-1(K537R) showed abolished rhythms of *Per1* in luciferase assays in MEF cells. Moreover, ChIP assay did not reveal any reduction in the recruitment of CLOCK-BMAL-1 (K537R) onto the clock genes promoter. This raised an important question to explore the perturbed physiological outcome. Interestingly CLOCK-BMAL-1 (K537R) in combination with CRY1 overexpression reveal decreased repression in reporter activity of *Per1* luciferase constructs, therefore, indicating that CLOCK mediated BMAL-1 acetylation leads to recruitment of CRY1 towards CLOCK-BMAL-1 and perform negative limb function. In contrast to HAT acetylation function, histone deacetylases (HDACs) compensates its effect in terms of gene regulation. HDACs discharge the acetyl group from histone tails to increase the affinity of histones towards DNA and allow the spatial tightening of the chromatin which leads to a decline in gene expression. HDACs are also involved at the level of TTFL, where paired amphipathic helix protein (SIN3A) and HDAC1 are being recruited to *Per1* promoter, leading to down-regulation of *Per1* finally closing the loop [43], while SIN3B and HDAC1/2 have been shown to interact with CRY [44,133]. SIN3A and SIN3B protein in concert with HDAC functions as corepressor and are a major constituent of the transcriptional inhibitory complex as observed by its interaction with core clock repressors. To understand the negative limb function of TTFL through PER/CRY, a thorough co-immunoprecipitation followed by tandem mass spectroscopy was carried out in mice overexpressed with FLAG-tagged PER1 and PER2. Peptide analysis at CT20 from mouse liver revealed several circadian proteins precipitated with PER including some RNA binding proteins such as PTB associated splicing factor (PSF). PSF is well known to function as transcriptional corepressor acting through recruitment of SIN3-HDAC complex. Further, mice liver nuclear extract affinity purified with PER2 showed binding of PSF, SIN3A, and HDAC at CT18. To test the functional recruitment of this chromatin repressor onto the circadian gene, ChIP assay was done and which revealed peak occupancy at CT10–14 onto E-box of *Per1* promoter coincides with a decline in *Per1* mRNA level. However, reduction in SIN3A and HDAC recruitment at E-box accompanied with an increase in H3K9 acetylation was observed in *Per1/Per2* double mutant at CT10 in mice liver and lung tissue thus indicates the recruitment of SIN3A and HDAC is PER dependent during repression phase of TTFL. An *in-vitro* report in cell culture confirmed the loading of HDAC1 and HDAC2 by mCRY1 onto promoter as immunoprecipitated in SIN3B overexpressed cells. Although overexpression of deletion construct of PAH domains of SIN3B showed no precipitation with mCRY1 indicates that SIN3B binds with CRY1. Treatment with HDAC inhibitors such as Trichostatin-A (TSA) showed increased expression of *Per* genes immediately after induction. Moreover, *in-vitro* experiments in SCN and MEF cultures with drugs such as Valproic acid and Trichostatin-A which blocks HDAC have also

shown to perturb *Per2* expression [45]. This result thus confirms the role of HDAC and SIN3B in suppressing the gene expression through CRY1. Further, Shi *et al.* documented the dual contrasting role of HDAC3 in the TTFL mechanism, which is separated temporally is independent of deacetylases activity. The study included the genetic depletion of *Hdac3* in *Hdac3*$^{fx/fx}$; *Alb*:*Cre* mice with *Per2*:*Luc* knock-in showing perturbed bioluminescence from liver slices and altered circadian genes mRNA profiles at different time points. Additionally high amplitude of *Per2* in *Per2*:*Luc* MEF, high amplitude of *Per2* and *Dbp* mRNA profiles in wild type (WT) MEF constitutively overexpressed HDAC3 by adenovirus signifies the requirement of HDAC3 in an amplitude of clock genes. ChIP assay revealed the exact mechanism for a high amplitude of those mRNA as *Hdac3*$^{fx/fx}$; *Alb*:*Cre* mice showed reduced BMAL-1 enrichment on *E*-box of *Per2* and *Dbp* promoter. Interaction of BMAL-1 with HDAC3 was confirmed in both *in-vitro* as well as *in-vivo* studies. Additionally *Hdac3*$^{fx/fx}$; *Alb*:*Cre* mice exhibited reduced BMAL-1 levels. Therefore detailed studies in determining the role of HDAC3 surprisingly displayed increased ubiquitylation of BMAL-1 when HDAC3 expression was increased. Furthermore the ubiquitylated BMAL-1 level was more pronounced after MG132, which inhibited UPS-mediated degradation. Pinpointing the ubiquitylation timing in relation to HDAC3 led to the finding that the levels were corresponded to the peak enrichment of BMAL-1 onto E-box. Therefore, it concludes the activation phase where HDAC3 promotes the recruitment of BMAL-1 at E-box and stabilizes the ubiquitylated BMAL-1 onto the promoters. In regard to low CRY1 level during subjective night (CT12–24 repression phase) but abundant *Cry1* mRNA level exhibited by *Hdac3*$^{fx/fx}$; *Alb*:*Cre* mice led the belief of HDAC3 regulating the CRY1 level at post-translational level by inhibiting the degradation. This interesting phenomenon was confirmed by co-transfecting *Fbxl3*, *Cry1*, *Hdac3* in HEK293T cells and inhibiting *de novo* protein synthesis by different concentration of cycloheximide showed stable CRY1 protein levels even in the presence of FBXL3 (F-box E3 ligase, assists UPS degradation). Hence during the repression phase, it prevents degradation of CRY-1 and thereby allowing PER and CRY to interact and inhibit the CLOCK and BMAL-1 function [46]. Genome-wide studies in chronobiology and epigenetics have shown that one of the auxillary loop protein REV-ERBα/β recruits HDAC3 and nuclear receptor corepressor (NCOR) onto chromatin of hepatic genes associated with lipid metabolism to regulate rhythmic histone acetylation. > 100 lipid metabolic genes were co-localized with REV-ERBα and HDAC3 in mice liver tissue at ZT10 and repressed at this phase. This decrease in expression of lipogenesis pathway is the normal outcome of the circadian cycle to keep the level of lipids in the liver at bay. Whereas, genetic deletion of HDAC3 as well as, REV-ERBα show progression towards hepatic steatosis with increased triglycerides in the liver, establishing a seesaw relationship between clock and metabolic pathways. Genetic deletion of a domain (DAD) of HDAC3, which help in interaction with NCoR revealed increased histone acetylation and abrupt circadian mRNA profiles of metabolic genes in DADm (mutant DAD) mice and ultimately resulted in increased energy expenditure indicating the role of HDAC3 and NCoR in energy balance and metabolism [47–50]. Yet another HDAC cognate member, Sirtuin (SIRT1) an NAD^+- reliant deacetylase, functions on numerous clock genes as immunoprecipitation study with SIRT1 revealed an interaction with CLOCK, BMAL-1 and PER2 [133]. Strikingly this association with all core components did not turn into deacetylases function for all clock protein except for PER2, which displayed increased acetylation in *Sirt1* knockout (KO) MEF cells as compared to WT MEFs. Besides, SIRT1 circadian association with PER2 observed in MEFs cells were consistent with cyclic acetylation and deacetylation of PER2. Studies involved in the role of SIRT1 on PER2 revealed that in the absence of SIRT1 lead to an increase in the half-life of PER2 protein in presence of cycloheximide. Whereas nuclear extracts from *Sirt1* KO MEFs in presence of MG132 showed increased PER2 acetylation and protein levels in contrast to no acetylation and more PER2 levels in WT MEFs. This confirmed that SIRT1 encourages deacetylation and degradation of PER2 protein in mice [51]. SIRT1 being a metabolic sensor also connects metabolic pathways to the circadian system, as it requires NAD^+ to perform its function. Additionally, SIRT1 also play a pivotal role in neurodegeneration [52], aging, synaptic plasticity, and memory formation by maintaining the length of dendrites, branching [53] and density of dendritic trees, thus creates a crossroads between circadian epigenome and memory formation [53]. These aforementioned data was speculated from a series of exhaustive study in *Sirt1* KO mice indicating deficits in immediate memory by decreased spontaneous alternation of choices in visiting the arms of Y-arm maze. The decrease in freezing behavior in classical fear conditioning by electrical foot shock experiments and lower magnitude of long term potentiation of hippocampal slice culture of *Sirt1* KO mice indicating impaired short term and long term associative memory. Additionally, *Sirt1* KO mice also showed less dendritic complexity and differentially regulated genes involved in membrane fusion, myelination, synaptic plasticity and metabolism in the hippocampus. SIRT1 regulates these genes by modulating IGF-I, ERK1/2 and IRS2 pathway, which are known to control these mechanisms. Importantly SIRT1 is also known to modulate core clock gene by directly deacetylating BMAL-1 in young mice, through peroxisome proliferator-activated receptor gamma coactivator −1 alpha (PGC-1α) and nicotinamide phosphoribosyltransferase (NAMPT) mediation while this function decays in aged mice [54]. PGC1-α is one of the crucial master regulators of mitochondrial biogenesis and energy metabolism and also known to interact with CREB for initiating gene transcription. While NAMPT controls the level of NMN by salvage pathway for maintenance of energy homeostasis. SIRT1 regulates clock genes circadian rhythmicity, which are found to be perturbed upon knockdown of *Sirt1* in N2a cell lines. PGC1-α, the master coactivator positively regulates *Bmal-1* transcription and also well-known SIRT1 substrate led the conjecture of PGC1-α involvement through SIRT1 in clock regulation. Indeed knockdown of PGC1- α resulted in a decline in clock gene transcripts level at similar time points as was observed in SIRT1 knockdown. Moreover, overexpression of PGC1-α also showed a similar enhanced mRNA profile of clock genes compared to SIRT-1 overexpression. Additionally ChIP assay revealed the cooperative binding of both the protein onto *Bmal-1* promoter, which indicates both the protein are tightly coupled in same pathway. *Nampt* a clock output gene regulated by CLOCK-BMAL-1 relays activity to SIRT1 by providing NAD^+ displays synchronous oscillation with *Sirt1* and *Pgc1-α* in SCN and shows a decline in levels upon aging. Thus led to hypothesizing that *Nampt/Sirt1/Pgc1-α* acting through another loop and amplifies the clock regulation. The decrease in NAD^+ levels upon aging is the evidence for decreased mitochondrial metabolism and SIRT3 has been documented to be involved in circadian deacetylation of mitochondrial proteins which provide convincing evidence of circadian control over aging [55]. To govern robust regulation of metabolism and homeostasis, the clock indirectly regulates the redox state of the cell via maintenance of NAD^+ level. NAD^+ levels of cell are maintained by the NAMPT enzyme which is in direct regulation of the clock and shows circadian rhythmicity throughout 24 h. NAD^+ levels in turn, renders activity to SIRT3 enzyme, which is known deacetylase of many of the crucial mitochondrial enzymes involved in the fatty acid oxidations and respiratory chain. Whole cell proteome from *Bmal-1*$^{-/-}$ mice liver immunoblotted with acetyl-lysine Ab showed differential acetylation and deacetylation level of many proteins of mitochondria. In particular, the SIRT3 targets such as urea cycle protein manganese superoxide dismutase (MnSOD), isocitrate dehydrogenase 2 (IDH2) and ornithine transcarbamylase showed increased acetylation. In addition to that lipid metabolism proteins long chain acyl dehydrogenase (LCAD) and medium and long chain acyl CoA dehydrogenase (MCAD) also displayed increased acetylation in *Bmal-1*$^{-/-}$ mice. Moreover, these increased acetylation levels were correlated with decreased activity in *in-vitro* enzyme activity assays from liver mitochondrial extracts. Therefore, acetylation of mitochondrial proteins is indirectly regulated through SIRT3 by clock components. Additionally upon aging the low

levels of NAD^+ declines the activity of mitochondrial proteins through increased acetylation ultimately leading to perturbation in circadian mitochondrial homeostasis.

Besides acetylation and deacetylation, histones may also undergo methylation at H3K4 me3, an active mark for transcription, has been found to be associated with various clock genes and clock output genes [35]. Chromatin modifiers like histone methyltransferase (HMT), myeloid/lymphoid or mixed–lineage leukemia (MLL) namely, MLL1 and MLL3 have been shown to play an important role in functioning together with CLOCK-BMAL-1 complex onto the chromatin and modify H3K4 by methylating it [56,57,133]. *Dbp* promoters in dexamethasone-induced MEFs cells show circadian rhythmicity in H3K4 trimethylation (me3) with peak at 18 h and trough at 30 h of dexamethasone induction. Additionally HMT MLL1 transiently co-expressed with CLOCK-BMAL-1 show increased signal by *Dbp* luciferase-based reporter expression and E-box containing promoter in MEFs cells. Nevertheless, absence CLOCK protein decreased the recruitment of BMAL-1 and MLL1 thus concerted with abolished rhythms of H3K4me3 in MEFs cells. Interestingly mice liver also displayed circadian changes of H3K4me3 at thousands of genomic loci with a peak in the night. However, ChIP seq data confirmed the occupancy of MLL3 on 13% of such genomic loci that exhibit circadian transcription. In particular genomic loci were found to be mapped with various clock-controlled genes and core clock genes such as *Rev-erbα*, *mPer1*, *mPer2*, *mCry1*, *mCry2* and *Bmal-1* [57]. Remarkably, MLL3 deletion perturbed the circadian rhythmicity of clock genes as indicated by MLL3 null MEF cells by reporter constructs. Similarly, chromatin at *mPer1* promoter has been found to be enriched with H3K27me3 marks by CLOCK-BMAL-1 mediated recruitment of another HMT enhancer of zeste homolog 2 (EZH2) [58,133]. *In-vivo* Co-IP with CLOCK co-purified BMAL-1 with EZH2. Recruitment of EZH2 concerted with an increase in H3K27me3 onto *mPer1* promoter leading to suppression of *mPer1* expression in the repression phase. However, knockdown of EZH2 by shRNA exhibited inhibition in mCRY mediated suppression and also perturbs the *mPer1* oscillations in MEF cells. Hence, EZH2 mediated chromatin alteration is critical for controlling the repression phase of TTFL. Though, chromatin modifying proteins like lysine demethylase JARID1A which can also inhibit HDAC1 activity on chromatin and can bind to CLOCK-BMAL-1 complex, mediating gene transcription [59,133]. JARID1A showed circadian enrichment at *mPer2* promoter in mice liver tissue with a peak at CT8 to CT10 synchronous with peak of BMAL-1 occupancy and gene expression. This increase in gene expression was consistent with the relative luciferase activity 3T3 cells co-transfected with *Per2*:*Luc* constructs and *Jarid1a*. To gain insight into the mechanism by which JARID1A controls gene expression, levels of H3K9ac were detected in WT and *Jarid1a*$^{-/-}$ cells which revealed decreased acetylation in *Jarid1a*$^{-/-}$ cells. However, HDAC1 deacetylation activity was reduced in presence of JARID1A in *in-vitro* cell culture studies with a similar level of acetylation as compared to control. Though inhibiting relationship to HDAC1 was more prominently seen in the liver nuclear extract with lesser occupancy of HDAC1 onto *Per2* promoter between CT6 to CT12 when JARID1A occupied the promoter. HDAC1 recruitment was more in between CT12 to CT18, which indicates that JARID1A controls gene expression in association with CLOCK-BMAL-1 by inhibiting HDAC1 recruitment onto promoter at activation phase. In addition to conventional histone modifications, phosphorylation on H3S10 has been reported in SCN neurons upon light induction [60,133]. Immunofluorescence on mice SCN cryosections at different time points showed enhanced signals of H3S10 phosphorylation upon light induction for 15 min light pulse to the animals. H3S10ph levels also found to be correlated with induction of *c-fos* gene expression between CT15 to CT21 after light pulse. This indicates that H3 phosphorylation is linked to an immediate early gene response after photic induction. However, kinases downstream to CREB are thought to be phosphorylating the H3S10 for further chromatin modification during immediate early gene responses. Thus, H3S10ph holds an important link of epigenetic changes in response to environmental stimuli for regulating circadian rhythm. Furthermore, circadian phosphorylation by Protein Kinase C (PKCα) of FAD-dependent demethylase activity of lysine-specific histone demethylase-1 (LSD1) activates CLOCK-BMAL-1 mediated transcription [61,133]. *In-vitro* studies in MEFs cell showed LSD1 circadian phosphorylation by PKCα, which allows LSD1 to bind to CLOCK-BMAL-1. However, LSD1 mutant (*Lsd1*$^{SA/SA}$, serine 110 to alanine) mice exhibited defect in circadian behavior through disrupted locomotor activity and also showed attenuated gene expression of clock genes. Subsequently, the mutation leads to low enrichment of BMAL-1, decline in activation marks (H3K9ac) and no change in repression marks (H3K4me2) onto *Dbp* and *Per2* promoter in mice. Furthermore, on upon photic induction, *Lsd1*$^{SA/SA}$ mice failed to display induction in *Per1* gene expression. Moreover, PKCα is induced by light stimuli, this reveals the fact that phosphorylation of LSD1 by PKCα is important for clock gene expression through activation of CLOCK-BMAL-1 by LSD1. In short, robust regulation of circadian rhythms are governed by a wide array of epigenetic modifiers or vice versa, are involved directly or indirectly in TTFL or auxiliary loop ultimately controlling substantial transcriptome of the cell. Interestingly, epigenetic regulation also interconnects energy metabolism, mitochondrial biogenesis, aging and neurodegeneration with core components of clock, deciphering its role in enormous physiological processes.

4. DNA methylation and circadian rhythms

Apart from histone modifications DNA methylation is another epigenetic mechanism which plays a profound role in controlling the gene expression. To regulate gene expression DNA methylation largely occurs at single base cytosine which is widespread as CpG signatures throughput the genome. Nonetheless, it shows reversible biochemical changes mainly in gene rich regions to direct development, differentiation and organ genesis depending on the genes to be turned on or turned off during respective stages. Inside gene rich regions CpG signature marks are distributed mostly in the regulatory elements and upon methylation on cytosine, it allocates various chromatin modifiers further packaging the local regions into a condensed form. DNA methyltransferase (DNMT), adds methyl group onto 5th carbon of cytosine base [62] whereas ten eleven translocase (TET) is the major demethylase which removes it. Interestingly, adult nervous system also shows increased levels of *Dnmt1* and *Dnmt3* and conditional gene deletion studies have shown its functional significance in lineage specification of neural progenitor cell, memory formation and neuronal maturation [62–64]. Group of researchers have observed the presence of methyltransferase activity in many biological processes such as circadian rhythms, neuronal plasticity, memory and learning [62,65–68]. As anticipated, strong synchronizer light also triggers rhythmic DNA methylation at clock genes in SCN [69] which indicates the influence of environmental signal on methylation. Methylome studies in mammals show that light-dark cycle can affect global methylation pattern on promoter in SCN of mice subjected to a shortened time of 22 h. Particularly, shortening of cycle to 22 h instead of 24 h lead to transcriptional changes in *Dnmt* and *Tet* as observed in SCN of mice. Interestingly, these changes were reversible after sustained entrainment to normal time period [69]. A study in *Neurospora* involved in the understanding role of chromatin remodelers chromodomain helicase DNA binding protein 1 (CHD1) on negative core clock gene expression, *frequency* (*frq*) revealed the importance of normal light-dark cycle [70]. Using Δchd1deletion strain and lighting conditions such as constant light light and constant dark dark, they demonstrated that CHD1 regulates the chromatin at *frq* gene and has a role on decrease in methylation pattern. However, the methylation status of *frq* loci was controlled by DNA methyltransferase DIM-2 (defective in methylation) in normal light-dark cycle and decreased in Δdim-2. More importantly, the absence of light dark cycle has shown diminished DNA methylation at the promoter clock gene *frq* defining the role of light in DNA

Table 1
Role of DNA methylation in circadian rhythms in various tissues and their associated experimental methods.

S. no.	Tissue/model	Involved methods	Associated functions/summary	Reference
1.	Endometrial cancer tissue	MS-PCR	Alteration in *Per1* promoter methylation and uncoupling of gene expression causes abnormal changes in circadian rhythms	[84]
2.	Breast cancer tissue	MS-PCR	Alteration in 5mC of *Per1*, *Per2* and *Per3* genes may lead to an abnormal changes in circadian rhythms	[93]
3.	Chronic myeloid leukemia tissue	MS-PCR	Altered DNA methylation of *hPer3* gene causes downregulation of its associated genes	[94]
4.	Endometrial samples	MS-PCR	Observed frequent DNA methylation in clock genes	[95]
5.	Cervical cancer cell lines	BGS	Hypermethylation of *Per1* with uncoupling in the expression of *Per1* and methylation of its promoter	[83]
6.	Cell lines	MS-PCR	Hypermethyltion of *Bmal-1* promoter causes impaired circadian changes in c-Myc, catalase, and p300	[82]
7.	Clinical samples	Bead chip assay	Long term shift workers show hypomethylation of both *Clock* and hypermethylation of *Cry2* patterns, similar to breast cancer patients	[86]
8.	Clinical samples	Sequenome Mass Array	DNA methylation of *Clock*, *Bmal-1*, and *Per2* are associated with metabolic syndrome and obesity	[90]
9.	Clinical samples	Methylation bead chip Assay	Altered amplitude of DNA methylation rhythms are associated with aging and Alzheimer's disease (AD) pathology	[81]
10.	Mouse SCN tissue	MeDIP and CpG hybridization array	Effect of light on DNMT and TET proteins expression and clock genes methylation pattern	[69]
11.	Lymphoma cell line	MS-PCR	Hypermethylation in CpGs of *Bmal-1*, and hypomethylation of *Per2* promoter	[96]
12.	Mouse tissue	BGS	Oscillation of modified cytosine levels are related to aging	[91]

MS-PCR, Methylation Specific PCR; BGS, Bisulfite Genome Sequencing; MeDIP, Methylated DNA Immunoprecipitation.

methylation of clock genes [70]. High throughput methylome study in mice liver indicated daily variation in the methylation profile of animals entrained in the light-dark cycle or dark-dark cycle. The rhythms of daily methylation level were in morning and evening phases, having the highest levels at the end of the day while lowest at the beginning of the day. Moreover, the daily variation was disturbed in *Per1*$^{-/-}$/*Per2*$^{-/-}$ double knockout (DKO) mice and found to be higher levels as compared to wild type animals. Increase in DNA methylation level in DKO mice indicates the role of clock output genes in methylation. In addition to this, 24 h rhythmic profile in DNA methylation levels were correlated with an increase in the expression levels of methylation machinery such as *Dnmt* and *Tet* levels [71]. Although committed investigations have failed to detect strict rhythmic cytosine methylation patterns in mice, in contrast to the anticipation [72]. Dedicated studies were attempted in investigating the methylation pattern of large fractions of oscillating genes (704 loci) between late afternoon (CT9) and late night (CT21) in mouse liver as they reach their highest levels. Contrastingly, the DNA methylation at the promoter of 18% active loci was not correlated inversely to their transcript levels. Nevertheless, these transcripts have shown > 10 fold change between peak and trough levels. The direct implication of clock gene into DNA methylation and reciprocal regulation, came from the evidence through DNA methylation-dependent regulation of *Bmal-1* transcription and rhythmic elevated enrichment of BMAL-1 onto the promoter of *Dnmt3a* concerted with transcription [73–75]. Apart from the presence of RORE in the promoter region of *Bmal-1*, it also contains many putative core motifs and binding motif for Sp2/Sp3. The CpG islands near the Sp1 activator-binding site of *Bmal-1* undergoes hypermethylation and prevents another TF biding to Sp2/Sp3 motif. This steric hindrance influences the gene expression and thereby suppresses the *Bmal-1* expression [76]. Additionally, aza-dC treatment (inhibitor of DNMT) in RPMI4802 cell line (hypermethylated *Bmal-1* promoter) recovered the transcript level [74]. Altercation in DNA methylation at clock genes has been observed in blood samples of many human diseases like dementia with Lewy bodies in which *Npas2* promoter is hypomethylated [77,78,96]. Indeed, the *Npas2* expression was enhanced in the patient leading to higher expression of *Rev-erb*, ultimately suppresses the expression of *Bmal-1* which correlates in those patients [77]. Instances have shown that sleep deprivation (SD) changes CpG landscapes onto promoter of numerous genes linked to nerve transmission, synaptic plasticity, cell signaling and neurite outgrowth [41,79]. Synaptic functions are known to control the sleep and wakefulness, Neuroligin 1 (NLG1) a synaptic adhesion molecule, which controls the localization and activity of NMDA receptors. However, prolonged wakefulness impairs the activity of NMDA and noticeably NLG1 expression is mainly controlled by core clock TFs [80]. SD has shown to decrease the transcripts level of NLG1 whereas an increase in the transcript level of *Dnmt3a1* and *Dnmt3a2* indicates the influence of SD on methylation as well as the indirect role of clock regulation [79]. Concrete evidences from the human study have indicated 24 h cyclic pattern of CpG methylation signatures which are coinciding with the gene expression data from dorsolateral prefrontal cortex [41,81]. High throughput methylation analysis of clock genes and CCGs on human brain samples indicated altered rhythms and amplitude in DNA methylation with greater age. Although similar DNA methylation rhythm profiles were also observed in Alzheimer's disease patients sample establishing a connection between neurodegenerative disorders and rhythmic DNA methylation [67,81]. Disruption in the clock gene promoter methylation pattern has been associated with breast cancer and a variety of malignancies [82–85]. In hematological malignancies, hypermethylation of CpG regions of *Bmal-1* showed downregulation of this gene and the importance of circadian epigenome in cancer [82,96]. Though aberrant methylation on CpG islands of other core clock genes specifically *Clock* and *Cry2* essentially involved in DNA repair pathways could be linked to cancer occurrence in long-term shift workers [86]. Such chronodisruption often have been reported with obesity and metabolic syndrome (MetS). More often the symptoms and

diseases are associated with a drastic increase in body weight, arteriosclerosis, and diabetes as a result of compromised insulin sensitivity and rise in blood pressure [87–89]. In addition to that methylation studies in human female subjects show a strong correlation with the methylation levels of *Clock* gene CpGs with MetS characteristics (BMI, fat content, insulin resistance, and systolic pressure) [90]. Recently in 2018, a group of researcher has demonstrated oscillation of modified cytosine (Osc-modC) enriched specifically at E-boxes of circadian genes in mouse liver and lungs and also correlated the levels of Osc-modC to aging [91]. Mechanistically, Osc-modC includes the oxidation of 5mC to hmC meaning DNA demethylation followed by remethylation. Interestingly studying Osc-modC could tells us the temporal state of clock genes, which are undergoing epigenetic changes at any particular time. At last clinical studies in human blood have shown homocysteine concentration in plasma and global DNA methylation variation throughout 24 h in 15 males and 15 females [41,92]. In addition to this, there have been reports, which indicates changes in the methylation of clock genes and CCGs in many different types of cancer and diseased phenotypes may be acting as cause or effect of perturbation of circadian rhythms leading to phenotype (Table 1). Table 1 depicts the variation in methylation of clock genes in different cancer cell lines and clinical samples and model system used for the study. Additionally to understand the reliability of variation observed we also depicted the methods used in this study.

Overall, epigenetic modification such as methylation at DNA level also runs deeply at TTFL level, plays an important role at clock genes and CCGs expression ultimately defining the controlled behavioral and physiological outcome.

5. Non-coding RNA in circadian rhythms

In recent years, the landscape of rhythmic gene regulation has become more pronounced with the emerging evidences indicating towards the other cellular mechanism in addition to the classical TTFL model [16]. MicroRNAs (miRNA) have emerged as another avenue of circadian gene regulation. These small RNA (22–26 nt) binds to the complementary regions in the 3′UTR of the target genes inhibiting their translation and enhancing the degradation of mRNA down the lane. Recently various groups have studied miRNA mediated control of one of the pivotal pathway such as MAPK/CREB which is receptive to photic entrainment cues [97]. It is admissible that inherent post-transcriptional events in regulating the clock or induced by any external stimuli might induce miRNAs to regulate the circadian protein expression by key transactivation mechanism [97]. Two of the very important brain-specific miRNAs are miR-132 which is involved in the modulation of chromatin remodeling and translational control of circadian clock, whereas miR-219 is implicated in period determination [97]. *In-silico* target prediction of miR-132 revealed numerous chromatin remodelers such as *Mecp2*, *Ep300*, and *Jarid1a* as well as proteins involved in translational control like *Btg2a* and *Paip2a*. *In-vitro* reporter assay co-transfected with miR-132 and 3′UTR:Luc construct of targets (*Mecp2*, *Ep300*, *Jarid1a*, *Btg2a*, and *Paip2a*) showed a decrease in relative signals. Moreover, this finding was more evident in miR-132 conditionally expressed transgenic mice (tTA:miR-132), which showed a decline in these target protein levels. Photic induction majorly drives expression of miR-132, *Per1*, *Per2*, *Btg2a*, and *Paip2a*. MeCP2 and CREB mediate the transcriptional activation of these genes via regulating the chromatin modification upon light induction. BTG2a and PAIP2a regulate the translation of *Per1*, *Per2* and may also of CCGs in the cytoplasm and thus involved in the period length. Whereas the MeCP2, EP300, and JARID1A control the chromatin events required for the regulation of circadian rhythmicity of CCGs transcripts. Upon induction miR-132 then controls the expression of *Mecp2*, *Ep300*, *Btg2a*, and *Paip2a* at the post-transcriptional event. Hence the rhythmicity is maintained within the cells throughout 24 h [16]. Both of the miRNAs showed time of the day dependent variation in terms of expression observed at CT6 (subjective mid-day) and CT19 (subjective mid-night). However, the direct target of these two miRNA has not been identified yet but lack of *mCry1/Cry2* did not reveal the time of the day dependent variation, therefore, indicating the control of molecular clock over rhythmic miRNA expression [97]. Increasing evidences also implicated about different miRNAs in the regulation of clock genes such as *Per1* and *Bmal-1*, but the mechanism of integration into the circadian clock regulation system has not been known. Recently Chen *et al.* group in 2014 have indicated very remarkable mechanism of time delaying of *Per2* translation and control of period determination by a group of miRNAs targeting *Per2* directly through consensus seed sequence [98]. *Dicer* is one of the regulatory components of miRNA processing which produces mature miRNAs from the pre-miRNA. *Dicer* mutant mice showed shortening of period length, which is evident regulation of clock by miRNA. The turnover of PER1 and PER2 protein contributes to the period length and *Dicer* mutant mice showed the upswing in PER1 and PER2 translation thereby shortening the period length. Moreover, three miRNAs such as miR-29a, miR-30a, and miR-24 were shown to control the *Per1* and *Per2* mRNAs in the cytoplasm confirms the RNAi integration in clock. Vollmer's group also showed 53 miRNAs endure through robust circadian oscillations out of 258 expressed miRNA from Small RNA seq experiments. These also included a wide array of miR family miRNAs involved in liver functions such as Let-7, miR-33, miR-103 and miR-122 [72]. Additionally, there are number of miRNAs which are either involved in regulation of core component and vice versa and thereby govern rhythmicity in various physiological outputs as mentioned in Table 2. Table 2 depicts a wide array of miRNAs involved into circadian rhythmicity of clock genes or CCGs involved in various processes. Tissue/model depicts the finding was observed in these systems.

Besides small miRNAs, long non-coding RNAs (lncRNAs) have also emerged as a meaningful transcriptional regulatory mechanism over these years. Though, how these long non-coding RNAs controls gene regulation is yet to be discovered but fraction of literature indicate that these long non-coding RNAs are pervasively transcribed from enhancers and help to recruit many regulatory transcription factors and also plays a substantial role in enhancer-promoter (EP) interaction loop. LncRNAs mainly focuses on the recruitment of chromatin modifiers, mRNA decay, mRNA transport and RNA maturation recommends its dynamic role at pre-transcriptional and post-transcriptional steps [115,116]. Studies have demonstrated that lncRNAs display robust spatiotemporal expression patterns indicating lncRNAs expression tightly regulated [117]. Perturbation in structural and expression levels of those may lead to neurodegenerative diseases and cancer [118,119] suggesting the impact of lncRNAs on physiological and metabolic pathways [117]. Additionally, lncRNA functions as a junction for clustering of many gene regulatory proteins interacts with mRNA to increase its half-life [120], sponges miR-372 [121] and could act as cis-trans regulators as well [122,123]. Genome-wide studies in rat pineal gland have shown differential expression of 112 lncRNAs throughout 24 h and have shown to increase the nocturnality phenotype. Studies have indicated that light exposure in night promptly reverses the levels of lncRNAs indicating a possible connection between lncRNAs and circadian rhythm. *In-vitro* organotypic pineal cultures have suggested norepinephrine (NE)-induced expressions of lncRNAs through cAMP induction. However, various cAMP analogs have also induced the lncRNAs, which indicates that NE in night acts through cAMP induction [124]. Recently, an involvement of lncRNA hepatocellular carcinoma upregulated long non-coding RNA (HULC) in controlling hepatocellular carcinoma (HCC) by disturbing the circadian rhythm through heightening the phase of *Clock* periodic expression has been well documented [117]. Both *in-vitro* and *in-vivo* functional experiments such as reporter assay and knock down experiments indicted higher *Clock* gene expression in hepatoma cell lines probably due to through mRNA stability by HULC. CLOCK is known to modulate nuclear factor-κβ (NF-kβ), energy metabolism and inflammatory processes and thus explains the increased

Table 2
Involvement of various miRNAs (Non-coding RNAs) in circadian rhythms in different tissues/model and their associated functions.

S. no	Tissue/model	miRNAs	Associated functions/summary	Reference
1.	Mice SCN and PC12 cell line	miR-132 and miR-219	Regulates light dependent clock resetting and also Period length and lost function in mCry mutants is observed	[97]
2.	Mouse retina tissue	miR-182 and miR-96	Targets rhythmicity of *Adcy6* and *clock genes*	[99]
3.	Mouse liver tissue	miR-122	Targets ccg *Nocturinin* and controls lipid metabolism	[100]
4.	HeLa cell line	miR-194 and miR-192	Inhibits *Per* gene	[101]
5.	Mouse liver tissue	miR-122	REV-ERBα drives miR-122 expression, controls metabolism through PPARβ/δ and SMARCD1/BAF609 circadian regulation	[102]
6.	Mouse SCN tissue	miR-132	Regulates chromatin remodelers such as MeCP2, EP300, and JARID1A	[16]
7.	Mouse skeletal Muscle tissue and Mathematical model	miR-206	Regulates *Clock* through CLOCK-BMAL-1-MyoD axis	[103]
8.	Mice Serum and HEK293T	miR-152 + and miR142-3p	Involvement with *Bmal-1* gene	[104]
9.	Human blood cells and 293ET cells	miR-142-3p	Regulates *Bmal-1* in circadian rhythms	[105]
10.	Mouse Embryonic Fibroblasts (MEF) cells	miR-30, miR-24, and miR-29	Controls translation of mPER1/mPER2	[98]
11.	Mouse liver tissue	miR-24, miR-29, miR-122, miR-22, and miR-25/92	Associated with rhythmic transcriptome of liver	[106]
12.	Mouse liver tissue	miR-27b-3p	Reduces endogenous *Bmal-1* and gluconeogenic proteins rhythmicity	[107]
13.	Mouse calvaria tissue	miR-433	Controls bone metabolism, targets HDAC6 and regulates clock genes and osteoblastic genes	[108]
14.	Mouse tissue and 3T3 cell line	miR-17-5p	Targets *Npas2* and *Clock genes*	[109]
15.	Mouse liver tissue	miR-378	Regulates time of day dependent expression of cell cycle gene *Cdkn1a* and *Pdk4* gene of glucose metabolism and also controls oxidation reduction pathway	[110]
16.	Mouse tissue	miR-21	Regulated by *Per2* gene which is accountable for cardioprotection from ischemia by reducing cell death of cardiomyocytes	[111]
17.	Mouse tissue	miR-132/212	Regulates circadian day length changes through regulation of spine morphogenesis in SCN by controlling MeCP2	[112]
18.	Mouse tissue	miR-24	Provides *mPer2* mRNA stability and translation	[113]
19.	miR-132 null mouse	miR-132	Rhythmic regulation of miR-132 involved in learning and memory formation in time of day dependency	[114]

expression during tumorigenic development. Role of lncRNA in circadian system has been found in many species including *Neurospora*, wherein *qrf* located to antisense of core component of clock *frq* shows antiphase oscillation and upsets *frq* gene expression by chromatin modification and premature terminating the nascent transcript. *Qrf* expression is light inducible as well as light independent. In which light dependent *qrf* expression represses *frq* expression and resets the clock while light independent expression regulates circadian rhythmicity [125]. Similarly in mice lncRNA located on antisense as *Per2* to *Per2* shows antiphase oscillations to *Per2* [73], however, the detailed function of as *Per2* is not known yet.

High throughput deep sequencing with better depth and coverage such as strand-specific RNA seq (SSR seq) in concert with ChIP seq for chromatin marks unveiled as much as 102 rhythmic non-coding RNA which went undetected in high-density array studies in mice liver. However, only 19 lncRNA showed strong oscillatory profile. SSRseq also helped in discovering number of overlapping pairs of oscillatory transcripts which are located in close proximity such as long non-coding RNA AC 148977.1 and an unannotated lncRNA located near the TF Klf-3 locus. The expression levels of *Klf-3* transcript was found to be highest in evening while lncRNA expressions showed antiphase peak timing mostly in early morning [72]. eRNA is another type of lincRNA with cryptic or spurious functions despite their robust association between active histone marks on enhancer and synthesis of eRNA. Over these years several groups have investigated the role of eRNA as transcriptional activators [126–128] and enhancing EP looping by interacting with mediator and Cohesin complexes [128]. High resolution GRO seq and ChIPseq data obtained for genome-wide circadian regulators from mice liver have provided induction of over tons of circadian eRNAs and TF binding at those motifs. The possible eRNAs thought to be expressed from enhancer due to presence of active histone marks such as H3K27ac, H3K4me1, Pol II binding and DNase I hypersensitivity around the enhancer. However, the phase (peak activity) of circadian eRNAs were unevenly distributed (ZT18 to ZT24) as compared to evenly distributed phase of circadian rhythmic transcription which may arise due to control of promoters instead [129]. The enhancer's activation marks leading to eRNA expression which occurs at different phases as compared to core component expression with one high phase and one low phase. Therefore, this led to speculation that specific circadian TFs binding at different phases to eRNA loci driving the multiple phases of circadian gene expression. Particularly repressive action of REV-ERB peaks at ZT-10, hence shaping the circadian transcription via sub-loop in reverse phase at ZT-20 in mice liver by binding to some eRNA loci at that time [47]. Over thousands of REV-ERBα binding sites, only < 10% of them are characterized by transcription of rhythmic eRNAs (ZT18-24) and their target gene expression upon recruitment of corepressor (HDAC3) of REV-ERBα. Furthermore, this binding leads to marked decrease in H3K9ac after ZT20 to ZT10 on those sites delineating the role of eRNAs in regulation of liver cistrome [129]. Several groups have identified circadian lncRNA transcribed from enhancer regions such as lnc-crot lncRNAs which controls the temporal partitioning of fatty acid oxidation (light phase) and fatty acid synthesis (dark phase) in RNA dependent manner [130]. The involvement of non-coding RNAs with the likes of miRNAs and lncRNAs in clock regulation, it is evident that how clock regulates multiple phases of circadian rhythm though the main TTFL mechanism is constant in every cell. Since miRNA and lncRNAs are known to target many genes, which widen our understanding of clock being tightly regulated; and are embedded into many pathways at the same time. Thus, it is important to enhance our knowledge on tiny players of clock, which could help in apprehending cross talk and interrelationship between many molecular pathways and know the cause of diseased outcome.

6. Circadian 3D nuclear territories

Circadian behavior governed by the rhythmic epigenetics and

transcripts of clock and CCGs, which also suggests us common epigenetic marks on promoter and enhancer of those oscillatory genes. As genes are dispersed onto different chromosomes throughout the nucleus, then to confer co-regulation, CCGs must coalesce their regulatory elements at one place. Therefore it is evident that at the finer scale an additional layer lies within the nucleus, which separates chromosomes in permissive and repressive compartments. Decade of research studying chromosomal organization in nucleus interior have unraveled functional compartmentalization within the nucleus [131–133], majorly nucleus center serving as transcription factories whereas periphery of the nucleus marks permissive compartments. Hence, 3D folding of chromosomes sufficing the regulatory elements, a ground for another epigenetic layer of regulation is gaining prominence in genome regulation at the moment [134,135]. Nonetheless, chromosomes territories are compelling evidence for the constitution of nucleus architecture [136]. This phenomenal spatial non-random positioning of chromosomes in the nucleus generates chromatin architectural station for the interaction of diverse regulatory elements such as interactions of promoter, enhancer, and insulator of genes. EP looping interactions are the major driving force for gene regulation which has been extensively studied by the discovery of fascinating chromosomes conformation capture (3C) and 3D-FISH [137–140]. Mounting evidences have indicated about the dynamic nature of such DNA contacts conveying into correlated transcriptional readouts in developmental transitions, cellular differentiation and stimulated gene expression [141–143]. However, such interaction studies in the clock biology are at its primitive stage. Cell culture experiments have provided a hint on the role of nuclear compartmentalization, long-range DNA contacts on cyclic gene expression [144,145], short-range chromatin loops at certain loci [146]. Long range interaction study of *Dbp* genomic locus one of the 1st order CCGs has shown interaction with numerous circadian genes and is found to be dynamic in nature in MEFs cultures. Cells were investigated from every 4 h between CT22, the peak phase and CT34, the trough phase of *Dbp* and to be ensured of the second successive phase CT46 was also taken into consideration. Circular chromosome conformation capture (4C) study revealed the DNA contacts of *Dbp* locus with genes involved in lipid, carbohydrate, nucleotide metabolism, and xenobiotic degradation pathways. Most of the genes included enzymes involved in oxidation and reduction reaction, enzymes and protein which bind to metabolites (NADH, FAD, NAD^+, and Haem). *Dbp* itself is involved in the modulation of xenobiotic pathways and are controlled by binding of CLOCK-BMAL-1 to the E-Box. However, studies in BMAL-1 deficient MEFs showed *Dbp* circadian interactome dependency on intact clock components as it was significantly low in absence of BMAL-1 [10]. Therefore, enrichment of these pathways with *Dbp* genomic locus indicates the control of clock on metabolism and biological processes through dynamic interactions. These remodeled DNA contacts and loop formation in developmental transitions are found to be static for days, but studies on mice tissue have revealed plastic nature of dynamic chromatin contacts over 100 kb scale occurring within 24 h. Nevertheless, chromatin contacts (within 30 Kb range) between enhancer and promoter of circadian genes have indicated short-range interactions occurring rhythmically in 24 h [139]. In particular, the promoter of circadian clock gene *Cry1* and a liver-specific clock output gene glycogen synthase 2 (*Gsy2*), the regulatory regions downstream to TSS were investigated using 4C-Seq. These transcripts were expressed antiphasic to each other in mice liver, mostly *Cry1* peaking at ZT20 (during the night) while *Gsy2* at ZT08 (during the day). Interestingly, according to the transcript peaking time, the contact frequency between promoter of *Cry1* and +26 kb downstream intronic enhancer region showed higher contact frequency at ZT20. Similarly, for *Gsy2* promoter and enhancer in +21 kb downstream to TSS in exon-8 regions showed increased frequency at ZT08 versus ZT20. Genome-wide circadian TF enrichment studies, histone marks (H3K27ac) and Pol II bonding studies by ChIP seq on regulatory regions revealed the binding site for BMAL-1 and REV-ERBα for *Gsy2* gene. The investigation also led to the speculation of significant competing effects on circadian rhythms by BMAL-1 (ZT10) and REV-ERBα (ZT06) binding which shows a promising role of these interactions in circadian rhythms [72,73,147,148]. Nevertheless, in the case of *Cry1*, 300 bp enhancer located within the intronic regions of RRE element plays an important role via competitive binding of activator RORγ and repressor REV-ERBα at peak ZT20 and at trough ZT08. However, the absence of *Bmal-1* showed low levels of REV-ERBα in *Bmal-1* knockouts and high RORγ ultimately leading to closed EP loop, signifies perturbed sub-loop of clock mechanism (TTFL). This study was further substantiated by constitutively functioning *Cry1* loop producing low levels of EP interaction in *Cry1Δe* and converging into pronounced short periodic phenotypes in locomotor activity [139]. Also, earlier reports of variation in non-coding genetic elements have been linked to sleep phenotype and biological clock endorsing the infringement of regulatory elements in clock [149,150]. The sleep phenotypes such as self-reported morningness and variation in sleep duration were associated with SNP variants in gene bodies of *Per2*, *Per3*, and *Clock* throughout the different population. Decisively high-resolution single molecular RNA FISH studies investigated the circadian daily transcriptional burst frequency of *Cry1*, which was compromised after the abolishment of such EP interactions [139]. Though regulatory elements forms EP interactions in cis or trans manner, hence to restrict the interactions within the co-regulated regions and not exploding the bursting effect on nearby genes present in the adjacent locus, these EP interactions occur within the insulated regions topologically associated domains (TADs). TADs are regions within the nucleus and core component of compartmentalization with demarcated insulated boundaries often occupied by several protein complexes such as CCCTC binding factor (CTCF), Cohesin, and Mediator (MED1). Cohesin functions in concert with CTCF and insulates the gene preventing its transcriptions by excluding it to come within the EP interactome. Interestingly, recent investigation of super-enhancer upstream to clock gene *Nr1d1* bound by BMAL-1, discovered that the co-binding of CTCF-Cohesin in the interactome anticipates insulation of the circadian phase but Cohesin-non CTCF produce high circadian amplitude of transcription. To determine the higher order chromatin structure in regulating circadian rhythmicity, the super-enhancer region upstream to *Nr1d1* was used as bait in 4C experiment to generate interactome. This super-enhancer spans over 150 kb region and showed an enriched signal at CT6 and CT18. Out of thousands of CCGs, *Fbxl20*, *Cdk12*, *Med24*, *Thra*, and *Nr1d1* showed high interaction with enhancer at CT6, although Med24, *Thra* and *Nr1d1* showed the strongest interaction. DNA binding studies by NR1D1, BMAL-1, CTCF, Cohesin, and GABPA revealed the binding of Cohesin-non CTCF prefer between BMAL-1/NR1D1 binding site and random sites. Circadian rhythmicity of CCGs tested in the Cohesin-non CTCF domain showed positive influence on gene expression upon binding of BMAL-1/NR1D1. Whereas Cohesin-CTCF binding locked the expression of nearby CCGs within the binding domain. This study revealed long-range interactions playing a role in converging co-regulation of the circadian gene and maintaining the dynamicity in circadian rhythms [146]. Importantly this study also indicated the role of architectural protein in recognizing CCG from non-CCGs. *In situ* Hi-C followed by tandem 3C and ChIPseq data generated in mice liver discovered about 6510 dynamic sub-TADs within the static TAD regions, of which only 349 were ZT-22 specific whereas 527 were ZT-10 specific. The intronic enhancer of *Cry1* gene within ZT22 sub-TAD showed enhanced EP interaction with correlated high expression of *Cry1* mRNA. The intronic enhancer region also showed binding sites for REV-ERBα enriched at ZT10, which is antiphasic to the *Cry1* gene expression (low gene expression at ZT10). The binding of REV-ERBα to some sites lead to gene repression by destroying the EP loop and are known as engaged sites whereas other sites which do not repress expression are passive sites. The dynamic gene regulation mediated by REV-ERBα is associated by recruitment of corepressor complex NCoR and HDAC3 to engaged sites resulting in tightening of chromatin at ZT10. Interestingly genetic depletion of REV-ERBα showed increased EP interaction at ZT-

Table 3
Circadian 3D nuclear epigenome and their associated physiological outputs.

S. no	Tissue/model	Methods used	Associated functions/summary	Reference
1.	MEF cells	4C Seq and 3C	Long range interactome of CCG *Dbp* absent in MEF *Bmal-1* knockout	[10]
2.	Mouse liver	GRO Seq and ChIP Seq	Detection of eRNA from different enhancer regions which regulate many phases of circadian rhythmicity	[129]
3.	hESC	4C Seq and DNA/RNA FISH	Chromosomal recruitment towards nuclear lamina during repression phases mediated by PARP-1 and CTCF	[144]
4.	Mouse liver	4C Seq and ChIP Seq	Long range chromatin looping involves CTCF mediated insulation of circadian phase and non-CTCF-Cohesin mediated high rhythms	[146]
5.	Mouse liver and lungs	4C Seq and ChIP Seq	Deletion of intergenic enhancer nearby *Cry1* locus perturbs daily rhythms of *Cry1* burst frequency due to decreased DNA contacts of enhancer and *Cry1* promoter which displays shortening of locomotor activity period	[139]
6.	Mouse liver	Hi-C	Revealed dynamic circadian Sub-TADs regions. REV-ERBα modulates chromatin architecture to suffice repressive arm to function as controlling mechanisms of circadian gene expression	[145]
7.	Multiple bioinformatics data	Meta-analysis	CLOCK-BMAL-1 modulates chromatin landscapes and shapes permissive chromatin environment for circadian gene expression	[151]

4C, Circular Chromosome Conformation Capture; 3C, Chromosome Conformation Capture; GRO Seq, Global Run On Assay; Hi-C, High Resolution Chromosome Conformation Capture; hESC, Human Embryonic Stem Cells.

10 also within ZT22 sub-TAD indicates the role of REV-ERBα in circadian phasic regulation. Additionally consistent with circadian enhancer marks (H3K27ac) accompanied by circadian eRNA synthesis from these enhancer sites as compared to passive sites and was found to be abolished also in absence REV-ERBα [145], thereby providing evidence of REV-ERBα requirement and binding on engaged site. On top of that, we have incorporated few more of such circadian 3D changes governing the physiological outputs as a whole through circadian core components (Table 3). Table 3 depicts the summarized table containing recent advancement in the circadian 3D genome with state of art techniques used in detecting the chromatin interactions. Use of high end techniques defines the reliability and detection limit of the technique. Tissue/Model depicts the model system used for that particular study.

Owing to co-regulation of thousands of genes from every corner of nucleus, the circadian 3D genome undergoes remarkable folding and architectural changes throughout 24 h. This helps the nucleus to govern the robust regulation of CCGs at a specific time of day, when it is programmed to be needed by the cell with an increasing understanding on how this dynamic folding drives rhythmicity and impart controlled regulation of only clock genes and CCGs during a short period of time. Thus, circadian 3D genome could be useful as a tool to understand the mechanistic of folding or shaping, in different diseases or in cellular differentiation, in which this type of changes remains steady or occur less frequently.

7. Conclusion

Epigenetic regulation of circadian rhythms plays a decisive role in generating robust cyclic changes in clock and CCGs expression profile. However, reciprocal interaction of clock gene protein products and chromatin modifiers illustrates widespread involvement of the biological clock and rigidity of the regulation (Fig. 2). Recent studies in circadian rhythm developed an understanding of new avenue of regulatory prospects of clock genes and different order of CCGs expression, perturbation in which may instigate into several diseases and disease phenotype. On numerous occasions clock genes control the gene regulation of CCGs of uncountable metabolic pathways through the involvement of chromatin modifiers, hence studying the relationship between them is of utmost importance. Alteration in methylation pattern of clock gene has been designated in many hematologic malignancies and many other cancer phenotypes such as endometrial cancer, breast cancer *etc.* which opens a new version of chronotherapy in chemotherapeutic interventions. Divergence in numerous daily habits such as sleep reduction, night time eating and intense light illumination in night lead to chronodisruption which could prime variation in clock gene methylation and vice versa ultimately lead to obesity and metabolic syndrome. Besides altered DNA methylation has also been observed in CpG sites of enumerable genes of neuritogenesis, synaptic plasticity and neurotransmission which is further supported by the evidence of arrhythmic DNA methylation in aged and Alzheimer's patients' brain samples. Nonetheless, HDAC, SIRT1, and miR-132 are also associated with aging, neurodegeneration, synaptic plasticity and memory formation. Another Micro-RNA miR-21, a CCG, which is driven by *Per2*, show significant impact in reducing inflammation, apoptosis and increasing energy metabolism in cardiomyocytes and found to be a cardioprotectant. Therefore, studying this interesting regulatory axis of biological clock and epigenetics could help biologists in inventing biomarkers of metabolic syndromes, cancer, cardiomyopathy, and neuropsychiatric disorders and so forth. More importantly, epigenetic regulation of biological clock is environmentally influenced and show observable behavioral phenotypes hence it could also be used as a best model to study gene and environment interactions.

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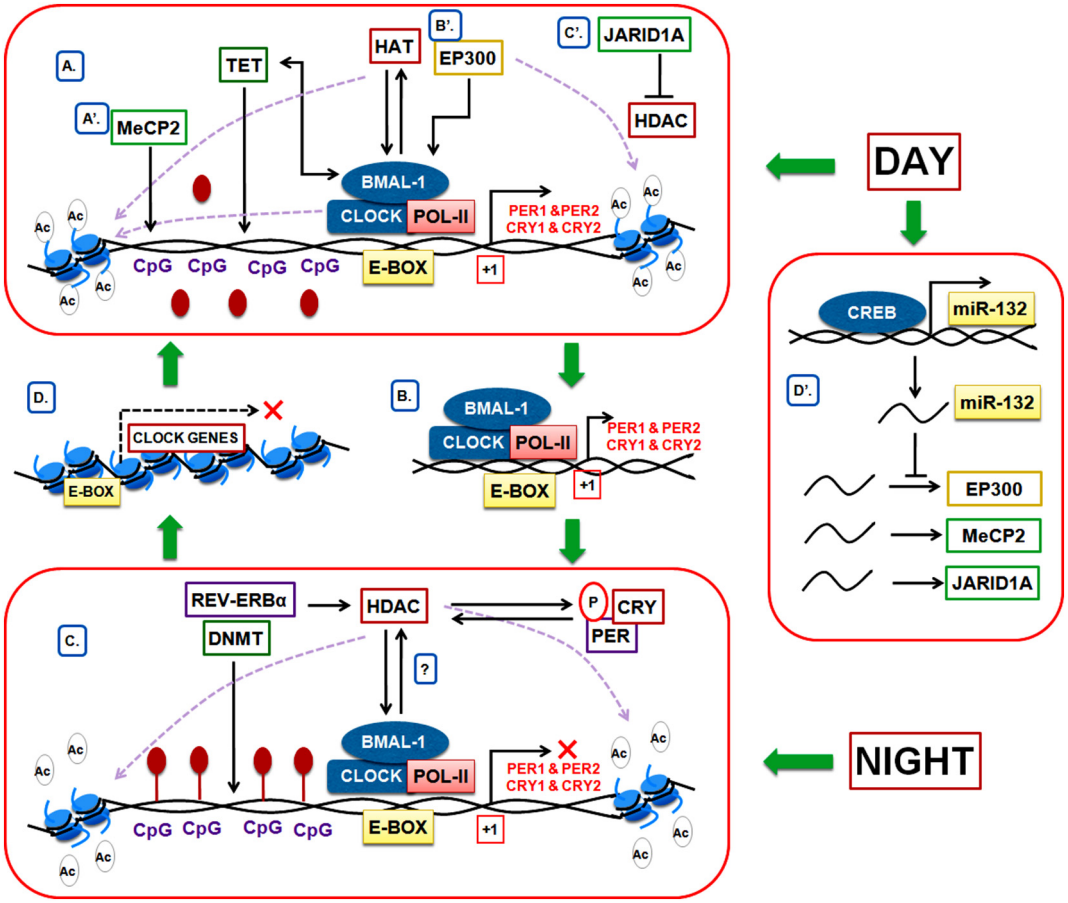

Fig. 2. Proposed model on the involvement of epigenetic regulation in circadian rhythm. (A and B) During subjective day CLOCK-BMAL-1 complex binds to the promoter of clock genes by opening the chromatin through interacting with HAT or by intrinsic HAT activity of CLOCK. Additionally TET demethylates the CpG islands at promoters; (A′, B′ and C′) Depicts additional chromatin modifiers recruited to open the chromatin; (C and D) By the end of the cycle the TTFL loop shuts down by darting in of HDAC and Co-repressor by REV-ERBα and PER/CRY complex to close the chromatin and simultaneous action of DNMT which methylates the promoter to follow the repression phase. HDACs may also be interacting with CLOCK-BMAL-1 to influence the gene expression (D′) miR-132 regulates the period length by controlling *Per2* expression at transcription level through regulating chromatin modifiers such as JARID1A, EP300, and MeCP2. miR-132 also controls proteins BTG2A and PIAP2A and are responsible for maintaining PER2 protein levels in the cytoplasm (Not depicted here). HDAC, histone deacetylase; DNMT, DNA methyltransferase; HAT, histone acetyl transferase; CLOCK, circadian locomotor output cycles kaput; JARID1, jumonji, AT-rich interactive domain 1; CRY, cryptochrome; PER, period; CREB, cAMP response elements binding protein; MeCP2, methyl-CpG-binding protein 2; BMAL-1, brain and muscle Arnt-like protein-1; TET, ten eleven translocation; REV-ERBα, nuclear receptor.

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Conflict of interest

The authors declare no conflict of interest in publishing this work.

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Glossary of terms used in circadian biology

Zeitgeber: It is an external signal helps to synchronize the circadian rhythms according to outside environment such as light dark cycle, food, temperature
Zeitgeber time (ZT): It is refereed as time (in hours) after light on in laboratory conditions having 12 h light and 12 hr dark cycle. Where ZT0 means light on and ZT12 is light off
Circadian time (CT): It refers to time in absence of light. Light is off from CT0 onwards
Period: Time required to complete the cycle
Subjective time: Subjective day means light phase and Subjective night meaning dark phase for animal
Circadian phase: An event functions or occurs at distinct stage of circadian rhythm taking place at 24 h interval