Perfect timing: Epigenetic regulation of the circadian clock

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A B S T R A C T

In mammals, higher order chromatin structures are critical for downsizing the genome (packaging) so that the nucleus can be small. The adjustable density of chromatin also regulates gene expression, thus this post-genetic molecular mechanism is one of the routes by which phenotype is shaped. Phenotypes that arise without a concomitant mutation of the underlying genome are termed epigenetic phenomena. Here we discuss epigenetic phenomena from histone and DNA modification as it pertains to the dynamic regulatory processes of the circadian clock. Epigenetic phenomena certainly explain some regulatory aspects of the mammalian circadian oscillator.

1. Some basic considerations concerning epigenetics and the circadian clock

Genetic information is encoded in a relatively simple fashion. An average protein of between 50 and 100 kDa requires between 1.35 and 2.7×10^3 nucleotides (nt). If we accept current estimates of circa 30,000 genes in the mammalian genome, then approximately 5×10^7 nt could represent real protein coding information. This is only about 1–2 percent of the entire amount of DNA present in a cell. This observation invites two simple hypotheses: the extra DNA must be somehow used for shaping the expression of the genome (else it would not survive evolution since so much extra-genic DNA is energy-expensive to build and maintain) and the vast amount of DNA necessitates an efficient packaging mechanism for considerations of both size and organization. The latter point is facilitated by the formation of nucleosomes, which are DNA wrapped around histone octamers [1]. These can then form higher order structures enabling further compaction of DNA [2–4]. Most of the mammalian genome is packaged as heterochromatin, which is very dense, but regions that are transcriptionally active are less dense and are referred to as euchromatin. As a simple correlation, the tighter the packaging of the local chromatin structure, the less probable it is that transcription and gene expression will occur.

The information on the local chromatin structure can be inherited to the next generation of cells. Thus, the local chromatin structure can exert epigenetic regulation, i.e., it modifies gene expression and consequently specific phenotypes in a heritable fashion without mutation of the DNA sequence. In mammals, the information reflecting the local chromatin structure is embedded in at least two distinct ways: (i) methylation of specific residues of the local histones [3,5], which are evenly distributed to the newly synthesized DNA during DNA replication, or (ii) methylation of specific cytosine residues in the DNA whose patterns can be enzymatically copied [6,7]. Hence, both kinds of marks can be easily copied to the newly synthesized DNA strands and transferred as such to subsequent generations. Note that temporal regulation of epigenetic modifications was initially not taken into consideration, as these marks were considered highly stable, even over generations. This time scale is obviously not relevant for dynamic processes such as the circadian clock.

Research into the molecular mechanism of circadian oscillators generally focuses on complex genetic networks based on transcriptional/post-translational feedback loops [8,9]. In essence, a gene product accumulates to a concentration upon which it represses its own transcription directly or indirectly via other transcriptional regulators. Consequently, the protein concentration is declining and a following cycle occurs about a day later. Combining these dynamic regulatory systems together with static, long-term epigenetic phenomena is on the first glance counterintuitive. However, recent research suggests that histone and DNA methylation are much more dynamic than previously thought. For instance, over a hundred DNA methylation sites oscillate in synchrony with the cell cycle [10] and the cell cycle is often gated by the circadian clock [11–14], an observation that brings epigenetic regulation into the range of circadian timing. Hence, these processes might play a

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role within the mammalian circadian oscillator as well. They may be fundamental to phenomena like hysteresis of repression at the regulatory regions of circadian target genes [15], tissue-specific gene expression, or passing of oscillator information to daughter cells during mitosis [14].

2. The histone code of repression: reversible methylation?

Nucleosomes represent the smallest unit of DNA compaction. There exist about 3×10^7 nucleosomes in the mammalian nucleus. They consist of a histone octamer, which is surrounded by about 1.67 loops of DNA. The 'DNA content' of a nucleosome is about 147 base pairs. Due to the specific surface properties of the histone octamer, DNA is relatively free to move around it [16]. In addition, about 3/4 of the DNA strand is accessible e.g., to transcription factors. Surprisingly, although they are bound to DNA, nucleosomes are not barriers to the general processes of DNA replication or transcription. During replication, the histones H3 and H4 are evenly distributed to the new DNA strands [17] and during transcription the DNA can loop out from the nucleosome to allow access to for instance RNA-polymerases [18].

However, nucleosomes can also exert regulatory effects on transcription and therefore gene expression [19,20]. This function is mediated by the amino-termini of histones, which protrude and are quite flexible [21]. Many of the amino acid residues in these histone tails are prone to post-translational modification. In general, acetylation of lysine residues or phosphorylation of serine residues is associated with an open chromatin configuration, which facilitates transcription. However, the precise mode of action of the modified histone tails is not known yet. Alternative hypotheses are that the chromatin configuration is either directly affected by the negative charges introduced into the histone tails via changing histone–histone interactions, or because specific proteins recognize and interact with these modifications.

A higher nucleosome density, depletion of acetylation and phosphorylation, or the presence of mono-, di- or tri-methylated lysine residues in the histone tails reflect a repressive transcriptional mode [5]. The latter modifications are specific binding sites for e.g., heterochromatin proteins (Hp) [22,23] or polycomb group (PcG) proteins [24], which assemble the chromatin even tighter. The number and kind of histone modifications on the histone tails manifests the histone code. Specific binding proteins can interpret this histone code as markers for transcriptional activation or repression. Since, as already mentioned, nucleosomes and consequently the histone modifications stay attached to the DNA even during DNA replication, the modification state of nucleosomes can be transferred to the subsequent generations of a cell.

A first hint concerning the involvement of histone modifications in circadian timing was found at the *Period1* (*Per1*) and *Period2* (*Per2*) gene promoters [25]. Activation of transcription at these promoters is mediated by BMAL1 and CLOCK heterodimers [8,9]. PER1 and PER2 participate in the so-called negative limb of the mammalian circadian oscillator where they repress BMAL1- and CLOCK-mediated transcriptional activation thus creating a feedback loop. In this manner, PER1 and PER2 are important to establish the typical *circa* 24-h oscillations of this molecular oscillator. Rhythmic acetylation of lysine 9 of histone H3 at the *Period1* (*Per1*) and *Period2* (*Per2*) gene promoters is observed with the phase of histone acetylation coinciding with the phase of transcriptional activity of both genes [25]. Interestingly, one of the transcriptional activators, CLOCK, interacts with the histone acetyltransferase (HAT) p300, which specifically adds acetyl residues to the lysine 9 of histone H3. The p300 enzyme increases the transcriptional read-out of a luciferase reporter under the control of the *mPer1* promoter [25]. These data suggest that time-of-day specific recruitment of HAT

activity leads to rhythmic histone acetylation. Similar experiments showed that CLOCK and also NPAS2 recruit various histone acetyltransferases to the *mPer1* promoter in vascular tissues [26].

We now know that CLOCK itself possesses HAT activity [27]. Hence, rhythmic binding of the transcriptional activators BMAL1 and CLOCK can directly affect the acetylation of specific lysine residues of histones nearby their DNA binding sites without the help of other HAT enzymes. In addition, the HAT activity of CLOCK also acetylates its heterodimerization partner BMAL1 [28]. The specific acetylated lysine residue of BMAL1, however, is involved in repression rather than activation. Hence, the definitive impact of the HAT activity of CLOCK on the circadian oscillator is still an unanswered question. Nevertheless, rhythmic acetylation of histones – regardless of the details – is broadly occurring in circadian clocks.

A rhythmic counterpart to HAT activity on circadian genes was identified [29,30] in SIRT1, a histone deacetylase. This enzyme apparently removes acetyl residues not only from the histone tails, effectively decreasing transcriptional activation, but also from the PER2 [29] and BMAL1 protein [30], affecting the activity of these proteins. Furthermore, this enzymatic activity is modulated by the intracellular ratio of NAD^+ to $NADH/H^+$, providing a link between the metabolic state of the cell and the circadian clock. Histone deacetylation is also recruited to the regulatory clock network by the interaction of the Cryptochrome1 (CRY1) protein with various histone deacetylases (HDACs) [31], or by the interaction of the circadian nuclear receptor REV-ERBα with NCoR1 to recruit HDAC3 [32]. Taken together, histone acetylation and deacetylation are part of the regulatory processes that generate circadian oscillations in gene expression. From a formal point of view, histone acetylation and deacetylation solely correlate with the state of transcription and are hence too transient to represent epigenetic phenomena.

Going more into the direction of epigenetic gene regulation, PER1 and PER2 associate with WDR40 [33], an adaptor targeting lysine 4 of histone H3 in conjunction with the histone H3-specific methyltransferase Set1/Ash2. A knock down of WDR40 in fibroblast cell lines leads to a loss of rhythmic histone methylation and alterations in rhythmic gene expression. In particular, the magnitude of *mPer2* expression is reduced. Overall, however, the impact of a knock down of WDR40 is modest with respect to the functioning of the circadian oscillator. An explanation for this discrepancy may be that circadian oscillators are largely resilient to fluctuations of transcription rate [34]. Differences in the level of gene expression may therefore be compensated at the post-translational level.

Additional histone methyltransferases are associated with circadian regulation. The Enhancer of Zeste 2 (EZH2), a histone H3-specific methyltransferase that tags the lysine 27, co-immunoprecipitates with either CLOCK or BMAL1 [35]. A rhythmic pattern of di-/tri-methylation of this residue occurs at the promoter of the *mPer1* but not the *mPer2* gene and occurs in anti-phase to transcriptional activity of this gene. In co-transfection assays, EZH2 enzymatic activity enhances (overexpression) or decreases (diminished by RNAi) the repressing activity of CRY proteins on a *Per1*-luc reporter gene in fibroblast cells. Thus, the role of methylation of lysine 27 of histone H3 was shown to affect expression of the *Per1* gene. Similarly, recent findings suggest that the methyltransferase myeloid/lymphoid or Mixed-Leukemia Lineage (MLL) rather than Set1A mediates the methylation of histone H3 lysine 4 to regulate BMAL1 and CLOCK target genes [36].

The clock-controlled *albumin D-site binding protein* (*Dbp*) gene was established as a model for the analysis of rhythmic histone modifications [37]. Highly dynamic, reversible histone methylations occur at this gene (Fig. 1). Methylation of lysine 4 and acetylation of lysine 9 of histone H3 are in phase with transcriptional activity of the *Dbp* gene. These histone modifications are observed
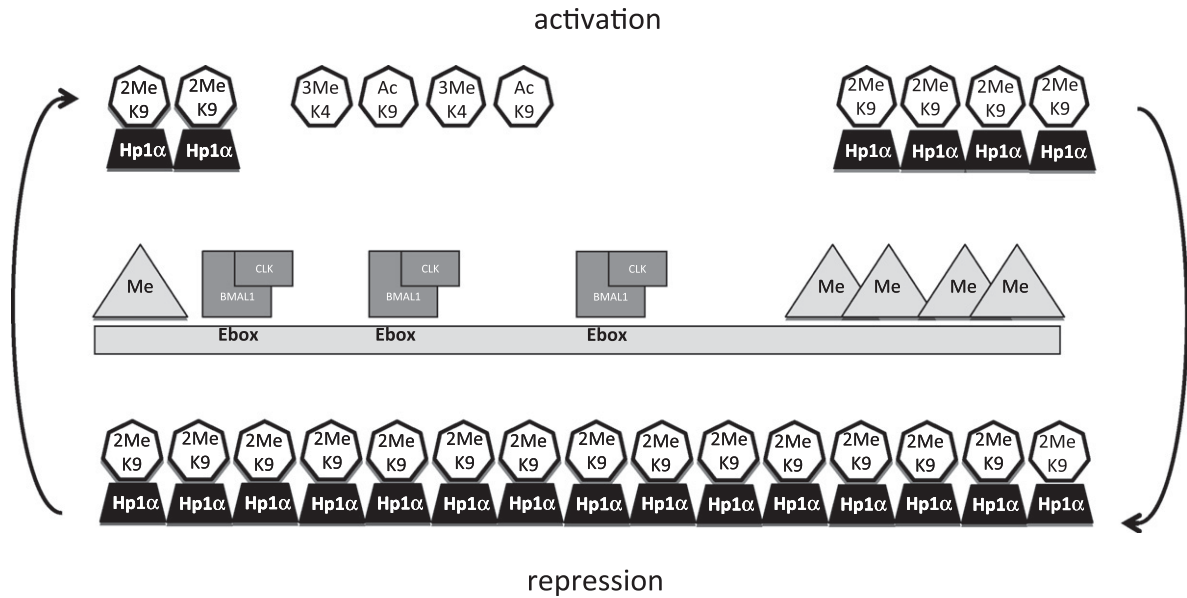
activation

repression

Fig. 1. Circadian regulation of the mouse *Dbp* gene. Upon binding of the two transcriptional activators BMAL1 and CLOCK, the local chromatin structure opens to allow transcription. This is accompanied by an increase in the typical markers for active chromatin, tri-methylation of lysine 4 and acetylation of lysine 9 of histone H3 around the promoter site (7 sided 'pac-man' units). Eventually, both transcriptional activators fall off their relevant binding sites and acetylation of histones is lost. This effect may be initiated by the recruitment of the SIRT1 histone deacetylase or related enzymatic activities. Finally, the chromatin starts to compact. Markers like di-methylation of lysine 9 of the histone H3 increase at the promoter region. This allows the spreading of HP1α□□□□□□*Dbp* gene, which may cause further chromatin compaction. Consequently, a heterochromatin-like state is generated to maintain repression (hysteresis) until a next cycle of transcriptional activation occurs on the following day. Note that stable methylated CpG sites (triangles) flank the *Dbp* gene, which may represent anchor points for surrounding heterochromatin. Adapted from [37].

prominently at the promoter region and rapidly decrease in 5′ to 3′ direction into the *Dbp* gene. This suggests that both types of modifications are performed by enzymatic activities associated with RNA-polymerase II and that these enzymatic activities dissociate from the elongating RNA-polymerase II during RNA synthesis [38].

At the nadir of *Dbp* gene expression, the picture is completely changed [37]. The above-mentioned histone modifications are strongly reduced and another modification, typical for a repressed state, increases throughout the *Dbp* gene. A di-methylated lysine 9 of histone H3 – the same residue that is acetylated during activation – becomes a binding site for Heterochromatin protein 1α (Hp1α) [22,23]. Consequently, this protein is found during repression all over the *Dbp* gene. In addition, the histone density, as judged by the presence of histone H3, increases. Altogether, the chromatin at the *Dbp* gene during repression resembles a heterochromatin-like state. Although these features have as yet only been analyzed for the mouse *Dbp* gene, it is tempting to speculate that reversible histone methylation would be part of many circadian regulatory regions.

What is the advantage of reversible histone methylation? The circadian transcriptional activators BMAL1 and CLOCK, and the transcriptional repressors PER1 and PER2 bind only temporarily to the relevant regulatory regions within their target genes. Recent evidence from the *Drosophila melanogaster* circadian oscillator suggests that the related Per protein is detached together with the analogous activating clock/cycle complex from the DNA [39]. From the kinetics of PER1 and PER2 binding relative to BMAL1-binding, a similar mechanism may be operative in mammals as well [40]. As a consequence, there must be mechanisms that continue the repressive state after the repressors have left the DNA. This concept is referred to as hysteresis [15]. The lysine 9 of histone H3-specific methylation and the binding of Hp1α signal a repressive state even in the absence of circadian repressors. Thus, they maintain the repressive state initiated by transcriptional repressors until a new cycle begins. However, to function properly, all of the histone modifications have to be temporally coordinated (Fig. 2).

3. Marks on DNA: reversible DNA methylation?

A very general epigenetic mechanism to inactivate genes is the methylation of cytosines, usually in the context of the sequence motif CpG [6,7]. There are about 5×10^7 potential CpG sites in the genome. Methylation of these sites either directly interferes with the binding of transcriptional regulators, or indirectly inactivates a gene by initiating the formation of a heterochromatic state. CpG sites within the genomes of mammals are drastically under-represented (about 20–25% of their theoretical abundance), and the remaining sites are enriched in functional subunits of the genome (e.g., CpG islands). A recent bioinformatics approach found that the regulatory regions of circadian genes are enriched in CpG motifs relative to other genes [41]. Therefore, an impact of CpG methylation on circadian gene regulation is conceivable.

In mammals, CpG methylation is performed by three different DNA methyltransferases (DNMTs). The isoforms DNMT3a and DNMT3b perform the de novo methylation of CpG residues during development, while DNMT1 is supposed to act as a maintenance methyltransferase that is responsible for copying the methylation pattern to the newly synthesized DNA strand during DNA replication [7]. Methylation of CpG residues provides binding sites for Methyl-CpG-binding proteins (MBPs). Of particular interest are the Methyl-CpG-binding proteins MBP1, MBP2, and MBP3 that have been shown to act as transcriptional repressors by serving as anchor points to recruit components of the repression machinery to a defined locus [42–44]. This machinery, which consists of histone deacetylases and methyltransferases, compacts the DNA around the CpG site into stable heterochromatin [45].

The CpG methylation status of a cell is epigenetically inherited over many generations. In non-proliferating adult tissues, DNA methylation is considered even more stable than histone methylation patterns in the absence of DNA replication. However, DNA demethylation has been demonstrated to occur in somatic cell nuclei [46] and according to cell cycle stage at some sites [10]. In addition, a publication describes diurnal changes of CpG methylation in
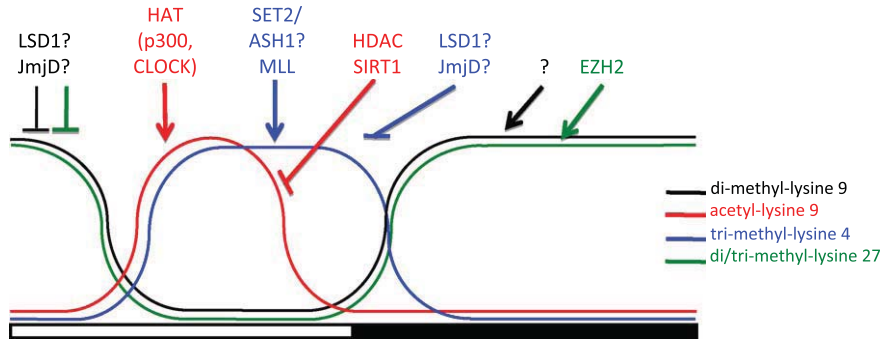
Fig. 2. Model of dynamic chromatin transitions at circadian genes over the 24 h day (day, open bar at bottom; night, closed bar at bottom). Activation of BMAL1 and CLOCK regulated genes coincides with acetylation of histone residues by HATs, and tri-methylation of lysine 4 of histone H3 by Set2/Ash1 (via a WDR5 adapter) or MLL, and the removal of repressive histone methylations by LSD1 or JmjD-domain proteins. These activities are counterbalanced by the recruitment of HDACs or SIRT1, and LSD1 and JmjD-domain proteins, respectively. Similar scenarios may occur at RORα/REV-ERBα and DBP regulated circadian target genes as well. Indeed, REV-ERBα was recently identify as the main anchor point for HDAC3 [64].

circulating white blood cells [47]. The authors report a nadir of homocysteine, a degradation product of S-adenosylmethionine, which is the donor of the methyl group transferred to the DNA, and a peak of DNA methylation in the evening in the blood of healthy subjects. The low amplitude of oscillation, which would affect only about 5% of the total CpG methylation sites, still translates into a huge number of CpG sites (2.5×10^6 potential sites). However, to date there is no single CpG motif known in a mammalian genome that shows circadian oscillations in methylation.

A defined role for CpG methylation of circadian genes may occur during development. A recent study compared CpG methylation in the promoter regions of the *mPer1, mPer2* and *mCry1* genes. At certain positions within the regulatory region of the *mPer1* gene the CpG methylation changes dependent on the developmental state [48]. Interestingly, in vitro methylation within the *mPer1* promoter has an inhibitory effect on reporter gene expression. This is a first experimental hint that CpG methylation could influence circadian rhythms. In addition, one of the strongest associations between

the circadian clock and DNA methylation concerns cancerous growth. The expression of many key circadian regulators (e.g., *Per1* and *Per2*) is lost during cancer development. In some cases, restoring expression of circadian clock components prevents malignant growth. CpG methylation is linked to this phenomenon because of a study that finds inactivation of *Per1, Per2* and *Cry1* in endometrial cancers by methylation [49]. Other studies describe hyper-methylation of *Per1* in cervical cancer cells [50] or non-small cell lung cancer [51], and of *Per3* in chronic myeloid leukemia cells [52], whereas hyper-methylation of sites within the *Clock* gene reflects a reduced risk of breast cancer development, possibly due to a reduction of the expression of the *Clock* gene [53].

Taken together, CpG methylation could exert a function within the mammalian circadian oscillator. Since CpG methylation is stable, may affect many potential regulatory sites and generates a tight repressive state, it is appealing to speculate that this mechanism provides tissue-specific gene regulation (Fig. 3). Mechanisms that provide reversible methylation of DNA remain to be discovered.
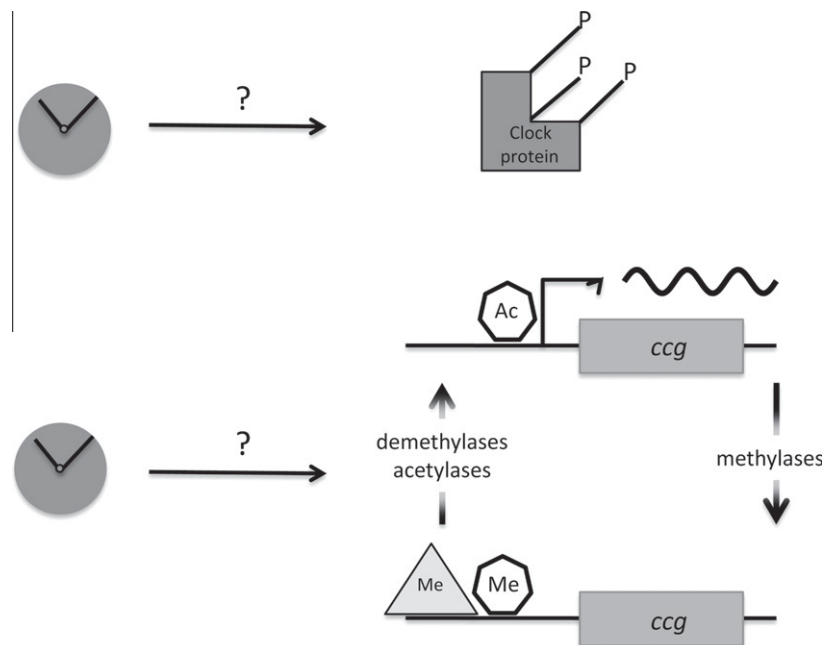

Fig. 3. Propagation of epigenetic information over cellular generations. To transmit temporal information to daughter cells, either circadian oscillator components bearing time-coding post-translational modifications (e.g., phosphorylation, P-P) are evenly distributed to the daughter cells, or methylation patterns (Me) are copied. Changes in the methylation patterns of DNA (triangles) or of the modifications (methylation, Me; acetylation, Ac) on histones allow tissue-specific activation or inactivation of circadian genes. Adapted from [6,7].

However, there is no doubt that CpG methylation impinges on the expression of circadian regulatory genes in cancer cells.

4. Perspectives

Until a couple of years ago, histone and DNA methylation were believed to be more or less irreversible. However, numerous enzymatic activities have been identified that remove methyl groups from e.g., specific histone residues. One of the first of these is the lysine specific demethylase 1 (LSD1) [54]. This protein possesses two opposing functions: depending on which complex it is associated with, it can either repress genes by removing the di-methylation of histone H3 lysine 4 or activate transcription by demethylating lysine 9 [55]. Via specific binding properties, it can be exquisitely targeted to specific genetic loci. Due to its bipartite function, LSD1 is an attractive candidate for a time of day dependent transcriptional regulator. Similarly, another demethylase family is the JmjD-domain containing proteins [56,57]. These demethylases remove tri-methyl groups from lysine residues. The function of these enzymes has been analyzed mainly during the early stages of development, with the JmjD2 sub-family involved in the demethylation of various lysine residues in undifferentiated and differentiated embryonic stem cells [58]. Concerning regulation of posterior development in animals, JMJD3 counterbalances the activity of PcG group proteins with respect to the state of lysine 27 of histone H3 [59]. It acts similarly during inflammation [60] and development [61]. Concerning circadian regulation, JMJD3 may, for instance, directly counteract the activity of EZH2, thereby leading to circadian changes in methylation status of the lysine 27 of histone H3 at the *mPer1* gene. To remove CpG methylation from DNA, Gadd45a has been identified [62]. However, it is currently unclear, whether this protein is a genuine enzyme to remove methyl groups from cytosine, or whether it is involved in a more general DNA repair mechanism. Interestingly, hydroxymethylation of cytosine bases is a quite abundant modification of DNA in the brain [63]. Hydroxymethylation may represent either an independent mark with yet to be specified function(s), or reflect a degradation intermediate of yet to be identified DNA demethylases.

Whether any of these demethylases are expressed in a circadian fashion or involved in circadian regulation remains to be investigated. The opportunities for precise and specific circadian regulation are manifold (Fig. 3). They may acquire specificity via their specific binding partners (e.g., clock transcription factors would bring them rhythmically to the proximity of clock gene promoters or other epigenetic regulatory enzymes) or by the timing of their expression, sub-cellular localization or post-translational modifications. Since many of these processes may be restricted to particular tissues or cell types, it is conceivable that these kinds of phenomena mediate tissue-specific gene expression.

An example of a situation where it would be an advantage to have a memory of circadian time encrypted as an epigenetic mark is cell division (Fig. 3). Experiments in tissue culture cells reveal that identical oscillator information is transferred to daughter cells. The mechanism behind this observation is unclear. Either the components of the circadian oscillator, whose concentrations and secondary modifications reflect circadian time, are evenly distributed to the daughter cells, or epigenetic marks fulfill this task. Clearly, further experiments are required to distinguish between these options.

Acknowledgements

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