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The Genetics of Mammalian Circadian Order and Disorder: Implications for Physiology and Disease

Joseph S. Takahashi[1,2,3,*], **Hee-Kyung Hong**[2,3], **Caroline H. Ko**[2,3], and **Erin L. McDearmon**[1,3]

[1]Howard Hughes Medical Institute, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3520

[2]Center for Functional Genomics, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3520

[3]Department of Neurobiology and Physiology, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3520

Abstract

Circadian cycles affect a variety of physiological processes, and disruptions of normal circadian biology therefore have the potential to influence a range of disease-related pathways. The genetic basis of circadian rhythms is well studied in model organisms and, more recently, studies of the genetic basis of circadian disorders has confirmed the conservation of key players in circadian biology from invertebrates to humans. In addition, important advances have been made in understanding how these molecules influence physiological functions in tissues throughout the body. Together, these studies set the scene for applying our knowledge of circadian biology to the understanding and treatment of a range of human diseases, including cancer, and metabolic and behavioural disorders.

Introduction

Circadian rhythms control a variety of biological processes in living systems, ranging from bacteria to humans [1, 2]. Perhaps the most obvious function regulated by circadian rhythms is the daily sleep and wake cycle in animals; however, many other physiological processes are regulated by circadian rhythms, including body temperature, feeding behavior, hormone secretion, drug and xenobiotic metabolism, glucose homeostasis and cell cycle progression. When circadian cycles are disrupted, either by genetic or environmental insults, disorders of diverse physiological processes can occur [3]. Links between circadian physiology and other physiological processes therefore have implications for human biology because it is likely that genetic variation in circadian clock genes can contribute significantly to physiological variation, and therefore potentially to variation in disease susceptibility. In addition, in modern societies, humans are increasingly ignoring natural circadian cues, so it is important to understand how the resulting perturbations of our circadian biology might affect our physiology and susceptibility to disease – both to understand the disease processes and to identify new targets for treatment.

In this Review, we bring together findings from recent studies that have begun to provide a molecular understanding of how circadian biology influences physiological processes that are relevant to human disease. We first briefly describe the generally accepted models of

[*]Correspondence: Joseph S. Takahashi, Howard Hughes Medical Institute, Dept. of Neurobiology and Physiology, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3520, 847 491-4605, j-takahashi@northwestern.edu.

how our circadian rhythms are regulated at the neural, genetic and molecular levels, an understanding that has come from extensive work in model organisms. We then highlight recent findings from genetic studies of human circadian disorders, which illustrate the striking conservation of clock gene function in model organisms and humans and highlight implications of human clock gene variation for human circadian biology. We will also discuss recent studies that have revealed a number of surprisingly direct molecular links with the cell cycle, metabolism and behavioral disorders, providing a basis for understanding how the circadian system impinges on diverse aspects of mammalian physiology of relevance for human diseases and their treatment that include regenerative medicine and cancer, obesity and diabetes, and mental health.

Neural Control of Mammalian Circadian Rhythms

The ability of living systems to sustain ~24 hour rhythms in the absence of environmental cues shows that most daily oscillations are not responses to the diurnal cycle, but rather are generated by an internal clock (Text Box 1). The 'master' internal clock in mammals is located in the hypothalamic suprachiasmatic nuclei, or SCN — bilateral nuclei comprised of about 10,000 neurons each[4, 5] (Figure 1). The primary environmental synchronizer of circadian rhythms in mammals is the daily light-dark cycle. A novel class of intrinsically photosensitive retinal ganglion cells that express the photopigment melanopsin integrates photic information for entrainment within the retina and projects to the core region of the SCN via the retinohypothalamic tract (RHT) [6, 7].

Text Box 1

Circadian Terminology and Activity Rhythms

In the natural environment, an organism's circadian system synchronizes a broad array of biological processes and behavior, such as sleep-wake cycle, periodic activity bouts, and blood hormone levels, to the prevailing light/dark cycle by phase advancing or phase delaying these daily rhythms. The time interval between phase reference points (e.g. two peaks) is called the *period*. Expression of endogenous circadian rhythm when subjects are isolated from the external cues, such as the light cycle, is described as *free running*. The timing of a reference point in the cycle (e.g. the peak) relative to a fixed event (e.g. beginning of the night phase) is known as the *phase*. The difference in the level between peak and trough values of the rhythm referred as *amplitude* (e.g. amount of activity or a circadian gene expression either in mRNA or protein levels).

To examine the effects of genetic disruption on behavioral circadian rhythms, long-term activity measurements are the most widely used method. When housed in a light:dark cycle, a normal mouse will exhibit nocturnal behavior by consolidating the majority of activity during the night, or dark, interval of the 24 hour day. When rodents are placed in constant darkness for extended periods of time, "free-running" circadian behavior persists and activity remains consolidated to the subjective night; however, the experimentally determined day length is usually different from 24 hours. For example, the internal clock in the inbred C57BL/6J strain of mice runs with an extremely precise free-running period of 23.6 hours. When individual genes involved in circadian rhythms are disrupted in mice, changes in free-running period, phase or amplitude of rhythms can be observed.

Both neuronal and humoral signals originating from the SCN regulate output pathways, which control diverse physiological functions. SCN neurons project to many regions (Figure 1), and these output pathways are responsible for proper timing of hormone release, feeding behavior and body temperature fluctuations [8]. In addition to these neuronal pathways, much

evidence points to SCN-secreted circulating factors in mediating physiological rhythms in both the brain and the periphery [4]. These factors are likely to include proteins such as TGFα [9], the cytokine CLC [10] and Prokineticin 2 [11–13].

Molecular components of the mammalian circadian clock

The circadian clock mechanism involves a cell autonomous transcription-translation feedback loop comprised of a core set of genes that are highly conserved among animals [1, 2]. In mammals, the circadian clock is composed of a primary negative feedback loop involving the genes, *Clock* and its paralog *Npas2, Bmal1, Period1 (Per1), Per2, Cryptochrome1 (Cry1)* and *Cry2* (Figure 2). During the day, the basic helix-loop-helix (bHLH) PAS-domain containing transcription factors CLOCK/NPAS2 interact with BMAL1 to activate transcription of the *Per* and *Cry* genes, resulting in high levels of these transcripts. The resulting PER and CRY proteins heterodimerize, translocate to the nucleus and interact with the CLOCK:BMAL1 complex to inhibit their own transcription [14]. During the night, the PER-CRY repressor complex is degraded, and CLOCK:BMAL1 can then activate a new cycle of transcription. The entire cycle takes approximately 24 hours to complete; however, little is known about the stoichiometry and kinetics of this feedback loop. In addition to the primary feedback loop, there is a second negative feedback loop involving the nuclear hormone receptor, *Rev-erbα*, which is a direct target of CLOCK:BMAL1 and which strongly represses *Bmal1* transcription [15, 16]. This secondary loop is not essential, but is thought to add robustness to the molecular clock. Finally, there are a number of other candidate clock components such as *Timeless, Dec1, Dec2* and *E4bp4* (reviewed in [2]), whose roles remain to be more clearly defined.

Recent work shows that post-translational modification and degradation of circadian clock proteins are critical steps for determining circadian periodicity of the clock [14, 17]. As seen previously in Drosophila, mammalian PER1 and PER2 proteins are progressively phosphorylated as they accumulate during the late afternoon and night. Key kinases involved in PER (and CRY) phosphorylation are Casein kinase 1δ and 1ε. The hamster *tau* mutant has a short 20-hour circadian period, which is caused by an A178C missense mutation in *CK1ε* leading to a dominant negative mutant allele [18]; this and further studies of this mutant have revealed a key role for CK1ε and protein phosphorylation in regulating circadian period in mammals [19]. One of the roles for phosphorylation of clock proteins is to target them for polyubiquitination and degradation by the 26S proteosomal pathway. *In vitro* studies have implicated both β-TCRP1 and FBXL3 E3 ubiquitin ligase complexes in directly targeting the PER and CRY proteins, respectively, for degradation [20–24]. Two recent studies have demonstrated that mutations in the *Fbxl3* gene result in long period phenotypes in mice, due to a defect in targeting CRY1 and CRY2 for degradation [24, 25]. Thus, the turnover of PER and CRY, respectively, are specifically regulated by two different SCF E3 ubiquitin ligase complexes.

Recent work has shown that circadian transcription of CLOCK-BMAL1 target genes is accompanied by circadian changes in histone H3 acetylation and chromatin remodeling [26]. Circadian transcription of the mouse *Dbp* gene is accompanied by rhythmic binding of CLOCK:BMAL1 to E-box *cis*-regulatory elements, acetylation of Lys9 of histone H3, trimethylation of Lys4 of histone H3, and a reduction of histone density [27]. Interestingly, the CLOCK protein itself has been shown to possess histone acetyltransferase (HAT) activity [28], and can acetylate its own partner, BMAL1, on Lys537 [29]. The histone deacetylase (HDAC), SIRT1, interacts with CLOCK and can deacetylate Lys537 of BMAL1 as well as Lys9/Lys14 of histone H3 [30]. SIRT1 is expressed in a circadian manner and regulates circadian gene expression of *Bmal1, Rorγ, Per2* and *Cry1* via interaction with CLOCK:BMAL1 and deacetylation and degradation of PER2 [31]. Thus, chromatin

remodeling is intimately linked to CLOCK:BMAL1-driven transcription both within the core clock autoregulatory feedback loop as well as downstream target genes of the CLOCK:BMAL1 complex. Since SIRT1 is an NAD+-dependent HDAC, it will be interesting to see if this pathway provides a link between cellular metabolism and the circadian clock.

Genetics of human circadian disorders

Given the molecular insights of the circadian clock mechanism from model organisms, an obvious question concerns the conservation and possible role of clock genes in human biology. Perhaps the most obvious circadian rhythm in humans is the daily pattern of sleep and wakefulness [32, 33] (Text Box 2). According to the well-accepted model of sleep regulation, the timing of sleep and wakefulness is controlled by two processes: a homeostatic process that underlies the rise of sleep propensity during wakefulness and its dissipation during sleep, and a circadian process that determines the thresholds for switching between sleep and wake [34]. Among the human sleep disorders, a subset of insomnias has been clearly linked to circadian alterations in the timing of sleep. These sleep disorders are known as: advanced sleep phase syndrome, delayed sleep phase syndrome, non-24-hour sleep-wake syndrome, and irregular sleep-wake pattern [35]. In an effort to understand the genetic basis of human circadian sleep disorders, both genetic linkage and association studies have been undertaken.

Text Box 2

Phenotyping Methods to Ascertain Circadian Rhythm Defects in Humans

Individual daily rest-activity rhythm parameters in humans can be measured with relative ease by a wrist actigraph, a small watch-like monitor that detects movements, used in conjunction with a sleep log/diary recorded by the subject [131]. Other phenotypic measures have been valuable in identifying individuals with altered sleep-wake cycle. These include self-report questionnaires, such as the Horne-Östberg Morningness/Eveningness Questionnaire (MEQ) [132], designed to quantitatively associate individuals to morning type (M-type) or evening-type (E-type) tendency of preferred activity time. The Horne-Östberg MEQ score has been shown to correlate well with an individual's intrinsic circadian period [133] and has been shown to be effective in identifying individuals/families with extreme diurnal preference [36, 37]. Additional physiological measures such as core body temperature, plasma melatonin, and saliva dim-light melatonin-onset time measurements have proven useful in determining the phase of the circadian clock with respect to the rest-activity cycle. Another self-report questionnaire, the Munich ChronoType (MCTQ) has been recently developed to include additional parameters to refine the assessment of an individual's diurnal preference and, thus, facilitate evaluation of individual's genetic chronotype [32, 33].

Familial Advanced Sleep Phase Syndrome

To date, there is one clearly established Mendelian circadian rhythm disorder in humans, known as familial advanced sleep phase syndrome (FASPS) [36, 37]. FASPS is inherited in an autosomal dominant fashion and characterized by persistent 3–4 hour advanced sleep onsets and awakening times relative to conventional and desired times [36, 37]. When patients are allowed to choose their preferred sleep schedule, sleep quality and duration are normal for age and only under the constraints of a forced bedtime and wake-time schedule does one become aware of this disorder. Thus, FASPS is thought to result from an abnormal phase position of the circadian cycle relative to the desired sleep-wake schedule. FASPS was the

first disorder to link known core clock genes directly with human circadian sleep disorders (Supplementary Table 1). Using linkage analysis, Toh and colleagues found that a missense mutation (S662G) in human PER2 (hPER2) cosegregated with ASPS [38]. Interestingly, the S662G mutation occurs in a phosphorylation site within a casein kinase 1 (CK1)-binding domain of hPER2. Biochemical analysis revealed decreased phosphorylation by CK1ε of an hPER2 peptide containing this site. Based on these results from the S662G hPER2 mutation and from other results from the hamster *tau* mutation in *CK1ε*, it was thought that decreased phosphorylation of PER could stabilize it leading to a premature accumulation of PER and a shortening of period. However, a recent report by Xu et al. showed that transgenic mice expressing the S662G mutant hPER2 revealed an unexpected reduction of PER2 [39]. These authors propose that PER2 is normally a positive regulator of its own transcription and with the S662G mutation, the level of *hPER2* transcription is decreased because the level of PER2 is decreased by increased turnover of PER2 [39]. However, independent work by Vanselow and colleagues suggests that the turnover of PER2 is not significantly affected by the S662G mutation and that nuclear retention of PER2 is reduced by the FASPS mutation [40]. As alluded to earlier, the hamster *tau* mutation in CK1ε also does not lead to a stabilization of PER as initially thought, but rather leads to a destabilization of PER by enhancing the turnover and degradation of PER via the proteasome [41]. Thus, the molecular basis of this form of FASPS appears to be caused by an increased turnover of nuclear PER2 either by enhanced degradation or by reduced nuclear retention of PER.

Given that expression of daily circadian rhythms involves a repertoire of circadian clock genes, it is not surprising to observe genetic heterogeneity in FASPS. Several independent studies of FASPS have failed to demonstrate mutations in *PER2* [37, 42]. Consistent with these observations, Xu and colleagues have identified a mutation in casein kinase δ (CK1δ), a paralog of CK1ε, in FASPS using candidate gene approaches[43]. Interestingly, although a transgenic mouse model carrying the human mutation (T44A) transgene showed shortened circadian period as expected, a *Drosophila* model carrying the same mutant transgene showed lengthened circadian period, suggesting different regulatory mechanisms between these species. It will be of great interest to see whether additional mutations in core clock genes will be found to underlie the additional cases of FASPS. The fact that the first two cases of FASPS are caused by mutations in core circadian clock genes is remarkable and strongly suggests that the clock mechanism of humans is highly conserved with that seen in rodent animal models.

Delayed Sleep Phase Syndrome and circadian rhythm disorders with a complex genetic basis

In humans, delayed sleep phase syndrome (DSPS) represents the most common circadian rhythm sleep disorder in the general population [35]. DSPS is characterized by a chronic inability to fall asleep or awaken at the desired “normal” times of day; the average onset of sleep in DSPS individuals occurs in the early morning (0300 to 0600 h) and their usual rise time occurs in the late morning to early afternoon (1100 to 1400h). Though rare, familial cases of DSPS have been described, suggesting that Mendelian inheritance of DSPS may exist [44, 45]. Several association studies of DSPS with sequence variants of known circadian genes have been reported. Both significant association and lack of association with the T3111C polymorphism in the 3′-untranslated region of *CLOCK* have been reported for DSPS and for diurnal preference [45–48]. One study reported that an amino acid substitution (S408N) in the *CK1ε* gene, which is a putative phosphoacceptor site, may play a protective role in the development of DSPS as well as Non-24-hour sleep-wake syndrome (in which loss of circadian regulation of sleep occurs) [49]. A single nucleotide polymorphism in the 5′-untranslated region of PER2 has also been reported to be associated with morning

preferences [50]. In both these cases the functional consequences of these sequence variants have not been reported and thus we do not understand their mechanism.

One of the more interesting sequence variants is a variable-number tandem-repeat (VNTR) polymorphism in the *PER3* gene, which encodes 18 amino acids repeating either four times (PER3–4) or five times (PER3–5); this coding region is predicted to harbor potential CK1 phosphorylation sites (which have not been analyzed) [51]. The shorter VNTR allele has been shown to occur with higher frequency in a study of 48 Japanese DSPS patients [51]. In a sample from an English population, one study showed that homozygosity for the shorter repeat allele (PER3–4/4) was strongly associated with DSPS [51]. However, in contrast to this finding, a study in a Brazilian population found that the frequency of the longer allele was higher in DSPS patients [52]. It has been proposed that the differences in the latitude of the two cities, which considerably influences temperature, the length of days throughout the year, and solar intensity, may have a role in the influence of *PER3* polymorphisms in DSPS [52]. Replication of this finding in equivalent latitudes is necessary to determine whether these associations are robust and if so whether there will be significant gene-environmental interactions for circadian traits in humans as seen in Drosophila [53].

The PER3 VNTR polymorphism may also contribute to sleep homeostasis; differences in sleep-wake structure, sleep propensity, and cognitive performance during sleep loss were noted between individuals who are homozygous for the shorter or longer allele in the general population [54]. It is noteworthy that mouse models of other clock genes, such as *Clock*, *Cry1, Cry2*, *Npas2*, *Dbp* and *Bmal1,* also have alterations in sleep duration and/or homeostasis [55–59]. Thus, in addition to the known phenotypic links between the circadian system and sleep, there are many examples in which circadian clock genes can clearly affect both the circadian control of sleep as well as the homeostatic sleep system. Given the interdependent relationships between the circadian and sleep systems, it will be important in future work to determine whether sleep and circadian phenotypes can be clearly distinguished at the genetic level. That is, can we find genes that selectively regulate sleep homeostasis independently of circadian rhythms and vice versa? Perhaps the only known genes definitively associated with sleep independent of the circadian system thus far are those genes causing narcolepsy, in which the loss of hypocretin/orexin ligand or hypocretin/orexin receptor 2, cause a defect in sleep stage entry into REM (rapid eye movement) sleep [60, 61]. Inroads into the genetics of sleep are emerging in Drosophila [62, 63], but there is still a paucity of work on the genetics of sleep in mammalian animal models where a ripe opportunity for genetic analysis exists [64, 65].

In future work it will be important to integrate our knowledge of circadian mechanisms in humans so that the ‘chronotype’ of individuals can be more easily and reliably assessed [33] (Text Box 3). Because there is such wide diversity in diurnal preference among people and because our 24/7 society demands such temporal variability in schedules, it likely that the genetically programmed internal clock of humans will be in conflict (e.g., phase desynchrony) with modern society [33]. Such desynchrony with the external world and the consequential internal desynchrony of the circadian system can contribute significantly to both the morbidity as well as the well-being of human health [3, 66]. If the chronotype of an individual can be utilized to inform and optimize adaptation to the 24-hour society, then it is likely that an understanding of clock genetics in humans should have direct application: a clock gene allelic profile should be predictive of an individual’s genetically programmed clock, and this information could then be used to optimize an individual’s schedule and treatment regime for many disorders [66].

Text Box 3

In vitro methods to dissect human circadian rhythm disorders

A major impediment in the study of human circadian rhythms is the difficulty and expense of assessing circadian rhythms in people. Because circadian clocks must be studied in temporal isolation, human circadian studies have demanded strict requirements of isolating subjects from environmental cues under constant conditions for prolonged period of time [134, 135]. Unlike a mouse, it is difficult to convince a person to spend weeks living in temporal isolation. Thus, only a few investigators have had the resources and facilities necessary for such experiments. Therefore, surrogate measurements for assessing human circadian rhythms are essential. These have taken two forms: One has been to assess 'diurnal preference' or 'chronotype' of subjects using questionnaires, hormonal markers and actigraphy to measure phase of entrainment of circadian rhythms while living in the real world [32, 131, 132, 136]. A second approach has been to develop cell-based and molecular approaches to assess circadian rhythms in humans by biopsy or blood sample [137]. In animal models, precise period and phase estimates of circadian rhythms can be made by real-time measurements using circadian reporter genes in cultured cells [70, 71, 73]. In humans, the challenge is to develop methods that are effective and robust in defining inter-individual phenotypic differences, but also that would be sufficiently cost-effective to screen large number of subjects.

Recently, Brown and colleagues [137] have developed a skin biopsy/culture method to measure inter-individual differences in circadian rhythms. By introducing a stable lentiviral-luciferase circadian reporter into cultured human fibroblasts, the authors showed that inter-individual differences of circadian period length were much more variable than different biopsies of the same individual. The same reporter system was implemented using known mouse circadian mutants and showed that circadian period measurements from fibroblast cultures were correlated with the behavioral rhythms of circadian clock mutants [137]. More recently, Brown and colleagues have gone on to show that there are individual differences in circadian amplitude and phase-resetting responses in fibroblasts from human subjects that are correlated with their diurnal phase preferences [138]. Thus cell-based assessments of circadian rhythms in fibroblast biopsies appear to be an excellent surrogate method to assess circadian periodicity in humans. It is important to note, however, that a recent study that examined different *Per* and *Cry* mutant mice has shown that SCN neuronal coupling can compensate for cell autonomous genetic defects [139]. The authors show that while fibroblasts and isolated SCN neurons derived from *Per1−/−* and *Cry1−/−* mice exhibited defective *Per2::luciferase* expression, intact SCN explants from the same mice showed robust oscillation of *Per2::luciferase* expression. Therefore, in some cases, examination of primary fibroblasts from patients with suspected circadian disorders may overestimate the effect of genetic mutations on circadian rhythms at the behavioral level. Nonetheless, the technique should be valuable in identifying affected individuals and indeed may be a more sensitive indicator of genetic variants in human circadian disorders.

Peripheral clocks and tissue-specific control

With the initial discovery of circadian clock genes in mammals, it became clear that the expression of these genes was ubiquitous and that the majority of tissues throughout the body expressed circadian oscillations in the gene expression (Figure 3) [67–69]. Indeed it is now well established that virtually every tissue in mammals has the capacity for generating circadian oscillations [70, 71]. Using single-cell measurements of fibroblasts engineered with either circadian-driven bioluminescent or fluorescent reporter genes, cell-autonomous

circadian rhythms can be observed that persist with undiminished amplitude for many days in vitro [72, 73]. Interestingly, there is no evidence for coupling of these cell autonomous circadian oscillators in fibroblasts. The existence of self-sustained circadian oscillators in peripheral tissues and cells raises a number of organizational questions. What is the role of these peripheral oscillators and how are they coordinated in the intact organism? Evidence suggests that peripheral oscillators are normally entrained by the SCN pacemaker, but that they are only weakly coupled to the SCN. For example, experimentally inducing 'jet lag' in rodents by advancing or delaying the light cycle by 6 hours reveals that the SCN can reset rapidly to these shifts while at the same time resynchrony of peripheral oscillators can take over a week [70]. Proximal cues such as feeding can override phase control of peripheral oscillators by the SCN under conditions of restricted feeding [74, 75]. It is thought that circulating factors such as glucocorticoids and the circadian variation in body temperature may be acting as entraining signals for peripheral clocks [76, 77]. Thus peripheral clocks are controlled at multiple levels. They receive entraining cues centrally from the SCN, and they have their own local cell autonomous oscillators that can operate independently from the SCN.

The relative role of central vs. local control of circadian rhythms in peripheral tissues can be addressed by tissue-specific conditional regulation of circadian gene expression in transgenic mouse models. For the regulation of circadian behavioral rhythms, it is clear that central SCN control is sufficient to generate circadian rhythmicity. For example, in *Bmal1* null mutant mice, circadian rhythms of behavior are abolished [78]; however, transgenic expression of *Bmal1* in the brain and SCN is sufficient to rescue circadian rhythms of behavior [79]. Interestingly, other phenotypic effects of the *Bmal1* null mutation (activity levels, morbidity, body weight loss) cannot be rescued by expression of *Bmal1* in the brain, but can be rescued by transgenic expression in skeletal muscle [79]. These experiments illustrate that the circadian behavioral effects of the *Bmal1* mutation can be partitioned from other physiological effects by tissue-specific rescue and show that *Bmal1* influences many other functions beyond circadian rhythmicity. Whether these pleiotropic effects of *Bmal1* are a consequence of *Bmal1*'s peripheral circadian function or whether they reflect independent functions remains to be determined.

To address the role of local cell autonomous oscillators in the liver, Kornmann and colleagues [80] used conditional overexpression of *Rev-erbα* in the liver to knockdown *Bmal1* in hepatocytes while maintaining normal circadian function in the rest of the animal. Disruption of cell autonomous clocks in the liver led to the abolition of cycling of the majority of genes in vivo; however, a subset of genes including *Per2*, *Nocturnin* and members of the heat shock protein family continue to oscillate suggesting that these genes may be responding to rhythmic systemic cues generated by SCN-driven rhythmic behavior of the animal [80]. These experiments suggest that cell autonomous oscillators in the liver play a dominant role in the local control of circadian gene expression in these cells; however, systemic cues in vivo can still drive rhythms in some genes in hepatocytes presumably to entrain these peripheral oscillators.

Taken together, the discovery of cell autonomous circadian oscillators throughout the body of mammals has radically changed our view of the circadian system. Instead of a model in which a dominant pacemaker in the SCN alone generates and drives rhythms throughout the organism, we now appreciate that almost every cell and tissue in the body contains circadian oscillators and that there is a hierarchy of control in which the SCN acts as a coordinator or synchronizer by centrally regulating activity, feeding and body temperature rhythms, all of which can then influence the phase of cell autonomous oscillators in the periphery.

Interaction with physiological processes

In addition to their primary role in the generation of circadian rhythms, recent work has shown that circadian clock genes can affect a wide variety of other physiological processes (Supplementary Table 2) [81–83]. The circadian system regulates these processes in two major ways; 1) outputs from the circadian clock originating either centrally from the SCN or locally from cell autonomous oscillators regulate key components in physiological pathways in a rhythmic fashion to drive circadian rhythms in these pathways; and 2) the core circadian clock components themselves appear to be elements within cellular pathways not traditionally thought to be under circadian regulation and may reflect additional roles for these genes. Emerging examples of circadian regulation of physiological pathways include diverse aspects of cellular metabolism, cell growth and DNA damage control, xenobiotic responses, as well as, the modulation of behavioral responses to drugs and alcohol.

Circadian Clocks and Metabolism

Perhaps the most striking discovery to be made from circadian gene profiling experiments was the observation that a significant fraction of the transcriptome (3–10%) is under circadian regulation and that the majority of the pathways regulated by the clock are imbedded in fundamental metabolic pathways [67]. In the SCN, genes involved in protein biosynthesis and trafficking, including ribosomal synthesis, translation initiation, folding, targeting, post-translational modification, and transport, were under coordinated circadian control. In addition, genes involved in energy metabolism, the redox state of the cell, and cell signaling also showed circadian variation in their steady state message levels. In the liver, basic cellular pathways such as glycolysis, fatty acid metabolism, cholesterol biosynthesis, and xenobiotic and intermediate metabolism were under circadian regulation. Importantly, rate-limiting steps in these various pathways were key sites of circadian control, highlighting the fundamental role that circadian clocks play in cellular and organismal physiology. Recent gene expression analysis of the nuclear receptor family has shown that of the 49 nuclear receptors in mice, 28 of them display tissue-specific circadian rhythms in various tissues [84]. Given the diverse roles nuclear receptors play in regulating metabolism, the circadian control of nuclear receptor expression provides a key nodal point for regulation of the overall physiology of the organism. Although there has been evidence for circadian modulation of metabolism previously, the pervasiveness of circadian control at a global level was not appreciated. These observations coupled with the cell autonomy of circadian oscillators in the majority of cells in the body suggest that circadian regulation may be a fundamental housekeeping function of the cell.

In addition to the circadian control of metabolism, circadian mutant animal models have been found to have metabolic disorders (Supplementary Table 2). For example, *Clock*$^{\Delta 19}$ mutant mice are hyperphagic and obese, and not only lose rhythms of core clock genes, but also have lower levels of *hypocretin/orexin* and *ghrelin* neuropeptide gene expression in the arcuate nucleus [85]. As a result, *Clock*$^{\Delta 19}$ mice exhibit metabolic syndrome with hyperlipidemia, hepatic steatosis, hyperglycemia and hypoinsulinemia. A preliminary study in humans suggests that *CLOCK* polymorphisms may be associated with obesity and metabolic syndrome [86]. In addition, both *Clock*$^{\Delta 19}$ and *Bmal1*−/− mice are hypersensitive to insulin shock, suggesting a direct role for the molecular circadian clock in regulating glucose homeostasis [87]. In humans, *BMAL1* has been associated with susceptibility to hypertension and type 2 diabetes [88]. In addition, BMAL1 appears to be involved in the control of adipogenesis and lipid metabolism in adipocytes [89]. During adipose differentiation, *Bmal1* is strongly induced in 3T3-L1 cells and both gain-of-function and loss-of-function experiments support a critical role for *Bmal1* in this pathway. *Bmal1*−/− mice also exhibit numerous other phenotypes, including decreased adult body weight and activity levels, increased tendon calcification, increased sleep duration and fragmentation,

and sarcopenia [58, 78, 90, 91]. Some of these may reflect a general tendency to age quicker than wildtype mice [91], but also result from the loss of tissue-specific BMAL1 functions [79]. Additional abnormalities have been characterized in other circadian mutants, for example the CK1ε (*tau)* mutant hamster exhibits reduced growth rate and elevated metabolic rate [92, 93]. In addition, mice deficient in *Nocturnin*, a gene with robust circadian expression that encodes a polyA deadenylase, are resistant to diet-induced obesity [94]. Thus, in these genetic models, we see a more direct link between circadian clock genes and metabolism. Mutations in a number of core clock genes are also associated with metabolic defects. Whether these effects are caused by alterations in circadian rhythmicity per se, or whether they are due to transcriptional targets of CLOCK and BMAL1 that are independent of the circadian clock system remains to be determined.

In addition to the circadian control of metabolism, it is also becoming clear that metabolism itself can have effects on circadian clocks [82]. It has been known for some time that restricted feeding cycles can entrain circadian rhythms in rodents. Although the SCN is generally resistant to the entraining effects of restricted feeding, a food-entrainable oscillator (FEO) in the brain and circadian oscillators in the periphery are strongly reset by restricted feeding [74, 75]. While it has not been established that restricted feeding entrains these oscillators by metabolic pathways, an hypothetical mechanism for how metabolism could have direct access to the clock mechanism has been proposed by McKnight and colleagues [82]. For example, in biochemical experiments it can be shown that the reduced forms of the metabolic products NADH and NADPH modulate CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers and enhance their DNA binding, while the oxidized forms NAD and NADP diminish DNA binding [95]. In addition, the PAS domains of NPAS2 can bind heme, and carbon monoxide can regulate the DNA-binding of NPAS2:BMAL1 dimers [96]. Recent work also shows that heme is the ligand for the circadian nuclear receptor, *Rev-erbα* [97, 98]. To make things more complicated, heme biosynthesis is also under circadian regulation so these pathways appear to feedback on each other [99]. It will be important to determine whether these redox state and heme interactions are physiological and operative in vivo. If so, then fluctuations in cellular metabolism could directly influence the transcriptional activity of circadian clock components, which in turn regulate the expression of many metabolic pathways.

Finally, additional evidence for effects of metabolism on circadian rhythms are experiments showing that alteration of metabolic state by genetic or environmental means can alter circadian rhythms in animals. For example, disruption of *PGC1a*, a transcriptional co-activator involved in energy metabolism, is also associated with abnormal circadian rhythms of activity, temperature and metabolic rate in mice [100]. Interestingly, environmental modulators of metabolic state (high fat diet) can subtly influence circadian period and the expression of cycling genes in mice [101].

Taken together, it is clear that circadian clocks and metabolism are intimately linked and reflect adaptive responses of living systems to the cyclic environment (Figure 4). Because metabolic processes vary with the physiological demands of the organism and many of these physiological demands occur on a daily basis, it is not surprising that direct circadian coordination of metabolism might have adaptive significance. On the other hand, disruption of the optimal tuning of rhythmic metabolic pathways could then have suboptimal consequences and important implications for metabolic disorders.

Circadian Clocks and the Cell Cycle

As alluded to above, the cell cycle has been observed to be under circadian modulation in many organisms and the selective factor for such modulation may have been to ‘escape from light’ and restrict DNA replication to the night [102] (Figure 5). Recently, it has been observed

that some circadian clock mutants in mice can affect cell growth and proliferation, and in some cases can predispose mice to cancer. For example, in regenerating liver following partial hepatectomy in mice, Okamura and colleagues have shown that the circadian clock system controls cell-cycle related genes that modulate the activity of Cyclin B1-Cdc2 kinase, a key mitotic regulator [103]. *Cry*-deficient mice exhibit delayed rates of liver regeneration after partial hepatectomy and the expression profiles of *Cyclin D1* and *Wee1* are deregulated. Importantly, the transcription of *Wee1* is circadian and appears to be a direct target of the CLOCK:BMAL1 transcriptional complex. Thus, in regenerating liver the expression of *Wee1* is co-regulated with *Per1* and the entry of the cell cycle into M phase is suppressed during this time of day. This may provide a molecular mechanism coupling the circadian clock to the cell cycle. Similarly, the *Clock* mutant strongly affects the expression patterns of many cell-cycle related genes and leads to reduction in cell proliferation in *Clock* mutant fibroblasts [104].

In other work, Fu and Lee [105] have shown that *Per2* mutant mice are predisposed to cancer following γ radiation treatment and that the expression of *c-myc* and *Cyclin D1* are regulated by the CLOCK/NPAS2:BMAL1 pathway. The *Per2* pathway involving *c-myc* and *Cyclin D1* also appears to be operative in leptin-dependent osteoblast proliferation in bone [106]. The *Per1* gene also has been linked to cell growth and DNA damage control [107], and overexpression of *Per1* sensitized cancer cells to DNA damage-induced apoptosis and caused significant growth reduction. The PER1 protein interacted with the check-point proteins, ATM and Chk2 [107], while related work has linked the TIMELESS protein with Chk1 and ATR-ATRIP [108]. Unexpectedly, activation of the DNA damage pathway itself has been shown to be able to reset the phase of the circadian clock in cells and in vivo [109, 110]. Thus, the circadian clock not only regulates key cell cycle regulatory proteins, but can also be reset by DNA damaging agents. While these links between the circadian clock and the cell cycle occur at the molecular level, there is also evidence that abolition of circadian rhythms in the host by SCN lesions can accelerate the growth rates of implanted tumors [111]. Thus, the circadian gene pathway can both interact directly with cell cycle pathways and influence cell growth through physiological system level interactions (Figure 5).

Circadian Sensitivity to Chemotherapeutic Agents

It has been know for many decades that the morbidity and mortality (drug-induced toxicity) of anticancer agents vary strikingly with time of day [66]. Using the chemotherapeutic agent, cyclophosphamide, we have recently reinvestigated this phenomenon and found that a treatment that causes 20% mortality when administered at dusk (ZT10-ZT14) results in 100% mortality when administered at dawn (ZT22-ZT02) [112]. To test the role of clock genes in this response, we used mouse mutants that have disrupted circadian rhythms. Both the *Clock* and *Bmal1* mouse mutants were hypersensitive to cyclophosphamide treatment, while *Cry*-deficient mice were resistant to the drug's toxicity [112]. These unexpected results point to a CLOCK:BMAL1 target underlying the sensitivity to cyclophosphamide. Contrary to conventional wisdom (changes in pharmacokinetics with time of day), there were no significant differences in the metabolism and turnover of cyclophosphamide that could explain the dramatic differences in circadian sensitivity. Instead, evidence suggests that the pharmacodynamic effects of cyclophosphamide-induced toxicity are best correlated with CLOCK:BMAL1-dependent modulation of B cell survival. The elucidation of the molecular mechanisms underlying the circadian control of B cell survival will provide a rationale not only for adjusting the timing of chemotherapeutic treatment to be less toxic but also for providing a basis for a search for pharmacological modulators of drug toxicity acting through circadian system regulators. This result may significantly increase the therapeutic index and reduce morbidity associated with anticancer treatment.

Thus, circadian rhythms and core clock components play crucial roles in many aspects of cell cycle regulation and morbidity of chemotherapeutic agents. These results, combined with epidemiological studies examining the link between rotating shift work and cancer risk, suggest a significant association between circadian rhythm disruptions and abnormal cell growth [113–115]. While some human studies also have shown the beneficial effects of timed chemotherapy delivery on both drug effectiveness and side effects, translation to recommended clinical practices has not been generally adopted [116, 117]. Further evidence that timed drug administration is beneficial will be needed to initiate this course of therapy for cancer treatment as well as therapies for a variety of other disorders. Indeed recent work demonstrates that even hematopoietic stem cell release is regulated by the circadian clock and that the efficacy of stem cell transplantation can vary by as much as two- to three-fold depending on the time of day [118]. Thus, chemotherapy and stem cell transplantation are two important targets for circadian optimization in clinical treatments in the future.

Circadian rhythms and affective disorders

In addition to physiological effects, circadian mutants can also exhibit behavioral dysfunctions associated with alcohol consumption, addiction and human mood disorders. The *Per2* gene affects the glutamatergic system in mice and *Per2* mutations enhance alcohol consumption [119]. Interestingly, *Clock* $^{\Delta 19}$ mutant mice display an overall behavioral profile that is strikingly similar to the manic state in human bipolar patients, including hyperactivity in a novel environment, decreased sleep, lower risk aversion, lower anxiety and an increase in susceptibility to reward by cocaine and alcohol [120, 121]. In humans, mood disorders are often associated with sleep disturbances and it has been suggested that circadian rhythms may influence susceptibility to depression (reviewed in [122, 123]). In support of this, *NPAS2*, a *CLOCK* paralog, has been implicated in seasonal affective disorder [124]. One study showed suggestive evidence for association of *PER3* and *ARNTL* (human ortholog of mouse *Bmal1*) with bipolar disorder [125]; however, association studies with *CLOCK*, *CRY1* and *PER2* genes in affective or bipolar disorders have so far been negative [126–128]. Whether circadian clock genes contribute to pathogenesis in non-seasonal affective/mood disorders in humans remains to be further investigated in additional patient populations. Thus far all of the association studies performed on circadian genes have been performed on small samples and few have been replicated. Clearly these small-scale, underpowered, candidate-gene studies need to be replaced by unbiased genome-wide association studies that are sufficiently powered and replicated. Thus, much caution must be taken in interpreting the existing reports on association of circadian genes with phenotypes in humans.

Perspectives

The elucidation of the molecular mechanism of circadian clocks in model organisms has revealed striking conservation of genes regulating human circadian rhythms. Emerging genetic studies on human clock genes suggest that genetic variation in these genes may be associated with metabolic, sleep and mood disorders. Given the strong links between circadian components and diverse physiological processes in animal models, genetic variation in human clock genes could also contribute to phenotypic variation relevant to disease pathways such as metabolic and mood disorders. As such, it will be important in future studies to consider the impact of circadian variation on the design and assessment of human phenotypes whether normal or disease-related. In addition, the clear role for circadian genes in sleep disorders such as FASPS provides new opportunities for addressing whether human circadian and sleep disorders, as seen in model organisms, are also associated with metabolic, cell cycle, or mood disorders.

An important issue to resolve concerning the mechanism of action of circadian alterations on physiological and disease processes is whether they reflect a primary effect on circadian (i.e., cyclic) function or modulation in pathways, or whether there may be functions of circadian clock components that are independent of the circadian system. These two scenarios could have important implications for therapeutic strategies because on the one hand treatments or lifestyles that perturb the normal patterns of circadian behavior could then have detrimental effects on metabolic and physiological pathways that are coordinated by the circadian system. On the other hand, if circadian clock proteins have other non-circadian functions, then therapeutic strategies that target them would need to consider whether the non-circadian functions are involved in the physiological processes linked to circadian genes, or whether they are off-target sites for side effects. In either case, delineating the mechanisms in which circadian clock gene products are linked to disease processes will be critical for therapeutic interventions targeting the circadian system.

Finally, with the knowledge that circadian clocks are cell autonomous and distributed throughout the body, it is important to target both central and peripheral circadian oscillators for therapeutic intervention. Because the circadian clock is so intimately linked to cellular metabolism and cell proliferation, new therapeutic indications such as the predisposition to type 2 diabetes and obesity may become amenable to treatments that modulate the circadian and sleep systems [129, 130]. Future exploration in these research areas, along with increased public awareness, may allow us to appropriately diagnose and treat human circadian disorders as well as improve lifestyle choices to best align our physiological systems with the daily clock.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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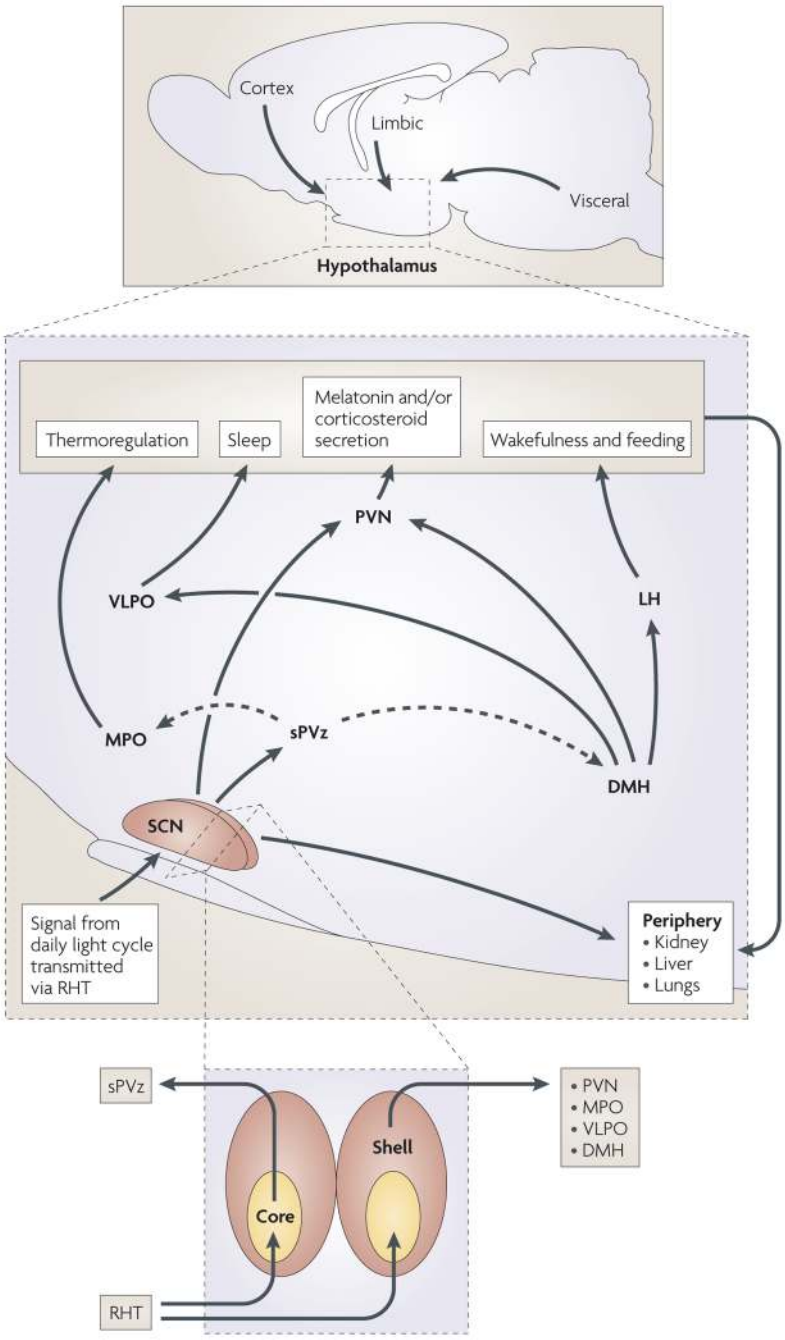

Figure 1. A schematic diagram of the SCN and its input and output pathways

The mammalian circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) is organized into two major subdivisions, the core and the shell. The core region of the SCN receives information about the daily light cycle via the retinohypothalamic tract (RHT). Both neuronal and humoral signals act as output signals from the SCN to other regions of the brain and the periphery. The SCN output pathways are responsible for proper timing of diverse physiological functions, including hormone release, sleep-wake cycle, feeding behavior, and thermoregulation. The SCN output to the subparaventricular zone (sPVz) is relayed to the medial preoptic region (MPO) to control circadian rhythms of body temperature; and a separate projection through the dorsomedial nucleus of the hypothalamus

(DMH) controls daily hormone secretion (via paraventricular nucleus, PVN) and sleep-wake cycles (via lateral hypothalamus, LH, and ventrolateral preoptic nucleus, VLPO). Figure modified from [8] [permission obtained].

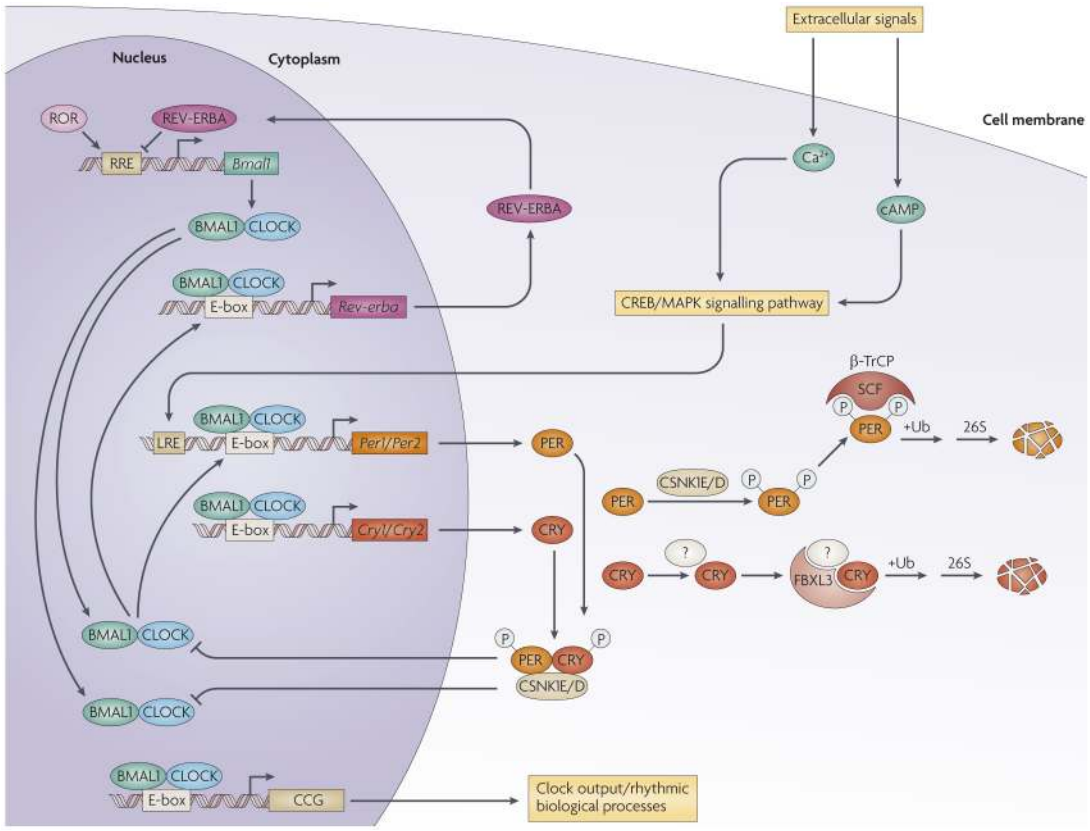

Figure 2. The mammalian circadian clock is composed of a transcriptional–translational feedback network

The circadian clock mechanism involves transcription-translation feedback loops comprised of a set of core clock genes. In mammals, the circadian clock is composed of a primary negative feedback loop involving the genes, *Clock/Npas2, Bmal1, Period1 (Per1), Per2, Cryptochrome1 (Cry1)* and *Cry2*. CLOCK/NPAS2 and BMAL1 are basic helix-loop-helix (bHLH) PAS-domain containing transcription factors that activate transcription of the *Per* and *Cry* genes. The resulting PER and CRY proteins heterodimerize, translocate to the nucleus and interact with the CLOCK:BMAL1 complex to inhibit their own transcription. With time, the PER-CRY repressor complex is degraded, and CLOCK:BMAL1 can then activate a new cycle of transcription. The secondary autoregulatory feedback loop is composed of REV-ERBα that is a direct target of CLOCK:BMAL1 transcription activator complex. REV-ERBα feeds back to repress *Bmal1* transcription and competes with RORa to bind retinoic acid-related orphan receptor response elements (RREs) in the *Bmal1* promoter. In addition to the transcriptional activators and repressors, post-translational modification and degradation of circadian clock proteins are critical steps for determining circadian periodicity. Key kinases for PER (and CRY) phosphorylation are Casein kinase 1δ (CK1δ) and CK1ε. One of the roles for phosphorylation of clock proteins is to target them for polyubiquitination and degradation by the 26S proteosomal pathway. Both β-TCRP1 and FBXL3 E3 ubiquitin ligase complexes have been implicated in targeting the PER and CRY proteins, respectively, for degradation. Figure modified from [140] [permission sought].

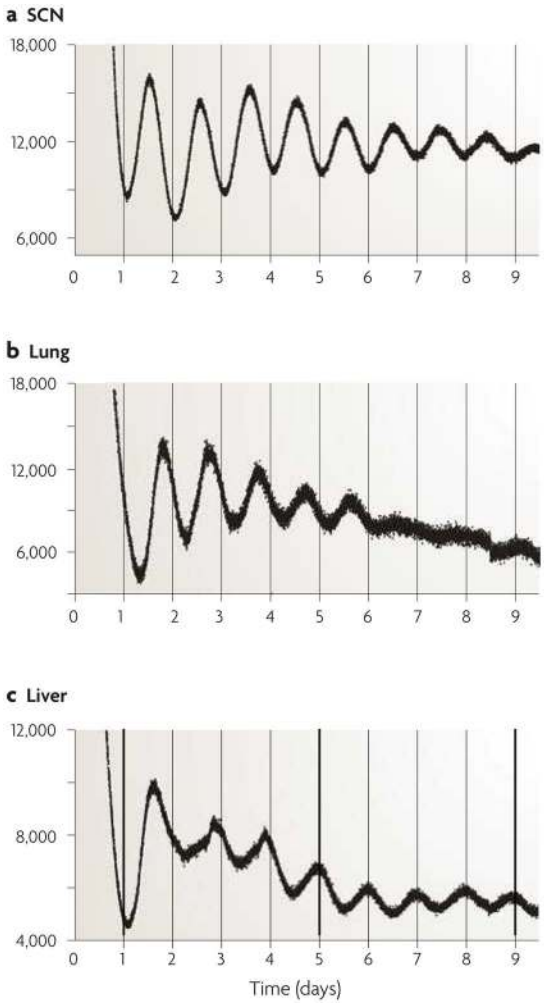

Figure 3. Central and peripheral oscillators

The expression of the core circadian clock genes is ubiquitous and reflects the presence of circadian oscillators in virtually every tissue and cell in the body. The SCN expresses robust oscillations of PER2::Luciferase activity when isolated in explant culture ex vivo, as do virtually every tissue in the mouse, such as the lung and the liver. Data from [71].

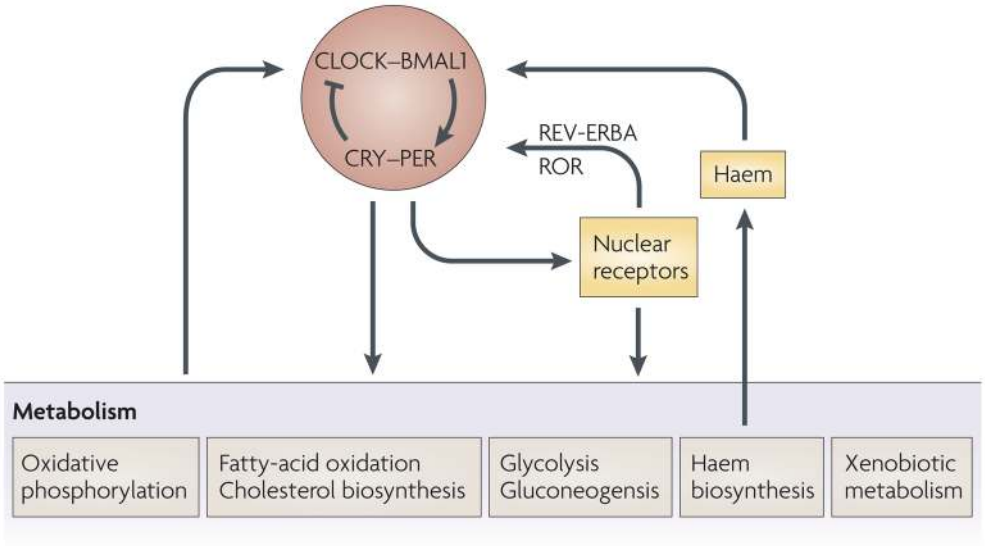

Figure 4. Interactions between circadian and metabolic systems

Fundamental metabolic pathways such as glycolysis, fatty acid metabolism, cholesterol biosynthesis, and xenobiotic and intermediate metabolism are all under circadian regulation. Metabolism itself can also have effects on circadian clocks: these include NADH and NADPH which can modulate CLOCK:BMAL1 and NPAS2:BMAL1 DNA binding; the PAS domains of NPAS2 which can bind heme and can be modulated by carbon monoxide; and, heme can also be a ligand for the circadian nuclear receptor, *Rev-erbα*.

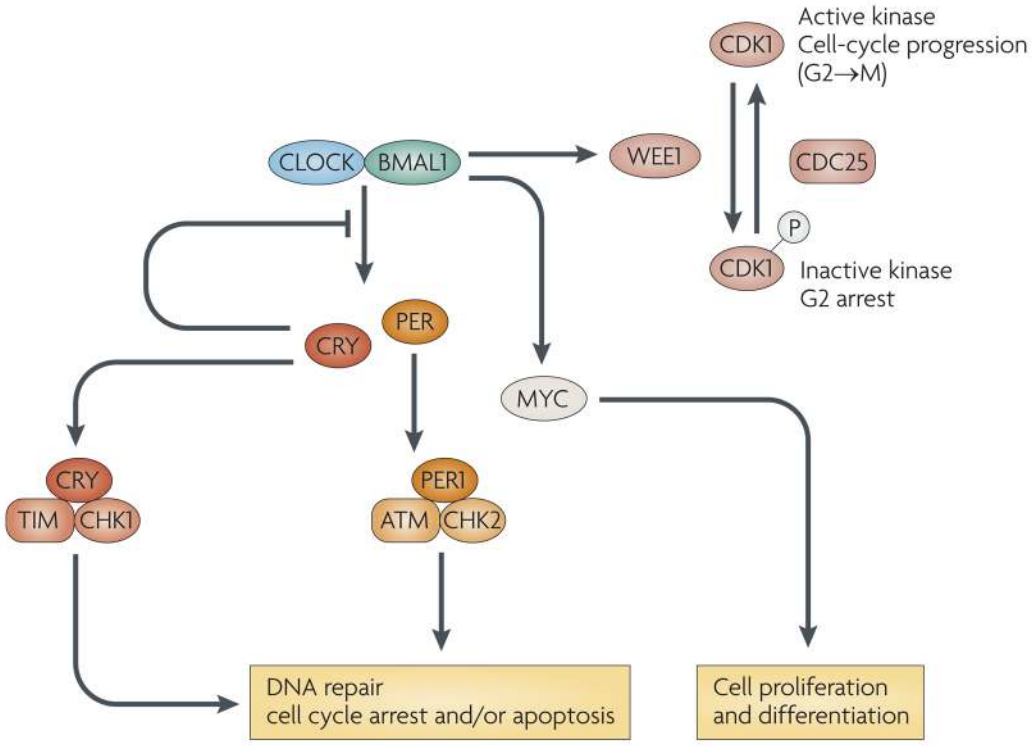

Figure 5. Circadian control of cell division cycles

Circadian system is linked to the cell division cycle via circadian control of gene expression and posttranslational mechanisms. The transcription of *c-Myc* and *Wee1* is circadian and appears to be a direct target of the CLOCK:BMAL1. The expression of *Wee1* is co-regulated with *Per* and the entry of the cell cycle into M phase is suppressed during the day time when the transcription of *Per* (and *Wee1*) is high. In addition, the PER1 protein interacts with the check-point proteins, ATM and Chk2; while related work has linked the TIMELESS (TIM) and CRY proteins with Chk1. Activation of the DNA damage pathway can also reset the phase of the circadian clock.