Published in final edited form as:
Mutat Res. 2009 September ; 679(1-2): 17–23. doi:10.1016/j.mrgentox.2009.07.015.

Pharmacological modulators of the circadian clock as potential therapeutic drugs

Marina P. Antoch[1] and **Mikhail V. Chernov**[2]

[1]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

[2]Small Molecule Screening Core, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

Abstract

Circadian clocks are molecular time-keeping systems that underlie daily fluctuations in multiple physiological and biochemical processes. It is well recognized now that dysfunctions of the circadian system (both genetically and environmentally induced) are associated with the development of various pathological conditions. Here we describe the application of high throughput screening approach designed to search for small molecules capable of pharmacological modulation of the molecular clock. We provide evidence for the feasibility and value of this approach for both scientific and therapeutic purposes.

Keywords

Circadian clock; genotoxic stress; CLOCK/BMAL1-mediated transcription; pharmacological modulators; chemical library; high throughput screening

1. Introduction

Given the important role the circadian system plays in synchronizing various aspects of metabolism and physiology both within an organism and between an organism and its environment, it is not surprising that disruption of proper synchronization is critical for an organism's well-being. Circadian desynchronization has been long associated with various sleep disorders [1] as well as with increased risk of different pathological conditions, including cancer and cardiovascular disease [2,3]. Recent data has also conclusively demonstrated the involvement of the circadian system or some of its molecular components in regulation of such basic processes as cell proliferation, response to genotoxic stress, and aging (reviewed in [4, 5]). However, despite the fact that the enormous significance of circadian clocks to human physiology is well recognized, no effective pharmacological approaches to modulation or re-adjustment of the circadian clock system have been developed so far.

Corresponding author: Marina P. Antoch, 1-716-845-3429 (ph), 1-716-845-8389 (fax), Marina.antoch@roswellpark.org.

Conflict of Interest Statement

The authors declare that they have no financial interest that might be viewed to inappropriately influence the work presented.

The following review discusses the potential for using high throughput screening to identify small molecules that can modulate basic parameters of circadian clock function. These molecules may represent invaluable research tools for dissecting circadian pathways by both refining the functional roles of known clock components and potentially discovering new ones. They also have the potential to be developed into therapeutic drugs, which can modulate various cell responses via the intrinsic clock mechanism.

2. Circadian clock organization

The circadian clock is an internal time-keeping mechanism which provides adaptation of living organisms to the 24-h periodicity of the Earth's rotation [6]. During the past decade, significant progress has been made in understanding the circadian clock at the molecular level. It is well accepted now that despite the differences in components of molecular clocks across species, the basic principles of clock function are similar in all organisms studied up to date and are based on tightly linked transcription/translation feedback loops [7] (schematically shown in Fig. 1). At the core of this mechanism are two basic helix-loop-helix (bHLH) PAS (*Per-Arnt-Sim*) domain-containing transcription factors, CLOCK and BMAL1. In the form of a heterodimer they activate transcription of various target genes through the E-box elements in their promoters. Among the transcriptional targets of the CLOCK/BMAL1 complex are genes encoding CRYPTOCHROME (CRY1 and 2) and PERIOD (PER1 and 2) proteins, which function as potent suppressors of CLOCK/BMAL1-dependent transactivation [8], and two nuclear receptors, REV-ERBα and RORα, which respectively repress or activate transcription of the *Bmal1* gene [9], forming an interlocked loop. In addition to the core components of the molecular oscillator, the CLOCK/BMAL1 complex drives rhythmic transcription of numerous clock-controlled genes either directly via the E-box regulatory element in their promoter region, or indirectly, via the rhythmic control of other transcription factors whose expression is under clock control [10]. The results of global temporal transcriptional profiling of various tissues using a microarray hybridization approach have estimated that as much as 10% of mammalian transcriptome oscillates in a circadian manner [11-13].

Mechanistically, daily rhythms in CLOCK/BMAL1-mediated transactivation have been attributed to circadian control of chromatin modifications, which may be promoter-specific. Thus, rhythmic histone acetylation has been shown in the *mPer1* promoter [14] whereas rhythmic patterns of methylation/demethylation and CLOCK/BMAL1 binding have been described in the *mDbp* promoter [15]. The link between circadian control and chromatin remodeling was further supported by the finding that the core circadian regulator CLOCK possesses intrinsic histone acetyltransferase (HAT) activity specific not only for histones [16] but for its transcriptional partner BMAL1 as well [17].

Both positive and negative regulators of the major circadian loop are subject to various posttranslational modifications, which are important for functional activity, nuclear/cytoplasmic shuttling and stability of clock proteins [5,18]. It is believed that various posttranslational modifications are imposing delay in CRY/PER-mediated repression, which results in prolonging of the circadian cycle to ~ 24 hrs [18]. The mechanism of PER/CRY-dependent repression is complex and is not yet well understood. It has been shown that CRYs regulate posttranslational modifications, nuclear/cytoplasmic distribution and stability of CLOCK and BMAL1 proteins as well as the interaction of the CLOCK/BMAL1 complex with chromatin modifying enzymes [19-21]. All these activities are likely to contribute to CRY-dependent inhibition of CLOCK/BMAL1 functional activity. Some data also suggest that CRY-induced posttranslational modifications regulate CLOCK/BMAL1 activity by converting it from a transcriptional activator to a transcriptional repressor; however, the molecular details of this process are still unknown [22].

In mammals, virtually all cells and tissues harbor a functional clock. Components of a molecular clock underlying rhythmic gene expression have been characterized in neurons of the suprachiasmatic nucleus (SCN)[23,24], in liver [23-27], heart[26], synchronized fibroblasts [28], adipose tissue[29], adrenal gland [30], skeletal muscle [31], pituitary [32] and pineal [33] glands, calvarial bone [34], as well as in epithelial cells of lung [35,36] and gastrointestinal tract [37-39]. In order to ensure proper temporal organization in metabolism and behavior at the level of an organism, the multiple tissue-specific transcriptional oscillations have to be synchronized with the natural 24-hr cycle. The current view on the mammalian circadian system suggests that it represents a hierarchy of multiple oscillators, where the SCN acts as major pacemaker, capable of sustaining oscillations and synchronizing peripheral clocks. The SCN itself is reset daily by changes in the light:dark conditions (reviewed in [40]) and upon synchronizing its phase to the light cycle, transmits this information to peripheral clocks by a combination of neural and humoral signals [41].

The complex relationships between central and peripheral oscillators have been studied best for metabolically active tissues, particularly for the liver (reviewed in [42]). It has been shown that under normal conditions the SCN plays a dominant role in synchronizing peripheral clocks to the light:dark cycle through combination of several mechanisms such as behavioral rhythms, body temperature rhythms and cyclic hormone secretion. However, under special circumstances (artificial feeding schedule with a phase opposite to the normal light:dark cycle), oscillations in the SCN and liver can be completely uncoupled [25,43,44]. Importantly, even in isolation, peripheral tissues are capable of self-sustained circadian oscillations for > 20 cycles [45]. Together, these findings demonstrate the existence of yet unknown synchronization signaling molecules that function independent of the SCN in a tissue-specific manner.

This concept was further supported by experiments performed in immortalized cell lines, where the effects of various signals on basic circadian parameters were tested. The pioneer work of Balsalobre et al [46] demonstrated that rat-1 fibroblasts are capable of generating circadian rhythmicity in gene expression when treated by 50% serum. This work initiated a series of experiments performed in different laboratories resulting in the identification of several compounds that could affect circadian parameters of cultured cells. In addition to serum, rhythmicity in cultured cells could be induced by the glucocorticoid receptor agonist, dexamethazone [47], forskolin, an activator of adenylate cyclase [48], phorbol-12-myristate-13-acetate (PMA), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, calcium ionophore calcimycin [49], endothelin [50], glucose [51] and prostaglandin E_2 [52]. Differences in rhythm inducing properties revealed by comparative quantitative analysis of ten individual signaling compounds [53] indicated that each of them could exert its action through different pathways. It is noteworthy that all of these compounds have an extremely broad spectrum of action, affecting many cellular and physiological processes, which restricts their usage as potential regulators of circadian behavior.

The fact that circadian parameters can be affected by external stimuli both *in vivo* and *in vitro* suggests that the components of the molecular clock can in fact be modulated pharmacologically and that *in vitro* cell-based assays represent an appropriate system for a high-throughput screening for such small molecules. Potential applications for circadian modulators are two-fold. First, they represent important research tools for identification and functional study of additional unknown clock components. Second, they may be developed into pharmaceuticals targeting the components of molecular clock. Below, we discuss the potential clinical applications of circadian modulators.

3. Molecular clocks and human health

3.1. Circadian-related disorders

The synchronization of all tissue-specific oscillators to ensure the correct phase of circadian-regulated processes plays an important role in normal physiology, while the disruption of rhythmicity may cause serious pathological conditions. Historically, circadian-related disorders were viewed mainly as sleep disorders, since the timing of sleep and wakefulness is at least in part mediated by a clock-dependent mechanism [54]. The elucidation of the molecular mechanism of circadian clock operation provided a background for more mechanistically linked connections. The best documented example is the genetic association of Familial Advanced Sleep Phase Syndrome (FASPS) with a defect (*missence* mutation) in the core circadian gene - *Period2*. The mutation results in a single amino acid change in CK1ε binding domain of the hPER2 molecule, which affects the level of its phosphorylation and stability [55]. More recently, a number of epidemiological studies have suggested that people who work in rotating shifts or at night show a higher incidence of carcinogenesis, heart disease, and metabolic syndrome caused by circadian desynchronization [56-58]. These people also have higher rates of errors and accidents and often experience a significant loss of alertness and ability to make decisions. Another common example of rhythm disruption is induced by travel between time zones, which often results in jet lag, a less serious, but often debilitating and disorienting condition [59].

There are two therapeutic approaches that have been used so far for treatment of circadian-related disorders - full-spectrum and bright light therapy and melatonin therapy [60,61]. Both light and melatonin, appropriately timed, have been shown to phase-shift human circadian rhythms. In addition, they both have acute physiological and behavioral effects. Depending on the dose, melatonin can reduce core body temperature and induce sleepiness. Conversely, light at night increases body temperature and enhances alertness and performance. However, there is currently no melatonin formulation approved for clinical use, nor are there consensus protocols for both therapies. Besides, their effect is too general and slow as they both act through the SCN and rely on the SCN's ability to synchronize the rest of the body while the desirable compound should directly affect the clock mechanism, preferably in a tissue-specific manner.

3.2. Clock proteins as modulators of genotoxic stress induced by anticancer treatment

Another important potential application for pharmaceuticals targeting the components of the molecular clock arises from recently discovered ability of key circadian proteins to modulate response to genotoxic stress. This was first demonstrated by testing the sensitivity of several circadian mutant mice to toxicity induced by the anticancer drug cyclophosphamide [62]. Despite similarities in their behavioral phenotype (the *Clock* mutant mice and *Bmal1*$^{-/-}$ and *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ knockout mice demonstrate arrhythmic behavior under constant environmental conditions) these mutants show significant differences in their response to cyclophosphamide-induced toxicity. Importantly, these differences correlate with the functional status of the CLOCK/BMAL1 transcriptional complex. Animals with a constant low level of CLOCK/BMAL1-mediated activity (*Clock/Clock* and *Bmal1*$^{-/-}$ mice) appeared to be extremely sensitive to drug. On the contrary, animals with a constant high level of CLOCK/BMAL1 transcriptional activity due to lack of circadian repressors (*Cry1*$^{-/-}$ *Cry2*$^{-/-}$ double-knockout mice) demonstrate high resistance. Consistently, wild-type animals show daily variations in their response, which correlates with normal daily variation of the CLOCK/BMAL transcriptional activity. The data suggest that the CLOCK/BMAL1 complex may directly control the molecular determinants of drug sensitivity at the transcriptional level [62]. Importantly, daily fluctuations in CLOCK/BMAL1-dependent transactivation might

provide mechanistic explanation for numerous examples of advantages of timed administration of chemotherapeutic drugs compared to conventional methods of delivery (reviewed in [63]).

The growing understanding of molecular links between the circadian and stress response systems provides a rationale for potential use of these mechanisms in clinical practice. The direct correlation between the functional activity of the CLOCK/BMAL1 and cellular sensitivity to genotoxic stress identifies the CLOCK/BMAL1 transcriptional complex as a target for pharmacological modulation. The ability to manipulate the molecular clock pharmacologically could potentially allow the resetting of the clock in drug-sensitive tissues to times of low toxicity, therefore minimizing the damaging effects of genotoxic treatments.

It is important to note, however, that in order to fully exploit this approach, more information is needed regarding the functional status of the molecular clock in tumor cells and tumors, which is still sporadic and sometimes controversial. Nevertheless, small molecules affecting core circadian regulators have the potential to be developed into drugs which may increase the therapeutic index of a given cancer treatment when used in combination with existing therapeutic schedules.

3.3. Non-circadian function of circadian proteins

Recent discoveries in the field of circadian rhythms, and particularly the detailed analysis of mice deficient in various components of the molecular clock, revealed a number of pathologies specific to each mutation (reviewed in [5]. For example, *Clock/Clock* mutant mice develop metabolic syndrome and obesity [64], animals with a targeted disruption of the CLOCK transcriptional partner, BMAL1 (*Bmal1*$^{-/-}$ knockout mice) display phenotype of premature aging [65], whereas an increase in carcinogenesis has been reported specifically for *Per2* mutants [66]. These examples lead to several important conclusions. First, they suggest that individual circadian proteins play unique roles in maintaining normal tissue homeostasis and that these roles could be relatively independent from their role in the molecular circadian oscillator. Second, they significantly broaden the potential translational applications of pharmaceuticals targeting circadian protein.

4. Search for pharmacological modulators of the circadian clock

4. 1. Chemical libraries and high throughput screening

In the pre-genomic era, small organic molecules remained the major tool for probing various biological processes. The situation changed with the progress of genomics and the development of multiple genetic and molecular biological methods. For several decades small molecules were viewed as secondary and less specific tools in molecular biology. However, fast advances in structural biology and the sequencing of the human genome facilitated the identification of targets for drug discovery, which in turn revived interest in chemical approaches in the biomedical field. At the same time, technological progress in automation and miniaturization of chemical screening and the reduction of price of high throughput screening (HTS) components made this platform accessible not only to the pharmaceutical industry, but to academic scientists as well. The technology is accessed mainly through the screening facilities of different scale opening in many medical and academic institutions. Chemical genetics, aroused as a result of this development, brings together molecular biology and chemistry. This area covers the discovery of new bioactive molecules and their use for probing biological processes or treating the disease. The major step of the drug discovery process is schematically shown in Fig. 2.

HTS of libraries of small organic molecules along with target-based rational design is one of the main tools for the discovery of bioactive compounds. Two main components of HTS are chemical libraries and readout assays.

Today's chemical/chemistry space or number of known organic molecules is close to 10^{13} [67]. It makes the compartmentalization of this space for the purpose of drug discovery very important. Such compartmentalization is achieved by the creation of relatively small collections of compounds or chemical libraries. All currently used libraries can be divided into two major groups based on the way they were manufactured: historical and combinatorial. Compounds in combinatorial libraries are built from a set of building blocks by the process of combinatorial chemistry. Combinatorial libraries of 1–10 million compounds are common in the pharmaceutical industry. Historical libraries are collections of premade compounds from different sources, mainly from the chemical labs.

Collections of natural compounds or known pharmaceuticals and bioactive compounds can be viewed as a special type of historical library. Examples of such libraries are the LOPAC (Library of Pharmacologically Active Compounds) from Sigma or the Spectrum collection from MSDSI. These libraries are frequently used for testing and validation of readout assays or for pilot screenings to obtain the proof of principle and justify the screening project [68, 69]. Besides this, the majority of these compounds have a well-characterized biological function, which increases the chances of making new discoveries even at this early stage.

One recent development in library production is the design of the focused libraries targeting a specific type of enzyme or a biological process. Such libraries can be handpicked from the volume of known bioactive compounds or built using combinatorial chemistry around the scaffold with the affinity to the specific family of enzymes [70]. Examples of such collections are the libraries of protease or kinase inhibitors; libraries of compounds related to apoptosis or cell cycle regulation and others.

4.2. Development of a readout assay for high throughput screening

In general, the readout assays used in HTS can be divided into two main categories: biochemical and cell-based. Historically, biochemical assays have been the main tool in drug discovery. These assays are often direct and target-specific. They can be based on *in vitro* enzymatic activity (kinase, protease, nuclease, etc.) or protein-protein interaction (receptor-ligand interaction). Biochemical assays are very suitable for HTS by virtue of high reproducibility, easy miniaturization and handling. But there are also certain problems associated with such assays. First of all, they presuppose the identification of the critical component of the pathway that can be used as a target, which may not be feasible. But even if known, some targets cannot be purified to work in *in vitro* reaction. Second, the activity of a small molecule in a biochemical reaction in which the target is taken out of the cellular context, does not necessarily translate into comparable activity *in vivo*. Thus, the selected compounds could be toxic *in vivo*, have permeability issues, or could be trapped inside of the cell in the location preventing their interaction with the target.

In contrast to biochemical assays, cell-based assays are considered to be more biologically relevant. These assays target the entire pathway or biological process in the context of all regulatory mechanisms in the cell and do not require the knowledge of a direct target. Therefore, multiple targets responsible for a given phenotype or the activity of a biochemical pathway can be aimed in one screening. The use of a cell-based assay also provides the advantage of filtering out cytotoxic compounds and compounds with low permeability at the stage of primary screening.

As described above, the circadian clock is a complex, multi-component system subject to many levels of regulation. It is present in each individual cell, and these cells could be synchronized by external stimuli. All these considerations make cell-based assays most suitable for targeting circadian pathways.

All cell-based assays can be roughly divided into several groups based on the readout used for quantification of the effect of compounds. Reporter-based assays rely on the activity of surrogate reporter genes, such as luciferase, GFP, β-galactosidase, placed under the control of a cellular process. Another group of assays is based on direct measurements or visualization of biological processes such as apoptosis, cell proliferation, cell cycle arrest, etc. Variety of different readouts is available for quantitation of these processes. Out of these, luciferase-based assays have been well developed to study circadian rhythms in diverse organisms (cyanobacteria, plants, fruit flies and mice) (reviewed in [71]). They rely on the relatively short half-life of luciferase, allowing assays to be performed on a circadian scale, and the robustness of circadian promoters. Among these, the most often used are the *mPer1, mPer2* and *mBmal1* promoter constructs of various length as well as the endogenous *mPer2* promoter expressed in cells isolated from *mPer2::luc* knock-in mice [45]. Each of them has its own advantages and the choice of promoter is dictated by the goal and design of each particular study.

In general, one can propose two ways of developing the circadian assay for HTS (schematically shown in Fig. 3). First, it could be based on a real-time recording of reporter activity in cells with synchronized circadian rhythms (by serum shock, dexamethasone or other treatment). In this experimental setup, chemical compounds are tested for their effect on basic circadian parameters (circadian period, amplitude and phase of rhythmicity) (Fig. 3A). This approach, which has been successfully used recently, requires a high level of automation, real-time recording and sophisticated data analysis [72].

In our own work, we used a complementary approach. Since our previous data have identified CLOCK/BMAL1 transcriptional activity as a potential target for pharmacological modulation of normal circadian function [62], we designed our readout system based on a steady-state level of circadian transcriptional machinery (Fig. 3B). For this, we generated a stable cell line expressing high levels of ectopic CLOCK and BMAL1 together with the *mPer1-luc* circadian reporter [73]. This cell line, which demonstrates constant high levels of luciferase activity, was used to screen two structurally diverse chemical libraries: manufactured by Sigma (Library of Pharmacologically Active Compounds, LOPAC, 1280 individual chemicals) and by Microsource Discovery, Inc (Spectrum, 2000 compounds). These two libraries are often used in small-scale screenings to validate and optimize assays. Since our assay is based on changes in the functional activity of circadian transcriptional regulators, both libraries were screened for chemicals that would activate or suppress CLOCK/BMAL1-mediated transactivation. Even though transcription factors are often considered as 'non-druggable' targets, there are examples of previous successful use of transcription-based assays [74,75]. Identification of known circadian regulators in our screening (see below) further confirmed the feasibility of the undertaken approach.

4.3. Assay optimization and validation

There are many parameters and elements of the HTS project that should be carefully thought through, designed and tested before the actual large-scale screening starts. The statistical characterization and justification of the readout assay is one of these elements. Dynamic range and variability of the signal are important for any biochemical or cell-based assay, but in the case of HTS it becomes critical for reliable selection of hits. The Z factor is the statistical parameter selected for characterization of HTS assays [76]. It is usually recommended that to determine the reliability of HTS, this parameter should be calculated for every assay before screening. The coefficient is defined as the ratio between the separation band and the dynamic range of the assay and can be calculated using the formula below where σs is the standard deviation of the sample and σc is the standard deviation of the control and μs and μc are means for signal and control.

$$Z=1-\frac{(3\delta s+3\delta c)}{|\mu s-\mu c|}$$

The closer the Z factor is to 1 (low standard deviations and high dynamic range) - the wider the separation band. It translates into a more reliable assay and more accurate hit selection. Usually, a Z factor of less than 1 but higher than or equal to 0.5 indicates an excellent assay.

Fig. 4 shows an example of a typical assay plate, in which 80 individual compounds from the LOPAC library were added to cells plated in columns 2–11, whereas the first and last columns were used for controls. The Z factor calculated for each plate falls into the range between 0.6 and 0.8, which according to [76], represents a statistically highly reliable assay.

Data analysis of screening results, which leads to the identification of primary hits, is the next important step in the process of HTS. The output of screening is an array of variable data points, reflecting the activity of different compounds. Statistically all data points located within 3 standard deviations around the mean of the control belong to the normal distribution. Consequently, any data point outside of this area can be regarded as a hit. In practice, there might be other considerations determining the value of the cut off score for each specific screening project. Thus, in our own screening protocol, in order to limit the number of potential hits to 1–2% of the number of screened compounds, it was considered a hit if the luciferase signal was outside of 4 standard deviations from the mean of the control.

4.4. Small molecules modulating CLOCK/BMAL1 transcriptional activity

Given the current knowledge of the molecular mechanisms of circadian transcriptional control, one can expect that chemical compounds identified in the process of HTS as regulators of CLOCK/BMAL1 transcriptional activity can exert their effect on multiple levels. They can affect the dimerization of CLOCK and BMAL1, DNA-binding properties of the complex or its interaction with components of general transcriptional machinery. Since posttranslational modifications of clock proteins are important for their function, compounds affecting phosphorylation (chemical regulators of kinases and phosphatases), SUMOylation or stability of CLOCK/BMAL1 are also expected to be identified as hits. In addition, the CLOCK protein harbors intrinsic HAT activity, which may also be targeted by drugs. Some of these effects could be non-specific (for example, general transcriptional regulators, such as Actinomycin D or compounds with high levels of cell toxicity), thus careful validation and characterization of primary hits are required. As a result of the primary screening of the two libraries, followed by data filtering and hit validation, we have identified 53 individual compounds belonging to different classes of biologically active molecules which could either activate or suppress CLOCK/BMAL1-mediated transcription. These compounds are now tested for the specificity of their action. Importantly, the list of confirmed hits includes several known regulators of circadian function (glucocorticoids, forskolin) supporting the validity of the screening approach**.**

In conclusion, we have developed and optimized a cell-based readout system that was successfully used to screen two small libraries of functionally characterized pharmacological compounds for their effect on transcriptional activity of the CLOCK/BMAL1 complex. The screening identified several compounds previously known to affect circadian function. In addition, it identified several novel chemicals that have not been previously linked to circadian function. These chemicals are in the process of more detailed study. Our data, together with a previously published study [72] convincingly demonstrate the feasibility of the proposed approach and its scientific and translational value.

Acknowledgments

We thank Victoria Gorbacheva and Anatoly Prokvolit for technical help in chemical screening and Dmitry Gudkov for the editorial help.

This work was supported by NIGMS grant GM550926 to M.P.A.

References

[1]. von Schantz M. Phenotypic effects of genetic variability in human clock genes on circadian and sleep parameters. J Genet 2008;87:513–519. [PubMed: 19147940]

[2]. Knutsson A. Health disorders of shift workers. Occup Med (Lond) 2003;53:103–108. [PubMed: 12637594]

[3]. Montagnana M, Salvagno GL, Lippi G. Circadian variation within hemostasis: an underrecognized link between biology and disease? Semin Thromb Hemost 2009;35:23–33. [PubMed: 19308890]

[4]. Kondratov RV, Antoch MP. Circadian proteins in the regulation of cell cycle and genotoxic stress responses. Trends Cell Biol 2007;17:311–317. [PubMed: 17644383]

[5]. Kondratov RV, Gorbacheva VY, Antoch MP. The role of mammalian circadian proteins in normal physiology and genotoxic stress responses. Curr Top Dev Biol 2007;78:173–216. [PubMed: 17338917]

[6]. Lowrey PL, Takahashi JS. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet 2004;5:407–441. [PubMed: 15485355]

[7]. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 2005;6:544–556. [PubMed: 15951747]

[8]. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. Hum Mol Genet 2006;15(2):R271–277. [PubMed: 16987893]

[9]. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 2002;110:251–260. [PubMed: 12150932]

[10]. Desvergne B, Michalik L, Wahli W. Transcriptional regulation of metabolism. Physiol Rev 2006;86:465–514. [PubMed: 16601267]

[11]. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. Coordinated transcription of key pathways in the mouse by circadian clock. Cell 2002;109:307–320. [PubMed: 12015981]

[12]. Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 2002;12:540–550. [PubMed: 11937022]

[13]. Duffield GE. DNA microarray analyses of circadian timing: the genomic basis of biological time. J Neuroendocrinol 2003;15:991–1002. [PubMed: 12969245]

[14]. Etchegaray JP, Lee C, Wade PA, Reppert SM. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature 2003;421:177–182. [PubMed: 12483227]

[15]. Ripperger JA, Schibler U. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat Genet 2006;38:369–374. [PubMed: 16474407]

[16]. Doi M, Hirayama J, Sassone-Corsi P. Circadian regulator CLOCK is a histone acetyltransferase. Cell 2006;125:497–508. [PubMed: 16678094]

[17]. Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, Sassone-Corsi P. CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature 2007;450:1086–1090. [PubMed: 18075593]

[18]. Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 2007;8:139–148. [PubMed: 17245414]

[19]. Dardente H, Fortier EE, Martineau V, Cermakian N. Cryptochromes impair phosphorylation of transcriptional activators in the clock: a general mechanism for circadian repression. Biochem J 2007;402:525–536. [PubMed: 17115977]

[20]. Kondratov RV, Kondratova AA, Lee C, Gorbacheva VY, Chernov MV, Antoch MP. Post-translational regulation of circadian transcriptional CLOCK(NPAS2)/BMAL1 complex by CRYPTOCHROMES. Cell Cycle 2006;5:890–895. [PubMed: 16628007]

[21]. Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, Lee KH. BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. Mol Cell Biol 2006;26:7318–7330. [PubMed: 16980631]

[22]. Kondratov RV, Shamanna RK, Kondratova AA, Gorbacheva VY, Antoch MP. Dual role of the CLOCK/BMAL1 circadian complex in transcriptional regulation. FASEB J 2006;20:530–532. [PubMed: 16507766]

[23]. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 2002;109:307–320. [PubMed: 12015981]

[24]. Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, Takasugi T, Nagano M, Nakahama K, Suzuki Y, Sugano S, Iino M, Shigeyoshi Y, Hashimoto S. A transcription factor response element for gene expression during circadian night. Nature 2002;418:534–539. [PubMed: 12152080]

[25]. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M. Entrainment of the circadian clock in the liver by feeding. Science 2001;291:490–493. [PubMed: 11161204]

[26]. Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ. Extensive and divergent circadian gene expression in liver and heart. Nature 2002;417:78–83. [PubMed: 11967526]

[27]. Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T, Ishida N. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem 2003;278:41519–41527. [PubMed: 12865428]

[28]. Grundschober C, Delaunay F, Puhlhofer A, Triqueneaux G, Laudet V, Bartfai T, Nef P. Circadian regulation of diverse gene products revealed by mRNA expression profiling of synchronized fibroblasts. J Biol Chem 2001;276:46751–46758. [PubMed: 11598123]

[29]. Ando H, Yanagihara H, Hayashi Y, Obi Y, Tsuruoka S, Takamura T, Kaneko S, Fujimura A. Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue. Endocrinology 2005;146:5631–5636. Epub 2005 Sep 5615. [PubMed: 16166217]

[30]. Oster H, Damerow S, Hut RA, Eichele G. Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. J Biol Rhythms 2006;21:350–361. [PubMed: 16998155]

[31]. Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, Takahashi JS. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. Proc Natl Acad Sci U S A 2007;104:3342–3347. [PubMed: 17360649]

[32]. Hughes M, Deharo L, Pulivarthy SR, Gu J, Hayes K, Panda S, Hogenesch JB. High-resolution time course analysis of gene expression from pituitary. Cold Spring Harb Symp Quant Biol 2007;72:381–386. [PubMed: 18419295]

[33]. Fukuhara C, Tosini G. Analysis of daily and circadian gene expression in the rat pineal gland. Neurosci Res 2008;60:192–198. [PubMed: 18067983]

[34]. Zvonic S, Ptitsyn AA, Kilroy G, Wu X, Conrad SA, Scott LK, Guilak F, Pelled G, Gazit D, Gimble JM. Circadian oscillation of gene expression in murine calvarial bone. J Bone Miner Res 2007;22:357–365. [PubMed: 17144790]

[35]. Burioka N, Takata M, Okano Y, Ohdo S, Fukuoka Y, Miyata M, Takane H, Endo M, Suyama H, Shimizu E. Dexamethasone influences human clock gene expression in bronchial epithelium and peripheral blood mononuclear cells in vitro. Chronobiol Int 2005;22:585–590. [PubMed: 16076656]

[36]. Takata M, Burioka N, Ohdo S, Fukuoka Y, Miyata M, Endo M, Suyama H, Shimizu E. Beta2-adrenoceptor agonists induce the mammalian clock gene, hPer1, mRNA in cultured human bronchial epithelium cells in vitro. Chronobiol Int 2005;22:777–783. [PubMed: 16147906]

[37]. Hoogerwerf WA, Hellmich HL, Cornelissen G, Halberg F, Shahinian VB, Bostwick J, Savidge TC, Cassone VM. Clock gene expression in the murine gastrointestinal tract: endogenous rhythmicity and effects of a feeding regimen. Gastroenterology 2007;133:1250–1260. [PubMed: 17919497]

[38]. Scheving LA, Russell WE. It's about time: clock genes unveiled in the gut. Gastroenterology 2007;133:1373–1376. [PubMed: 17919508]

[39]. Sladek M, Rybova M, Jindrakova Z, Zemanova Z, Polidarova L, Mrnka L, O'Neill J, Pacha J, Sumova A. Insight into the circadian clock within rat colonic epithelial cells. Gastroenterology 2007;133:1240–1249. [PubMed: 17675004]

[40]. Devlin PF, Kay SA. Circadian photoperception. Annu Rev Physiol 2001;63:677–694. [PubMed: 11181972]

[41]. Schibler U, Sassone-Corsi P. A web of circadian pacemakers. Cell 2002;111:919–922. [PubMed: 12507418]

[42]. Kornmann B, Schaad O, Reinke H, Saini C, Schibler U. Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. Cold Spring Harb Symp Quant Biol 2007;72:319–330. [PubMed: 18419289]

[43]. Le Minh N, Damiola F, Tronche F, Schutz G, Schibler U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. Embo J 2001;20:7128–7136. [PubMed: 11742989]

[44]. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 2000;14:2950–2961. [PubMed: 11114885]

[45]. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 2004;101:5339–5346. [PubMed: 14963227]

[46]. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 1998;93:929–937. [PubMed: 9635423]

[47]. Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler U. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 2000;289:2344–2347. [PubMed: 11009419]

[48]. Yagita K, Okamura H. Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts. FEBS Lett 2000;465:79–82. [PubMed: 10620710]

[49]. Balsalobre A, Marcacci L, Schibler U. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. Curr Biol 2000;10:1291–1294. [PubMed: 11069111]

[50]. Yagita K, Tamanini F, van Der Horst GT, Okamura H. Molecular mechanisms of the biological clock in cultured fibroblasts. Science 2001;292:278–281. [PubMed: 11303101]

[51]. Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T, Fukada Y. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. J Biol Chem 2002;277:44244–44251. [PubMed: 12213820]

[52]. Tsuchiya Y, Minami I, Kadotani H, Nishida E. Resetting of peripheral circadian clock by prostaglandin E2. EMBO Rep 2005;6:256–261. [PubMed: 15723041]

[53]. Izumo M, Sato TR, Straume M, Johnson CH. Quantitative analyses of circadian gene expression in mammalian cell cultures. PLoS Comput Biol 2006;2:e136. [PubMed: 17040123]

[54]. Dijk DJ, Czeisler CA. Contribution of the circadian pacemaker and the sleep homeostat to sleep propensity, sleep structure, electroencephalographic slow waves, and sleep spindle activity in humans. J Neurosci 1995;15:3526–3538. [PubMed: 7751928]

[55]. Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM, Ptacek LJ, Fu YH. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science 2001;291:1040–1043. [PubMed: 11232563]

[56]. Esquirol Y, Bongard V, Mabile L, Jonnier B, Soulat JM, Perret B. Shift work and metabolic syndrome: respective impacts of job strain, physical activity, and dietary rhythms. Chronobiol Int 2009;26:544–559. [PubMed: 19360495]

[57]. Stevens RG. Circadian disruption and breast cancer: from melatonin to clock genes. Epidemiology 2005;16:254–258. [PubMed: 15703542]

[58]. Scheer FA, Hilton MF, Mantzoros CS, Shea SA. Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci U S A 2009;106:4453–4458. [PubMed: 19255424]

[59]. Sack RL. The pathophysiology of jet lag. Travel Med Infect Dis 2009;7:102–110. [PubMed: 19237143]

[60]. Pandi-Perumal SR, Trakht I, Spence DW, Srinivasan V, Dagan Y, Cardinali DP. The roles of melatonin and light in the pathophysiology and treatment of circadian rhythm sleep disorders. Nat Clin Pract Neurol 2008;4:436–447. [PubMed: 18628753]

[61]. Prasko J. Bright light therapy. Neuro Endocrinol Lett 2008;29(Suppl 1):33–64. [PubMed: 19029878]

[62]. Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch MP. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. Proc Natl Acad Sci U S A 2005;102:3407–3412. [PubMed: 15689397]

[63]. Antoch MP, Kondratov RV, Takahashi JS. Circadian clock genes as modulators of sensitivity to genotoxic stress. Cell Cycle 2005;4:901–907. [PubMed: 15917646]

[64]. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J. Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005;308:1043–1045. [PubMed: 15845877]

[65]. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes Dev 2006;20:1868–1873. [PubMed: 16847346]

[66]. Fu L, Pelicano H, Liu J, Huang P, Lee C. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell 2002;111:41–50. [PubMed: 12372299]

[67]. Schuffenhauer A, Brown N, Selzer P, Ertl P, Jacoby E. Relationships between Molecular Complexity, Biological Activity, and Structural Diversity. J Chem Inf Model 2006;46:525–535. [PubMed: 16562980]

[68]. Rickardson L, Wickstrom M, Larsson R, Lovborg H. Image-based screening for the identification of novel proteasome inhibitors. J Biomol Screen 2007;12:203–210. [PubMed: 17208922]

[69]. Urban DJ, Zheng W, Goker-Alpan O, Jadhav A, Lamarca ME, Inglese J, Sidransky E, Austin CP. Optimization and validation of two miniaturized glucocerebrosidase enzyme assays for high throughput screening. Comb Chem High Throughput Screen 2008;11:817–824. [PubMed: 19075603]

[70]. Miller JL. Recent developments in focused library design: targeting gene-families. Curr Top Med Chem 2006;6:19–29. [PubMed: 16454755]

[71]. Welsh DK, Imaizumi T, Kay SA. Real-time reporting of circadian-regulated gene expression by luciferase imaging in plants and mammalian cells. Methods Enzymol 2005;393:269–288. [PubMed: 15817294]

[72]. Hirota T, Lewis WG, Liu AC, Lee JW, Schultz PG, Kay SA. A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. Proc Natl Acad Sci U S A 2008;105:20746–20751. [PubMed: 19104043]

[73]. Wilsbacher LD, Sangoram AM, Antoch MP, Takahashi JS. The mouse Clock locus: sequence and comparative analysis of 204 kb from mouse chromosome 5. Genome Res 2000;10:1928–1940. [PubMed: 11116088]

[74]. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, Gudkov AV. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285:1733–1737. [PubMed: 10481009]

[75]. Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, Melillo G. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 2002;62:4316–4324. [PubMed: 12154035]

[76]. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 1999;4:67–73. [PubMed: 10838414]

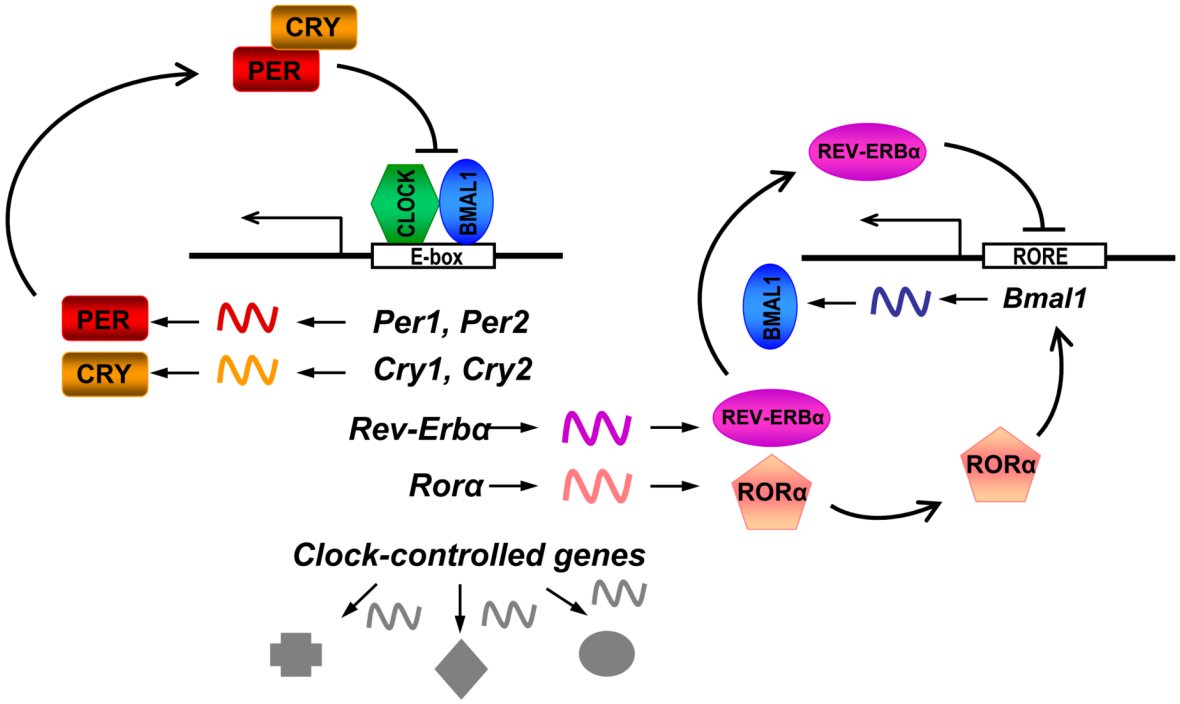

Fig. 1.

Schematic diagram of the circadian transcription/translation feedback loops showing the CLOCK/BMAL1 complex as an integral regulatory component. See text for details.

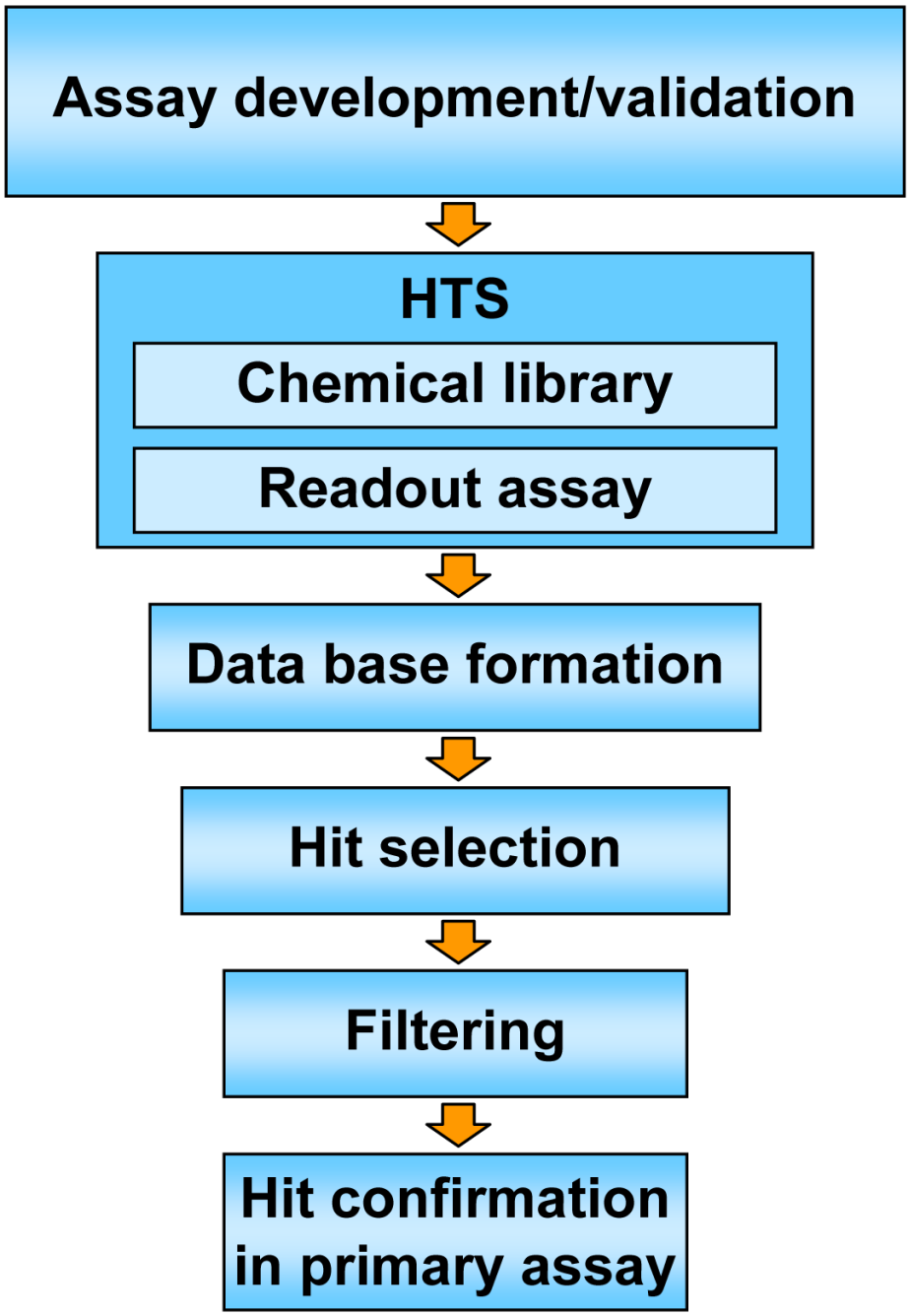

Fig. 2.
High throughput screening of chemical libraries

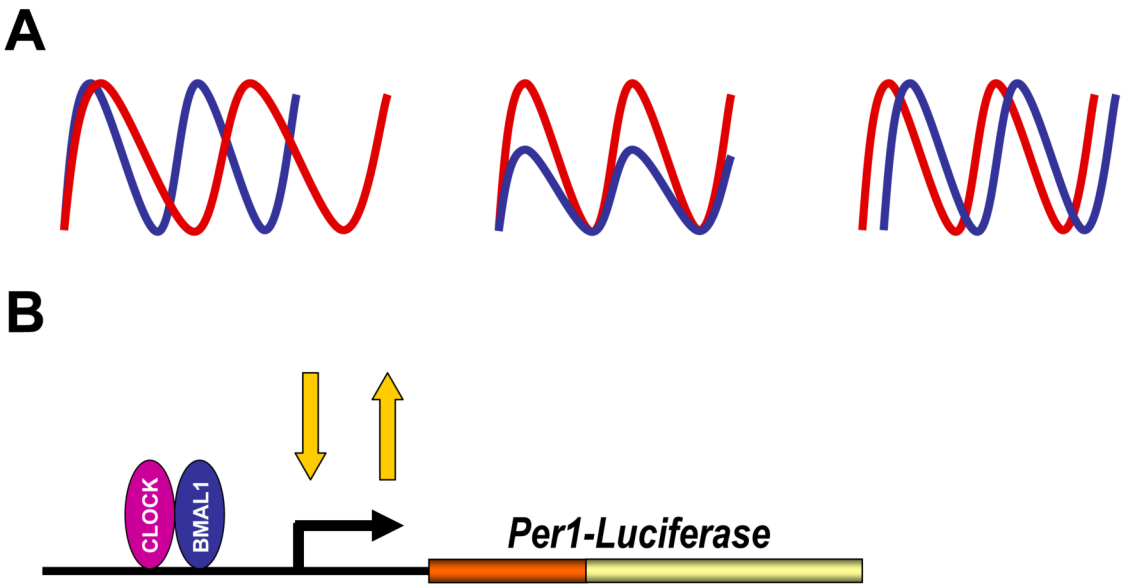

Fig. 3.
Cell-based readout systems for targeting the components of the molecular clock. (A) Search for modulators of basic circadian parameters (period, amplitude and phase of the oscillations); (B) Search for modulators of the steady-state activity of circadian proteins independent of the oscillation.

1964	1462	1920	2242	1409	1784	1477	2407	2139	1758	1784	2308
193	1755	1744	1565	1861	1708	1989	1538	2070	1912	2761	597
1643	1512	1448	1643	2473	1729	1695	2142	1769	2173	1980	1798
197	1218	1245	1761	2659	1375	1612	1752	1654	1605	1642	345
1501	1514	1288	1562	1605	546	1405	1680	1602	1632	1847	1784
200	2147	1661	315	1642	1539	1657	2372	1531	1501	1595	486
1540	1506	1340	1603	1393	1477	1532	1589	6432	1320	2124	1706
185	1511	1603	1298	1727	1481	1607	1673	1823	1588	1289	185

Fig. 4.

A typical example of an assay plate. Cells were seeded in 96-well clear-bottom luciferase assay plates in phenol red-free DMED supplemented with 10% FBS at a density of 3×10^4 cells/well (150μl/well). The compounds dissolved in DMSO were added to 80 wells (columns 2–11) in a volume of ~200 nl using pin tool (V&P Scientific, Inc) and automated liquid handling workstation JANUS by PerkinElmer. Columns 1 and 12 of each plate were used as a control; shown in lavender - had 200 nl DMSO added; shown in yellow - *Actynomycin* D (column 1 at 4mg/ml; column 12 at 1 mg/ml final concentration). After 24-hr incubation, 30 μl of BrightGlo luciferase substrate (Promega) was added, and after 5-min incubation, luciferase signal was measured using GloMax microplate luminometer (Promega). The compounds that decrease luciferase signal 4 standard deviations below the mean of a control were selected as inhibitors of CLOCK/BMAL1-mediated transactivation (shown in red); compounds that increased luciferase signal 4 standard deviations above the mean of a control were selected as activators (shown in green).