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High-Throughput Screening and Chemical Biology: New Approaches for Understanding Circadian Clock Mechanisms

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Most organisms exhibit daily changes in physiology and metabolism under the control of a cell-autonomous circadian clock. In the core clock mechanism, clock genes form a transcription factor network to generate circadian rhythms of gene expression. Clock protein phosphorylation and histone modifications are also important for the clock regulation. Pharmacological approaches have been making significant contributions to the clock research, for example, in characterizing the roles of protein kinases CKIδ, CKIε, CK2, and GSK-3β. Recently, high-throughput circadian functional assays have been established. Chemical biology approaches utilizing high-throughput compound screening together with RNAi-based genomic screening will open a new way for the circadian clock field. Finding a set of compounds that potently affect the clock function will lead to the identification of novel clock components and form the basis for therapeutic strategies directed toward circadian disorders.

Organization of Circadian Rhythms in Mammals

The circadian clock controls daily rhythms in a variety of physiological processes such as sleep/wake, body temperature, hormone secretion, and metabolism (Hastings et al., 2003; Green et al., 2008; Takahashi et al., 2008; Eckel-Mahan and Sassone-Corsi, 2009). The identification of clock-controlled processes is expanding and includes hematopoietic stem cell release (Mendez-Ferrer et al., 2008) and blood levels of hundreds of metabolites (Minami et al., 2009). Many of the rhythms persist even under constant conditions in the absence of any external time cues. Importantly, the intrinsic period length of the rhythms is strictly regulated by the circadian clock mechanism, and perturbation of clock function results in a change in period length. To synchronize with ambient 24 hr cycles, the clock has an ability to adjust its phase in response to environmental time cues primarily through light (Guler et al., 2008; Hatori et al., 2008).

The circadian clock mechanism resides at the cellular level, and single cells exhibit circadian rhythms in a cell-autonomous manner (Nagoshi et al., 2005; Welsh et al., 2005). These cellular oscillators are organized in a hierarchy, in which the suprachiasmatic nucleus (SCN), located in brain, constitutes the central circadian pacemaker controlling behavioral rhythms (Hastings et al., 2003; Liu et al., 2007a; Takahashi et al., 2008). In contrast, peripheral clocks in other tissues control local rhythmic outputs such as retinal visual processing, hepatic glucose regulation, and vascular regulation of blood pressure and heart rate (Storch et al., 2007; Lamia et al., 2008; Wang et al., 2008). Within the SCN, the cellular clocks are synchronized to form a coherent oscillator through intercellular coupling, making the SCN clock more robust against genetic and environmental perturbations than peripheral clocks (Liu et al., 2007b).

Transcription Factor Networks of the Circadian Clock

More than a dozen transcription factors and modulators constitute transcriptional feedback loops in the mammalian circadian clock mechanism (Figure 1A) (Reppert and Weaver, 2002; Gachon et al., 2006; Liu et al., 2008; Takahashi et al., 2008). In brief, bHLH-PAS proteins CLOCK (or its homolog NPAS2) and BMAL1 activate transcription of *Per* and *Cry* genes, and PER and CRY proteins (PER1, PER2, CRY1, and CRY2), in turn, inhibit their own transcription. This core loop is connected to two interlocking loops composed of bZIP proteins (DBP, TEF, HLF, and E4BP4) and nuclear hormone receptors (REV-ERBα, REV-ERBβ, RORa, RORb, and RORc). These factors act in a combinatorial manner on their three cognate *cis*-acting elements (E box, D box, and RORE) to form a network that generates robust rhythmic gene expression (Ukai-Tadenuma et al., 2008; Baggs et al., 2009). Importantly, many clock proteins bind to histone-modifying enzymes (Table 1), and histone acetylation and methylation show circadian rhythms on clock gene promoters (Etchegaray et al., 2003, 2006; Curtis et al., 2004; Naruse et al., 2004; Brown et al., 2005; Ripperger and Schibler, 2006; Liu et al., 2007c; Alenghat et al., 2008), providing another essential layer of control.

In addition to transcriptional regulation, posttranslational modifications of clock proteins by phosphorylation, ubiquitination, and acetylation play important roles. Most clock proteins undergo rhythmic phosphorylation (Lee et al., 2001), and many protein kinases are involved in the clock mechanism (see below). Upon phosphorylation of PER proteins, F box proteins β-TrCP1 and β-TrCP2 lead to regulated PER degradation thorough the ubiquitin-proteasome pathway that affects period regulation (Eide et al., 2005; Shirogane et al., 2005; Reischl et al., 2007; Maier et al., 2009). Another F box protein, Fbxl3, causes proteasomal degradation of CRY proteins, and mice harboring a missense mutation of the

Fbxl3 gene show a long-period phenotype (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). Acetylation of BMAL1 is regulated by CLOCK and SIRT1 and is necessary for normal clock oscillation (Hirayama et al., 2007; Nakahata et al., 2008). SIRT1 also deacetylates PER2 to facilitate proteasomal degradation (Asher et al., 2008). Intriguingly, SIRT1 activity is regulated by NAD^+, and the rate-limiting enzyme in NAD^+ biosynthesis (NAMPT) is under circadian control via a CLOCK-BMAL1-SIRT1 circuit, forming an interlocked transcriptional-enzymatic feedback loop (Nakahata et al., 2009; Ramsey et al., 2009). cAMP signaling also constitutes a novel feedback circuit: the transcription-based loops drive rhythms of cAMP signaling, and dynamic changes in cAMP signaling regulate transcriptional output cycles (O'Neill et al., 2008).

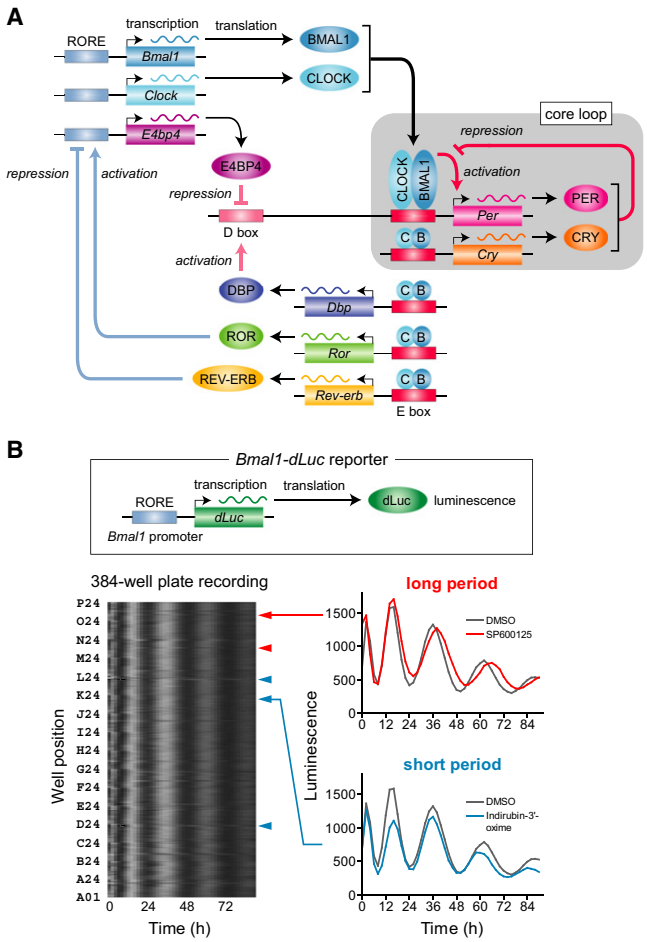

Figure 1. Mammalian Circadian Clock Mechanism and High-Throughput Circadian Assay

(A) Transcription factor feedback loops of the mammalian circadian clock. In the core loop, heterodimers of CLOCK (or NPAS2) and BMAL1 activate transcription from the E box element, and PER and CRY proteins inhibit the activation. In addition, DBP (or TEF, HLF) activates and E4BP4 represses D box-mediated regulation, and ROR proteins activate and REV-ERV proteins repress RORE-mediated regulation, forming interlocking loops. These feedback loops generate the rhythmic expression of not only clock genes, but also of output genes to control the circadian changes in physiology and behavior.

(B) Circadian high-throughput screening of a compound library. A clonal reporter cell line was established by using the circadian reporter *Bmal1-dLuc* (top panel). Luminescence intensity of the reporter cells showed circadian rhythm by reflecting *Bmal1* promoter activity. The rhythm was monitored in the presence of compounds (final 7 μM). One screening of the compound library LOPAC contained four 384-well plates, and profiles of one 384-well plate are represented in the bottom left panel. Each horizontal raster line represents a single well, and elapsed time is plotted to the right. Luminescence intensity data from each well are normalized for amplitude, and then indicated by gray scale: peak is white and trough is black. Red and blue arrowheads indicate the positions of long- and short-period compounds, respectively. Note that there are many compounds that change the phase of the rhythm without affecting the period. Bottom right panels indicate representative traces for a long-period compound (SP600125) and a short-period compound (Indirubin-3′-oxime).

Pharmacological Approaches for the Circadian Clock Mechanism

The application of well-characterized compounds has provided important insights into the molecular mechanism of the circadian clock. Here, we summarize protein kinase inhibitors that have been extensively used (Table 2). Of note, the effectiveness of a pharmacological approach is not only limited to kinases. For example, a set of pharmacological studies identified the interlocking loops of cAMP signaling and NAD^+ metabolism (O'Neill et al., 2008; Nakahata et al., 2009; Ramsey et al., 2009).

CKIδ and CKIε

Genetic and biochemical studies beautifully indicated the role of CKIδ/ε in the mammalian circadian clock. Hamster *tau* mutants showing a short-period behavioral rhythm have a missense mutation in the *CKIε* gene (Lowrey et al., 2000), and human familial advanced sleep phase syndrome (FASPS) with early sleep times and early-morning awakening is attributed to missense mutations of the *PER2* and *CKIδ* genes (Toh et al., 2001; Xu et al., 2005). Mouse models harboring these mutations show a similar short-period phenotype (Xu et al., 2007; Meng et al., 2008). Molecularly, CKIδ/ε phosphorylate PER proteins, causing proteasomal degradation, and *tau* and FASPS mutations lead to higher activity of PER degradation than wild-type, explaining the short-period phenotype (Gallego et al., 2006; Vanselow et al., 2006; Meng et al., 2008).

The functional importance of CKIδ/ε was successfully supported by the known CKIδ/ε inhibitors IC261, CKI-7, and D4476, all of which cause period lengthening in cultured cells (Eide et al., 2005; Vanselow et al., 2006; Reischl et al., 2007; Hirota et al., 2008). Furthermore, the CKIδ/ε inhibitor PF-670462 and the CKIε-selective inhibitor PF-4800567 have recently been developed, and their effects on the circadian period revealed a minimal role of CKIε and a much more prominent role of CKIδ in period regulation (Walton et al., 2009). This finding is consistent with a recent study with *CKIδ*- and *CKIε*-deficient mice (Etchegaray et al., 2009).

CK2

Our own compound screening strategy in human cells (see below) identified a CK2 inhibitor, DRB, as a long-period acting compound (Hirota et al., 2008). Similarly, another CK2 inhibitor, DMAT, causes period lengthening (Maier et al., 2009; Tsuchiya et al., 2009). Moreover, RNAi-based screening approaches have also identified the role of CK2 in the regulation of period length (Maier et al., 2009). These observations are consistent with previous

Table 1. Histone-Modifying Enzymes and Cofactors/Ligands Binding to Clock Proteins

Clock Proteins	Binding Partners	References
Histone Acetylation		
CLOCK/NPAS2-BMAL1	p300/CBP and PCAF	Etchegaray et al., 2003; Curtis et al., 2004
CRY1	mSin3B and HDAC1/2	Naruse et al., 2004
REV-ERBα	NCoR and HDAC3	Alenghat et al., 2008
RORa	PGC-1α, p300, and GCN5	Liu et al., 2007c
Histone Methylation		
CLOCK-BMAL1	EZH2	Etchegaray et al., 2006
PER1/2	WDR5	Brown et al., 2005
Cofactors/Ligands		
CLOCK/NPAS2-BMAL1	NAD	Rutter et al., 2001
NPAS2	Heme	Dioum et al., 2002
PER2	Heme	Kaasik and Lee, 2004
CRY1/2	FAD	Hitomi et al., 2009
REB-ERBα/β	Heme	Raghuram et al., 2007; Yin et al., 2007
RORa	Choresterol derivative	Kallen et al., 2002

findings in *Drosophila* that decreased activity of CK2 causes long-period behavioral rhythms (Lin et al., 2002; Akten et al., 2003). Further biochemical studies revealed that CK2 phosphorylates PER2 to regulate its stability (Maier et al., 2009; Tsuchiya et al., 2009) and also phosphorylates BMAL1 to regulate its nuclear accumulation (Tamaru et al., 2009). Importantly, PER2 and BMAL1 proteins that have missense mutations at CK2-phosphorylation sites cause abnormal oscillation of the cellular clock, indicating the importance of these modifications (Maier et al., 2009; Tamaru et al., 2009; Tsuchiya et al., 2009).

GSK-3β

Biochemical studies revealed that GSK-3β phosphorylates PER2 for nuclear localization (Iitaka et al., 2005), CRY2 for proteasomal degradation (Harada et al., 2005), and REV-ERBα for stabilization (Yin et al., 2006). Lithium has been proposed to act through GSK-3 inhibition (Quiroz et al., 2004), and it robustly lengthens the circadian period in a wide range of experimental systems (Engelmann, 1987), suggesting that GSK-3 inhibition causes period lengthening. Consistently, reduction of GSK-3 activity by genetic manipulation causes period lengthening in *Drosophila* (Martinek et al., 2001). However, our chemical screening strategy identified two compounds inhibiting both CDK and GSK-3 (indirubin-3′-oxime and kenpaullone) as short-period compounds. Further analyses with GSK-3-selective inhibitors (Chir99021 and 1-azakenpaullone) and RNAi-mediated knockdown revealed that inhibition of GSK-3β clearly causes a short-period phenotype in mammals (Hirota et al., 2008). Similar period shortening was observed with newly developed indirubin derivatives that selectively inhibit GSK-3 (Vougogiannopoulou et al., 2008). Because lithium affects inositol monophosphatase and other phosphomonoesterases as well as GSK-3 (Quiroz et al., 2004), the long-period phenotype in mammals might be mediated by lithium-targeted protein(s) other than GSK-3. Identifying these additional targets is of current interest in the field.

Other Kinases for Period Regulation

Pharmacological studies revealed that a CDK inhibitor, roscovitine; a p38 MAPK inhibitor, SB203580; and a JNK MAPK inhibitor, SP600125, cause period lengthening in cultured *Bulla* eye (Krucher et al., 1997), chicken pineal gland (Hayashi et al., 2003), and mouse SCN (Chansard et al., 2007), respectively. Interestingly, all of them (or the analog) were identified as long-period compounds in our screening with human cells (Hirota et al., 2008). It should be noted that these compounds possibly inhibit CKIδ/ε as well as their primary targets (Hasegawa and Cahill, 2004; Fabian et al., 2005). Additional studies are necessary to clarify the role of CDK, p38, and JNK in the molecular clockwork.

Kinases for Phase Regulation

In addition to playing a role in period regulation studies, pharmacological approaches identified the involvement of kinases in the phase-shifting mechanism. A MEK (ERK kinase) inhibitor, U0126, attenuates light-dependent phase delays and advances in the SCN (Butcher et al., 2002; Coogan and Piggins, 2003) and serum shock-mediated rhythm induction in cultured fibroblasts (Akashi and Nishida, 2000). Similarly, a CaMKII inhibitor, KN-62, attenuates phase delays and advances in the SCN (Golombek and Ralph, 1994). KN-62 also inhibits light-dependent activation of ERK, implicating CaMKII as an upstream regulator of ERK (Butcher et al., 2002). A PKG inhibitor, KT5823, attenuates phase advances, but not phase delays (Ding et al., 1998), suggesting a time-of-day-specific function for PKG. Intriguingly, PKGII-deficient mice show impaired phase delays but normal phase advances (Oster et al., 2003), contrary to what was observed in the pharmacological study. In cultured fibroblasts, an ALK inhibitor, SB431542, attenuates alkaline shock-induced phase delays, and ALK-SMAD3-*Dec1* signaling was identified as a novel clock input pathway (Kon et al., 2008).

High-Throughput Screening: A New Avenue for the Circadian Assay

In addition to the individual approach with a small number of compounds, comprehensive screening of compound libraries will be effective in investigating the molecular clock mechanism. Advances in cell-based circadian assays and bioluminescence recording technology (Nagoshi et al., 2005; Welsh et al., 2005) enabled us to develop a high-throughput circadian functional assay (Hirota et al., 2008). This system consists of luminescent reporter cells, screening automation, and a data analysis pipeline. We utilized the circadian luciferase reporter *Bmal1-dLuc*, which expresses the rapidly degradable luciferase under the control of *Bmal1* gene promoter (Liu et al., 2008), to monitor circadian rhythms in cultured cells (Figure 1B, top panel). The GNF Automated Compound Profiling System (Melnick et al., 2006) was applied to record the luminescence every 2 hr over the course of 4 days. A unique aspect of the circadian screening is that the phenotype is not only a simple intensity change, but

Table 2. Kinase Inhibitors Changing the Circadian Period

Compounds	Primary Targets	Period Phenotypes	Cell Types	References	Phenotypes in U2OS Cells[a]
IC261	CKIδ/ε	Long period	Rat-1 fibroblasts	Eide et al., 2005	Not tested
CKI-7	CKIδ/ε	Long period	NIH 3T3 fibroblasts	Vanselow et al., 2006	Not tested
D4476	CKIδ/ε	Long period	NIH 3T3 fibroblasts	Reischl et al., 2007	Long period
PF-670462	CKIδ/ε	Long period	Rat-1 fibroblasts	Walton et al., 2009	Not tested
PF-4800567	CKIε	No change	Rat-1 fibroblasts	Walton et al., 2009	Not tested
DRB	CK2	Long period	*Aplysia* eye	Raju et al., 1991	Long period
DMAT	CK2	Long period	U2OS cells; NIH 3T3 fibroblasts	Maier et al., 2009; Tsuchiya et al., 2009	Not tested
Indirubin-3′-oxime	CDK&GSK-3	Not tested	Not tested	Not tested	Short period
Kenpaullone	CDK&GSK-3	Short period	Rat-1 fibroblasts	Vougogiannopoulou et al., 2008	Short period
Chir99021	GSK-3	Not tested	Not tested	Not tested	Short period
1-azakenpaullone	GSK-3	Not tested	Not tested	Not tested	Short period
Indirubin derivatives	GSK-3	Short period	Rat-1 fibroblasts	Vougogiannopoulou et al., 2008	Not tested
Roscovitine	CDK	Long period	*Bulla* eye	Krucher et al., 1997	Long period
SB203580/SB202190	p38	Long period	Chicken pineal gland	Hayashi et al., 2003	Long period
SP600125	JNK	Long period	Mouse SCN	Chansard et al., 2007	Long period

[a] Hirota et al., 2008.

also an alternation in a repeating cycle. Because of this uniqueness, a specialized algorithm is necessary for identifying the "hits." We developed a curve-fitting program, CellulaRhythm, to calculate rhythm parameters such as period length from large amounts of luminescence data of 384-well plate recordings. The program can also visualize the luminescence rhythms as traces and heat maps for manual inspection of the validity of the calculated parameters. A more sophisticated software, MultiCycle (Actimetrics), has recently been developed, and it works in a manner similar to CellulaRhythm for the circadian parameter estimation. Between the parameters (period, phase, amplitude, and damping rate) we focused on the period, because a deficiency of the core clock mechanism can be reflected to an alternation of the period. A more severe phenotype is arrhythmicity, but it is difficult to differentiate from the effect on the general health of the cells. Importantly, the period is the most robust parameter, stemming from the repeating characteristics of the clock.

The success of the screening may depend on the robustness of the system. Especially, the cellular rhythmicity is a key factor for the circadian screening. Among all cell lines tested, human U2OS cells showed prominent and highly reproducible rhythmicity even in a 384-well format. In control (untreated) conditions, more than 97% of the wells are within the period range of mean ± 0.5 hr. We applied this system to further dissect the circadian clock mechanism by using a chemical biology approach. A structurally diverse chemical library, LOPAC (Library of Pharmacologically Active Compounds), containing 1280 pharmacologically active compounds was initially used to analyze the effect on the circadian period length in human U2OS cells (Figure 1B, bottom panels). We identified 11 compounds causing reproducible period changes of $\geq$ 0.5 hr. Among them, 7 compounds are protein kinase inhibitors/activators, including roscovitine, SP600125, and SB202190 (an analog of SB203580), previously known to change the circadian period in other organisms (Table 2). Importantly, the period effects of indirubin-3′-oxime, kenpaullone, and DRB predicted the novel roles of GSK-3β and CK2 in the mammalian circadian clock mechanism (Table 2). Together, these observations demonstrate the validity of the high-throughput circadian assay system and the effectiveness of chemical biology in exploring unidentified mechanisms of the circadian clock.

Our group and others recently developed 96- or 384-well format high-throughput circadian assays for RNAi-based genomic screening (Hirota et al., 2008; Vollmers et al., 2008; Maier et al., 2009; Zhang et al., 2009). The screening of RNAi libraries for human kinases suggested the involvement of more than 22 kinases in the cellular clock mechanism (Maier et al., 2009; Zhang et al., 2009). For example, knockdown of MAPK8 (JNK1) causes period lengthening (Zhang et al., 2009), which is consistent with the effect of a JNK inhibitor, SP600125 (Chansard et al., 2007; Hirota et al., 2008). A combination of genomic approaches and additional screening of kinase inhibitor libraries will likely reveal novel roles for many kinases in the mammalian clockworks.

Future Outlook of the Chemical Biology Approach for the Circadian Clock

In the screening of limited numbers of well-characterized compounds, many of the hits were related to the pathways already known to affect the circadian clock function. Therefore, it is interesting to expand the circadian screening for more comprehensive, large-scale compound libraries containing hundreds of thousands of compounds. A wide variation of chemical structures has the advantage of probing many classes of potential targets, which may include not only kinases, but also other proteins such as histone-modifying enzymes, metabolic enzymes, and even clock proteins. An attractive possibility of a chemical biology

approach will be the identification of novel clock components that cannot be easily achieved by forward and reverse genetic screens because of lethality, pleiotropy, and functional overlapping of closely related proteins. Although our high-throughput compound screening approach is very powerful, there are several important points to keep in mind. First, generally, it is technically challenging to identify proteins specifically affected by the novel compound. In addition, compounds (even well-characterized ones) may have multiple targets that give rise to the observed circadian effect. After the identification of candidate proteins, additional studies with other compounds targeting the same protein(s) (if available) and/or RNAi-mediated knockdown are necessary. The confirmation of the phenotype by multiple reagents may lead to the determination of the responsible protein(s). Simultaneous knockdown of multiple genes will be required if there is functional overlap, and this approach cannot be easily achieved in RNAi screening, which targets each gene one by one. Second, the phenotype may arise not only from a direct effect on the clock, but also from indirect effects such as alterations of general transcription/translation and the overall health of the cells. Although it is difficult to exclude the possibility of indirect effects, biochemical studies play important roles in exploring the mechanism of direct effects. For example, CK2 directly phosphorylates clock proteins for rhythm regulation (Maier et al., 2009; Tamaru et al., 2009; Tsuchiya et al., 2009), besides it has a general effect on cell health and transcription (St-Denis and Litchfield, 2009). Third, there are potential differences in the phenotypes between different candidate cell lines that can be used for screening. Given that the majority of the genes showing circadian expression are tissue specific (Duffield, 2003), clock modifiers could be cell type specific. Immortalized cell lines may have aberrant signaling pathways that possibly affect the circadian phenotype. Thus far, however, many of the period-changing compounds are effective in a variety of cell types, including primary cells and even other organisms (Table 2). In contrast, a phase-changing compound, dexamethasone, is effective in peripheral tissues, but not in the SCN because of the absence of glucocorticoid receptor (Balsalobre et al., 2000). Testing the effect of compounds in the SCN and peripheral tissues ex vivo as well as in vivo will provide new opportunities for common and tissue-specific clock mechanisms. Taken together, chemical biology will play an increasingly important role along with genome-wide RNAi screening in dissecting the molecular mechanism of the circadian clock.

Furthermore, compound screening will generate novel proof-of-concept probes for manipulating clock functions in a dose-dependent and inducible manner. Such proof-of-concept probes will provide the chemical starting points for the identification of small-molecule therapeutics designed for circadian disorders. For example, a novel CKIδ/ε inhibitor might be useful in treating the observed short-period phenotype caused by FASPS and *tau* mutations. Interestingly, many clock proteins bind to cofactors or ligands (Table 1) (Rutter et al., 2001; Dioum et al., 2002; Kallen et al., 2002; Kaasik and Lee, 2004; Raghuram et al., 2007; Yin et al., 2007; Hitomi et al., 2009), and CLOCK protein has an acetyltransferase activity (Doi et al., 2006). Compounds affecting these activities may alter circadian clock function and can be used as specific modulators of circadian oscillations.

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