

REVIEW

AMP-activated protein kinase as a key molecular link between metabolism and clockwork

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Circadian clocks regulate behavioral, physiological and biochemical processes in a day/night cycle. Circadian oscillators have an essential role in the coordination of physiological processes with the cyclic changes in the physical environment. Such mammalian circadian clocks composed of the positive components (BMAL1 and CLOCK) and the negative components (CRY and PERIOD (PER)) are regulated by a negative transcriptional feedback loop in which PER is rate-limiting for feedback inhibition. In addition, posttranslational modification of these components is critical for setting or resetting the circadian oscillation. Circadian regulation of metabolism is mediated through reciprocal signaling between the clock and metabolic regulatory networks. AMP-activated protein kinase (AMPK) in the brain and peripheral tissue is a crucial cellular energy sensor that has a role in metabolic control. AMPK-mediated phosphorylation of CRY and Casein kinases I regulates the negative feedback control of circadian clock by proteolytic degradation. AMPK can also modulate the circadian rhythms through nicotinamide adenine dinucleotide-dependent regulation of silent information regulator 1. Growing evidence elucidates the AMPK-mediated controls of circadian clock in metabolic diseases such as obesity and diabetes. In this review, we summarize the current comprehension of AMPK-mediated regulation of the circadian rhythms. This will provide insight into understanding how their components regulate the metabolism.

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INTRODUCTION

Circadian rhythms are under the direct influence of environmental cues, most notably the day–night cycles and controlled a genetically determined endogenous clock.¹ Circadian (derived from the Latin *circa diem*; about a day) clock could adapt organisms to the natural environment and then enable them to anticipate the environmental changes that the behavior and physiology of organisms can be adjusted to proper time a day.

Circadian rhythms are widely distributed in plants, animals, fungi and cyanobacteria, and are regulated by endogenous molecular oscillators called circadian clocks.^{2,3} In mammals, important daily activities such as sleep/wake cycles and metabolic homeostasis are governed by the endogenous circadian clock.^{4–6}

Available data suggest that the major mechanism of the molecular clock is a transcriptional negative feedback loop containing CLOCK (or NPAS2), BMAL1, PERIOD (PER), and CRYPTOCHROME (CRY). The CLOCK (or NPAS2)-BMAL1

heterodimer activates transcription of the negative elements, PER and CRY, as well as circadian output genes, through E-box enhancer elements.^{3–8} As PER levels increase in the cytoplasm, PER associates with CRY, the complex enters the nucleus to shut down transcription driven by CLOCK-BMAL1 complex. Thus, temporal accumulation and degradation rates of PER predominate in determining the timing of the negative feedback loop. There is one more regulatory feedback loop, in which RORs activate the transcription of BMAL1 and CLOCK, whereas Rev-Erbs repress BMAL1, CLOCK and NPAS2 through retinoic acid-related orphan receptor response element (RORE; Figure 1).^{9–11}

In MEFs, CLOCK, BMAL1 and CRY1 are similarly abundant, which is different from liver where BMAL1 is far less abundant than the other two. CRY2, PER1 and PER2 are less abundant than the other core clock proteins. The levels of PER1/2 (the limiting component in the negative complex) to BMAL1 (the limiting component in the positive complex) are almost 1:1. The combined levels of PER1 and PER2 are only

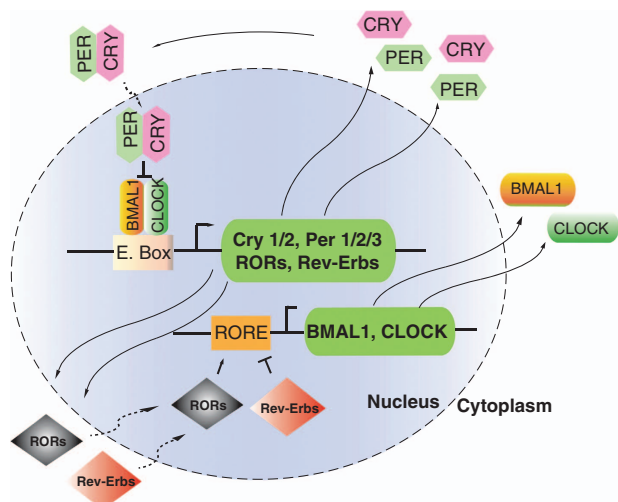


Figure 1 Feedback loops control the mammalian circadian clock. Mechanism of the molecular clock is a transcriptional negative feedback loop containing CLOCK, BMAL1, (PERIOD) PER and CRY. The CLOCK-BMAL1 heterodimer binds to enhancer E-box located in the promoter region of *Per* and *Cry* genes to activate their transcriptions. After translation, PERs and CRYs perform nuclear translocation and inhibit CLOCK-BMAL1, resulting in decreased transcription of their genes. There is other regulatory feedback loop. CLOCK-BMAL1 also induces the expression of Rev-Erbs and RORs, and in turn RORs activate the transcription of BMAL1 and CLOCK, whereas Rev-Erbs repress BMAL1 and CLOCK through retinoic acid-related orphan receptor response element (RORE) binding.

about half of those of CLOCK and BMAL1, implying that CLOCK-BMAL1 would be twice as abundant as PER-CRY, a negative complex.¹² This finding suggests that the ratio between the negative and positive complexes must be important for the molecular oscillator and rhythm generation, and PER seems to be rate-limiting for the rhythms.^{7,12,13}

Some clock-controlled genes are transcription factors such as albumin D-box-binding protein, ROR α and REV-Erb α , which regulate cyclic expression of other genes.¹⁴ D-box-binding protein binds to D-boxes (TTA (T/C) GTAA), whereas ROR α and Rev-Erb α bind to the Rev-Erb/ROR binding element, or RRE [(A/T) A (A/T) NT (A/G) GGTC]. Approximately 10% of the transcriptome displays robust circadian rhythmicity.^{15,16}

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine protein kinase that works as a central sensor of metabolic signals.¹⁷ AMPK is activated by adenosine triphosphate (ATP) exhaustion, which causes an increase in the AMP/ATP ratio.¹⁸ Once activated, AMPK switches on catabolic pathways to produce ATP while simultaneously shutting down energy-consuming anabolic processes.

AMPK is a heterotrimeric protein kinase consisting of a catalytic (α) subunit and two regulatory (β , γ) subunits. The N-terminus of the subunit contains a typical serine/threonine protein kinase catalytic domain. The C-terminal half of the α subunit contains a region of approximately 150 amino-acid

residues at the extreme C-terminus required for association with the β and γ subunits, whereas a region immediately downstream of the catalytic domain appears to have an inhibitory function.¹⁹ The β subunit has two conserved domains located in central and C-terminal region. C-terminal region is required to form a functional $\alpha \beta \gamma$ complex that is regulated by AMP, whereas the central domain is recognized for a glycogen-binding domain.^{20,21} The γ subunits ($\gamma 1$, $\gamma 2$ and $\gamma 3$) contain variable N-terminal regions followed by four tandem repeats of a 60-aa sequence termed as a CBS motif.²² The γ subunit contains four CBS domains, which bind AMP or ATP.^{23,24}

AMPK activated by increases in adenosine diphosphate (ADP) and AMP signals that the energy state of the cell is compromised. It is active when phosphorylated at T172 on the catalytic (α) subunit. Activation of AMPK by liver kinase B1-mediated²⁵ or calcium-calmodulin-dependent protein kinase, kinase β -mediated²⁶ phosphorylation is increased in the presence of high ratios of AMP/ATP or elevated intracellular calcium, respectively. Binding of ADP and AMP to the γ subunit cause conformational changes that inhibit T172 dephosphorylation and cause further allosteric activation.¹⁷

AMPK has been recognized as a key regulator of mammalian metabolic function. Nutrient-regulated diurnal phosphorylation of AMPK substrates in rat livers²⁷ makes AMPK an attractive candidate contributor to peripheral clock entrainment. Biochemical and bioinformatics studies have established the optimal amino-acid sequence context in which phosphorylation by AMPK is likely,^{28,29} which has facilitated prediction of novel substrates.

In this review, we will discuss the comprehensive studies on the catalytic activity of AMPK regulating circadian rhythms that affect behavior, energy metabolism and gene expression. We seek to further explore the connection among circadian rhythms, AMPK and metabolism.

A CONNECTION BETWEEN CIRCADIAN RHYTHMS AND AMPK

AMPK activation has recently been linked to regulation of the circadian clock, which couples daily light and dark cycles to the control of physiology in a wide variety of tissues and the hypothalamus through tightly coordinated transcriptional programs.³⁰ Several master transcription factors are involved in orchestrating this oscillating network.

THE ROLE OF AMPK IN THE PHOSPHORYLATION AND DEGRADATION OF CRYPTOCHROME

In mammals, the maintenance of circadian clock function depends on clock genes and their protein products in autoregulatory transcriptional feedback loops. In an autoregulatory feedback, the cyclic translation of *Per* and *Cry* messenger RNA leads to cyclic levels of PER and CRY proteins. These proteins form complexes and accumulate in the nucleus where they inhibit expression of their genes by acting on CLOCK-BMAL1 heterodimers.⁴

AMPK was shown to regulate the stability of the core clock component CRY1, which acts as energy sensors and can convert nutrient signal to circadian clocks. This process is performed by phosphorylation of CRY1-Ser 71, which stimulates the direct binding of the F-box and leucine-rich repeat protein 3 (FBXL3) to CRY1, targeting it for ubiquitin-mediated degradation.³¹

The importance of CRY stability for determining the speed of mammalian clocks became apparent when the most prominent mutants identified in each of two forward genetic screens for circadian rhythm perturbation in mice where alleles of the E3 ligase component FBXL3³² that catalyzes the polyubiquitination of CRY1 and CRY2 and thus stimulates their proteasomal degradation.³³

Previous studies showed that FBXL3 interacts with CRY1 and CRY2, promoting the degradation of both proteins by the ubiquitin/proteasome system, thus contributing to period length determination. Mutation of FBXL3 (C358S or I364T), a component of a SKP1-CUL1-F-box (SCF) E3 ubiquitin ligase complex, results in ~26-h period phenotypes in mice, indicating that FBXL3 has an important role in circadian period determination.^{34–36} However, overexpression of CRY1 protein does not lead to period alteration,³⁷ suggesting that the FBXL3 mutation might affect additional clock components.

Thus, loss of AMPK signaling *in vivo* stabilizes CRYs and disrupts circadian rhythms, consistent with the hypothesis that this pathway contributes to the metabolic control of light-independent peripheral circadian clocks. Given that AMPK is a central regulator of metabolic processes, the rhythmic regulation of AMPK has implications for the circadian regulation of metabolism. Genetic alteration of circadian clocks either ubiquitously³⁸ or in a tissue-specific manner³⁹ elicits dramatic changes in feeding behavior, body weight, running endurance and glucose homeostasis, each of which is also altered by manipulation of AMPK.^{40–45} The abilities of AMPK to mediate circadian regulation and of CRY1 to function as a chemical energy sensor suggest a close correlation between metabolic and circadian rhythms (Figure 2).

THE ROLE OF AMPK IN THE PHOSPHORYLATION OF CASEIN KINASE I

CKI represents a unique group within the superfamily of serine/threonine-specific protein kinases that is ubiquitously expressed in eukaryotic organisms.⁴⁶ The molecular weight of mammalian CKI isoforms (α , β , γ 1, γ 2, γ 3, δ and ϵ) varies from 37 kDa (CKI α) to 51 kDa (CKI γ 3). There are highly conserved sequences within their kinase domains in all CKI isoforms, but differ significantly in the length and primary structure of their N-terminal (9–76 aa) and C-terminal non-catalytic domains (24 aa up to more than 200 aa).^{47,48}

Casein kinases are important modulators of circadian clock function in mammals. A naturally occurring mutation in hamsters (Tau) that causes a long circadian period was determined to be a hypomorphic allele of casein kinase I epsilon (CKI ϵ).⁴⁹ In addition, genetic disruption or pharmacological inhibition of CKI ϵ and/or casein kinase I

delta (CKI δ) alters both cellular and behavioral circadian rhythms in mice. Casein kinases, preferentially phosphorylate serines located within negatively charged amino-acid sequence motifs and several serines in PER2 (which are conserved in PER1) have been identified as targets of CKI phosphorylation.⁵⁰ CKI-mediated phosphorylation of PER proteins is a primary determinant of their stability and circadian period.⁵¹

AMPK induces a phase advance of circadian expression of clock genes by degrading PER2 through phosphorylating CKI ϵ Ser389. AMPK phosphorylates Ser389 of CKI ϵ , resulting in increased CKI ϵ activity and degradation of PER2, leading to shortened period length (Figure 2).⁵²

PER proteins are progressively phosphorylated and disappear over a circadian day. Numerous studies using biochemical and genetic approaches showed that CKI δ/ϵ phosphorylates PER *in vitro* and in cultured cells.^{41–43} Phosphorylation of PER affects its cellular location and stability.^{9,43–45} In *Drosophila*, genetic studies have demonstrated that double-time, an ortholog of CKI δ/ϵ , is required for normal phosphorylation and turnover of dPER, and for behavioral circadian rhythms.^{46,53} However, in mammals, the known mutations in CKI ϵ or CKI δ , including null mutations,⁵¹ do not substantially disrupt the molecular oscillator and circadian rhythms to the extent seen in *Drosophila* mutants carrying the *dbt^P* or *dbt^{AR}* allele.^{51,53,54} This suggests that the two mammalian enzymes are at least partially redundant, or there are other kinases that can compensate for the loss of CKI δ/ϵ . In mutant mammals carrying mutations in CKI ϵ or CKI δ , PER still oscillates in abundance and phosphorylation. Interestingly, a CKI δ null mutation produced more severe phenotypes than did a CKI ϵ null mutation, suggesting that they may not be equally redundant.⁵¹ In mammalian cells, as in *Drosophila*, the dominant-negative form of CKI shows that

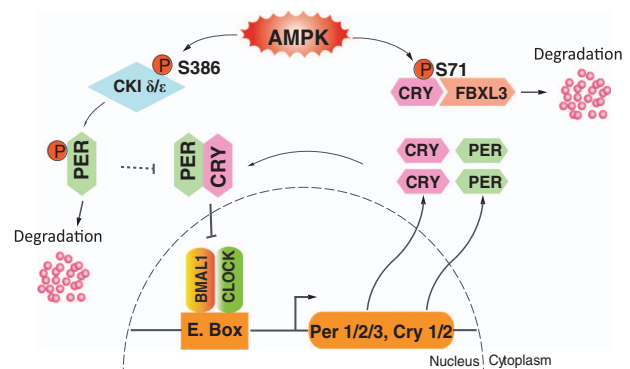


Figure 2 The regulation of AMP-activated protein kinase (AMPK) on CRY1 and CKI δ/ϵ in circadian clock. AMPK phosphorylates CRY1, leading to its interaction with FBXL3. This process promotes the degradation of both proteins by the ubiquitin/proteasome system, thus contributing to period length determination. AMPK also phosphorylates Ser389 of CKI ϵ , resulting in increased CKI ϵ activity and degradation of PER2, which lead to shortened period length.

general reduction of CK1 δ/ϵ activities results in slower oscillating of circadian rhythms.

Like in *Drosophila*, the phosphorylation of mammalian PER by CK1 δ/ϵ stimulates its degradation. In mammals, the CK1 δ/ϵ binding domain and phosphorylation sites of PER proteins have been identified.¹⁹ Phosphorylation of PER by CK1 δ/ϵ leads to conformational changes and masking of a nuclear localization signal.²³ However, CRY proteins form a complex with PER and CK1 δ/ϵ and protect PER from degradation that leads to nuclear accumulation of the CRY–PER–CK1 δ/ϵ complex.^{19,23} This complex inhibits the transcriptional activity of the DNA-bound BMAL1–CLOCK complex, thus the transcription of CRY and PER. It is thought that phosphorylation of different PER proteins by CK1 δ/ϵ has additional effects on the regulation of each PER protein. Furthermore, CK1 δ/ϵ is able to phosphorylate BMAL1 and CRY proteins thereby modulating the functions of these clock proteins.²⁰

THE ROLE OF AMPK ON SIRT1-MEDIATED REGULATION IN CIRCADIAN CLOCK

The regulation of gene expression that characterizes circadian physiology is involved in dynamic changes in chromatin transition.⁵⁵ Activation of clock-controlled genes by CLOCK:BMAL1 is associated with circadian changes in histone modifications at their promoters. CLOCK itself has a role as an enzyme with histone acetyltransferase activity, specifically targeting H3 K9/K14 in the chromatin and also non-histone targets, such as its own partner BMAL1.⁵⁶ The histone acetyltransferase activity of CLOCK is counterbalanced by silent information regulator 1 (SIRT1), a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent histone deacetylases.^{57,58} SIRT1 is a nuclear protein implicated in critical metabolic and physiological processes.

SIRT1 is also involved in the suppression of many age-related diseases such as cancer, Alzheimer's disease and type 2 diabetes.⁵⁹ At the cellular level, SIRT1 controls DNA repair and apoptosis, circadian clocks, inflammatory pathways, insulin secretion and mitochondrial biogenesis.⁶⁰

The reciprocal play between AMPK and SIRT1 is implicated in circadian clock and metabolic state through interacting with each other. AMPK enhances SIRT1 activity through increasing cellular NAMPT expression and nicotinamide adenine dinucleotide levels, leading to inhibition of CLOCK–BMAL1 complex. AMPK-mediated SIRT1 action also activates the deacetylation and modulation of the activity of downstream SIRT1 targets that include the peroxisome proliferator-activated receptor- γ coactivator 1 α , PGC-1 α and the forkhead box O1, -O3 (FOXO3a) transcription factors. The AMPK-induced SIRT1-mediated deacetylation of these targets explains many of the convergent biological effects of AMPK and SIRT1 on energy metabolism (Figure 3).^{61,62}

SIRT1 functions as an enzymatic controller of CLOCK function, transducing signals originated by cellular metabolites to the circadian machinery. Targeting SIRT1 with small molecule modulators has been a significant area of interest for several years. The study on the regulation of AMPK for

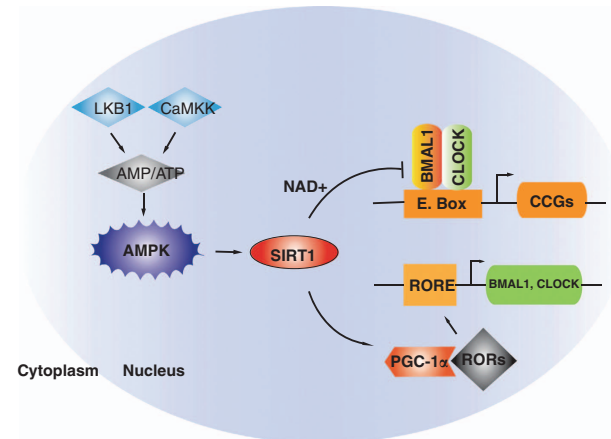


Figure 3 The mechanism of silent information regulator 1 (SIRT1) on circadian regulation. The reciprocal play between AMPK-activated protein kinase and SIRT1 is implicated in circadian clock and metabolic state. AMPK enhances SIRT1 activity through increasing cellular NAMPT expression and nicotinamide adenine dinucleotide (NAD⁺) levels, leading to inhibition of CLOCK–BMAL1 complex. AMPK-mediated SIRT1 action also activates the deacetylation and modulation of the activity of downstream SIRT1 targets that include the PGC-1 α .

SIRT1 is of importance primarily based on the promise that this approach holds for the discovery of new therapeutic agents for multiple diseases of aging.^{63,64}

THE INFLUENCE OF AMPK ON CIRCADIAN RHYTHMS IN METABOLISMS

The role of AMPK on the effects of energy balance by modulating the palmitate in the hypothalamus

Energy homeostasis of our bodies is under the regulated control of homeostatic hormones, nutrients and the expression of neuropeptides that alter feeding behavior in the hypothalamus.

AMPK has an important role in food intake and energy metabolism because it affects both the central nervous system and peripheral tissues. Many studies show that the activation of the hypothalamic AMPK is involved in the stimulation of food intake and the hypothalamic AMPK activity is increased during fasting and decreased during refeeding.^{65–67}

Constitutively, active AMPK led to increases in neuropeptide Y (NPY) and agouti-related peptide (AgRP) messenger RNA levels and subsequently caused an increase in the body weight of mice, whereas AMPK inhibition with dominant-negative forms of AMPK prevented increased weight gain and decreased the messenger RNA levels of orexigenic neuropeptides.⁶⁶ NPY gene expression is controlled by numerous signaling cascades. Phosphatidylinositol 3-kinase (PI3K), MAPK, mammalian target of rapamycin (mTOR) and AMPK have all been implicated in the control of NPY gene expression.

It was found that there is a link between excess palmitate concentrations and changes in clock genes and orexigenic neuropeptide messenger RNA levels at the level of a hypothalamic neuron.²¹ A high-fat diet leads to changes in the

expression of circadian behavior and transcripts such as CLOCK, BMAL1 and PER2 in the hypothalamus, liver and fat cells.⁶⁸ Elevated levels of palmitate, a predominant saturated fatty acid in diet and fatty acid biosynthesis, alter cellular function. It is likely that palmitate-induced signal transduction cascades lead to changes in circadian transcript expression such as an increase in BMAL1 and CLOCK and a decrease in PER2 and Rev-Erb α through AMPK-mediated regulation.

Taken together, AMPK might have a role in the palmitate-mediated regulation of clock genes in addition to regulation of orexigenic neuropeptide NPY in the hypothalamus.

Interplay between AMPK and metformin

Metformin is an important feedback to the circadian clock in the peripheral tissues, synchronizing it to the environmental cues such as food availability.⁶⁹ Metformin is a commonly-used treatment for type 2 diabetes whose mechanism of action has been linked, in part, to activation of AMPK. However, little is known regarding metformin effect on circadian rhythms.

Metformin leads to increased leptin and decreased glucagon levels.^{70,71} The effect of metformin on liver and muscle metabolism similarly leads to AMPK activation either by liver kinase B1 and/or other kinases in the muscle.⁷² Metformin blocks mitochondria complex I leading to increased NADH levels. An increase of NADH leads to enhanced activity of CLOCK-BMAL1-mediated expression. Metformin activates liver CKI α and muscle CKI ϵ , leading to CKI-mediated phosphorylation of PER2.⁷³ Growing results show the differential effects of metformin in the liver and muscle and the critical role that the circadian clock has in orchestrating metabolic processes. Those processes might be mediated by AMPK activation through metformin.

Circadian clock and metabolic diseases

Circadian rhythms are integral to the normal functioning of numerous physiological processes. The center of the biological clock exists in the suprachiasmatic nuclei. In addition to this central clock, each organ has its own biological clock system, termed the peripheral clock.

Each cardiovascular tissue or cell, including heart and aortic tissue, cardiomyocyte, vascular smooth muscle cell and vascular endothelial cell also has intrinsic biological rhythm.^{74,75} The peripheral clock system within each cardiovascular organ seems to have significant roles during the progression of cardiovascular disorders.⁷⁶ Loss of synchronization between the internal clock and external stimuli can induce cardiovascular organ damage. Discrepancy in the phases between the central and peripheral clocks also seems to contribute to progression of the disorders.

The important role of CKI δ/ϵ as one of the clock proteins is underlined by the fact that mutations of CKI or mutations of phosphorylation sites of their substrates are correlated with various diseases.

In mammals, similar defects have been described for a CKI ϵ mutant. Syrian hamsters homozygous for the Tau mutation in

the CKI ϵ gene resulting in an exchange of the conserved amino-acid residue 178 (R178C) have a shortened circadian period.¹⁰ The reason for this effect could be explained by the dominant-negative features of this CKI ϵ mutant. CKI ϵ R178C still associates with PER but its kinase activity is much lower compared to that of wild-type CKI ϵ . As a consequence, PER is hypophosphorylated and more stable. Although CKI δ partly compensates the mutant CKI ϵ , the preserved ratio of CKI ϵ and CKI δ binding to PER proteins still leads to hypophosphorylation of PER, resulting in shortening of the circadian rhythm in hamsters with Tau mutation.⁷⁷

In humans, a polymorphism of CKI ϵ at the autophosphorylation site serine 408 in which serine is substituted by asparagine (S408N) seems to have a protective role in the development of familiar advanced sleep phase syndrome (FASPS).⁷⁷ The lack of this autophosphorylation site results in an increased kinase activity of CKI ϵ and an elongation of the circadian rhythm. In addition, substitution of serine with glycine at amino-acid residue 662 within the CKI ϵ binding domain of mPER2, which reduces CKI ϵ -mediated phosphorylation of mPER, is found in FASPS patients.¹⁸ Furthermore, the polymorphism V647G of the hPER3 gene affects the CKI δ/ϵ binding site and correlates with delayed sleep phase syndrome (DSPS).⁷⁷

CONCLUSION AND FUTURE PERSPECTIVE

The master circadian oscillators in suprachiasmatic nuclei are principally entrained by the light/dark cycle through the retina stimulation, while pacemakers in peripheral organs, such as liver, are reset by food availability, hormone, metabolic and neuronal signals.

We expect that the communication of nutritional status to clocks is complex and that additional pathways contribute *in vivo*. The ability of AMPK to respond to metabolic cues and to directly modify circadian clock components suggests that it may be an important mediator of metabolic entrainment in peripheral clocks. AMPK-mediated phosphorylation of CRYs contributes to metabolic entrainment of peripheral clocks. Pharmacological activation of AMPK by intraperitoneal injection of either AICAR (5-aminoimidazole-4-carboxamide ribonucleoside)³¹ or metformin⁵² also caused a phase shift of the liver clock in mice, which suggests a possible ability to entrain the liver clock. These data suggest that AMPK activation may also have a role in circadian entrainment of muscle clocks.⁷⁸

Genetic alteration of circadian clocks either ubiquitously^{9,79} or in a tissue-specific manner³⁹ elicits dramatic changes in feeding behavior, body weight, running endurance and glucose homeostasis, each of which is also altered by manipulation of AMPK.^{40–42,44,45}

There have been many studies of the link between circadian rhythms and metabolism in brain and peripheral organs. The role of AMPK between the circadian clock and metabolism is essential for maintaining metabolic homeostasis and preventing metabolic disorders. The orchestration of circadian

rhythms and metabolic regulation is tightly interlocked at both physiological and molecular levels.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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