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AMPK at the crossroads of circadian clocks and metabolism

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Abstract

Circadian clocks coordinate behavior and physiology with daily environmental cycles and thereby optimize the timing of metabolic processes such as glucose production and insulin secretion. Such circadian regulation of metabolism provides an adaptive advantage in diverse organisms. Mammalian clocks are primarily based on a transcription and translation feedback loop in which a heterodimeric complex of the transcription factors CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle Arnt-like protein 1) activates the expression of its own repressors, the period (PER1-3) and cryptochrome (CRY1,2) proteins. Posttranslational modification of these core clock components is critical for setting clock time or adjusting the speed of the clock. AMP-activated protein kinase (AMPK) is one of several metabolic sensors that have been reported to transmit energy-dependent signals to the mammalian clock. AMPK does so by driving the phosphorylation and destabilization of CRY and PER proteins. In addition, AMPK subunit composition, sub-cellular localization, and substrate phosphorylation are dependent on clock time. Given the well-established role of AMPK in diverse aspects of metabolic physiology, the reciprocal regulation of AMPK and circadian clocks likely plays an important role in circadian metabolic regulation.

1. Introduction

Circadian biological phenomena — from the daily movements of plant leaves to human sleep cycles — have been recognized for centuries, but their underlying physical and biochemical mechanisms remained mysterious for most of that time. A 1972 study (Stephan and Zucker 1972) demonstrated that the suprachiasmatic nucleus (SCN), a collection of approximately 10,000 neurons at the base of the hypothalamus, is required for daily rhythms in animal behavior in response to light stimuli. For a long time mammalian clocks were thought to be confined to this small brain area. However, within the last decade, it has been shown that circadian clocks are widely distributed in mammalian tissues.

Genetic and biochemical studies in the late 1990s have identified the molecular components of the mammalian circadian clock: a molecular oscillator based on negative feedback, in which the transcription factors CLOCK (or the related neuronal PAS domain-containing protein 2, NPAS2) and its partner BMAL1 work together to drive the expression of many genes, including those encoding their own inhibitors — the period (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2) proteins (Green et al. 2008) (Figure 1). Additional loops and layers of control have been described but for the purpose of this review, we will focus on this simplified core clock machinery.

The ability to predict recurring daily changes in the environment could confer an adaptive advantage on organisms that have circadian clocks. Such a phenomenon has been demonstrated in bacteria and plants: mutants in which the circadian period is genetically altered to be either shorter or longer than 24 hours are less fit than wildtype organisms in a natural environment but they will out-compete their wildtype brethren under environmental conditions engineered to match their altered periods (Dodd et al. 2005,Woelfle et al. 2004). These advantages seem to depend on optimized metabolic function: for example, plant leaves store more chlorophyll when their endogenous period matches the period of the external environment.

Several lines of evidence suggest that mammalian clocks in peripheral organs also optimize the timing of metabolic processes and enable efficient energy storage and utilization. First, the expression of enzymes, transporters, and receptors that regulate metabolism robustly fluctuate throughout the day (Panda et al. 2002). Second, the timing of circadian clocks in tissues outside the SCN is set by feeding time rather than by the light-dark schedule (Damiola et al. 2000,Stokkan et al. 2001). Finally, disruption of clock function in either the liver or the pancreas leads to impaired glucose homeostasis while leaving behavioral rhythms intact (Lamia et al. 2008,Marcheva et al. 2010,Sadacca et al. 2011). Notably, liver-specific ablation of *Bmal1* caused lowered blood glucose only during the times of the day when mice are naturally fasting, suggesting that the liver clock predicts recurring daily changes in diet-derived nutrient availability, and increases glucose production during times of expected fasting. Thus, circadian clocks may reduce stress on the systems that mediate response to acute metabolic challenges.

AMPK is a heterotrimeric protein kinase that responds to cellular energy stress by virtue of adenine nucleotides binding to its gamma regulatory subunits (AMPKγ1, AMPKγ2, and AMPKγ3). AMPKγ binds to ATP, ADP or AMP and its AMP- or ADP-bound conformation enables activation of the catalytic alpha subunits (AMPKα1 and AMPKα2) such that AMPK activity increases in response to cellular ATP depletion. The beta regulatory subunits (AMPKβ1 and AMPKβ2) interact with glycogen (Polekhina et al. 2003), are modified by myristoylation (Oakhill et al. 2010), and seem to control the subcellular localization of the heterotrimeric AMPK complex. In the liver, AMPK is activated in response to prolonged fasting (Witters et al. 1994), and twenty-four hour oscillations in the phosphorylation of the AMPK substrates acetyl coA carboxylase (ACC) and Raptor have been observed (Davies et al. 1992,Lamia et al. 2009). AMPK has long been recognized as a central regulator of mammalian metabolic function (Kahn et al. 2005), and as such there has been a great deal of interest in identifying its phosphorylation targets (Mihaylova and Shaw 2011). As part of that effort, two groups have described amino acid sequence motifs that increase the probability of phosphorylation by AMPK (Gwinn et al. 2008,Scott et al. 2002) and several clock components contain potential AMPK target phosphorylation sites (Figure 2). Thus, AMPK has the properties required to act as a clock-resetting signal.

2. AMPK roles in clock function

2.1 Phosphorylation of Cryptochromes

Mammalian cryptochromes (CRY1 and CRY2) are transcriptional repressors that are required for circadian clock function (Sancar 2004). Fruitflies and plants also have cryptochrome proteins but instead of repressing transcription, they function as blue light photoreceptors that are destabilized upon exposure to sunlight and thus participate in light-induced clock resetting (Sancar et al. 2000). The importance of cryptochrome 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 were alleles of the E3 ligase component FBXL3 (F-box/LRR-repeat protein 3) (Godinho et al. 2007,Siepka et al. 2007) that catalyzes the polyubiquitination of CRY1 and CRY2 and thus stimulates their proteasomal degradation (Busino et al. 2007).

FBXL3 is a member of a large family of F-box proteins, which mediate regulated degradation by targeting Skp-Cullin-F-box (SCF) E3 ligases to phosphorylated substrates (Ho et al. 2006). In the case of cryptochromes and FBXL3, AMPK-mediated phosphorylation of CRY1 and CRY2 stimulates their interaction and the FBXL3-mediated degradation of cryptochromes (Lamia et al. 2009). Interestingly, the serines that are phosphorylated by AMPK are evolutionarily conserved in all cryptochromes that act as transcriptional repressors and are not present in those that function as blue light photoreceptors (Lamia et al. 2009,Yuan et al. 2007) (Figure 2). Furthermore, the presence of AMPK-dependent phosphorylation sites seems to be associated with increased body size, suggesting that AMPK-dependent degradation may have evolved to replace light-induced degradation as a clock-resetting signal in organisms in which light cannot penetrate to all cells.

2.2 Phosphorylation of Casein kinase I

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 (CKIε) (Lowrey et al. 2000). In addition, genetic disruption or pharmacological inhibition of CKIε and/or casein kinase I delta (CKIδ) alters both cellular and behavioral circadian rhythms in mice (Etchegaray et al. 2009,Hirota et al. 2011,Meng et al. 2008). 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 (Vanselow et al. 2006). CKI-mediated phosphorylation of PER proteins is a primary determinant of their stability and circadian period (Lee et al. 2011). Interestingly, the human sleep disorder familial advanced sleep phase syndrome (FASPS), characterized by an extreme “early bird” phenotype, is caused by mutation of serine 662 in human PER2 (S659 in mouse PER2) to glycine (Toh et al. 2001), which appears to prevent the phosphorylation of nearby serines by CKI (Xu et al. 2007). The kinase responsible for phosphorylating PER2 S662 has not yet been identified, but the surrounding amino acid sequence suggests the possibility that AMPK may be involved (Figure 2). Though a possible role of AMPK in directly phosphorylating PER proteins has not been investigated, AMPK was reported to be capable of phosphorylating CKIε at serine 389 and thereby increasing its enzymatic activity, indirectly leading to a destabilization of PER2 (Um et al. 2007).

2.3 AMPK in Entrainment

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. The results of preliminary investigations into this question are consistent with such a role but not conclusive. Pharmacological activation of AMPK by intraperitoneal injection of either AICAR (5-Aminoimidazole-4-carboxyamide ribonucleoside) (Lamia et al. 2009) or metformin (Um et al. 2007) caused a phase shift of the liver clock in mice, which suggests a possible ability to entrain the liver clock. In addition, acute AICAR stimulation altered the expression of clock genes in skeletal muscle in wildtype mice but not mice lacking the AMPKγ3 regulatory subunit, suggesting that AMPK activation may also play a role in circadian entrainment of muscle clocks (Vieira et al. 2008). However, glucocorticoids are similarly capable of causing phase shifts upon acute exposure in vivo but have subsequently been found to be dispensible for metabolic entrainment (Balsalobre et al. 2000,Le Minh et al. 2001). Further study is required to decipher the precise role of AMPK in

the entrainment of circadian clocks in peripheral organs. It seems likely that metabolic entrainment occurs through a multitude of pathways and may not absolutely require any single signal.

3. Other metabolic sensors implicated in clock regulation

3.1 SIRT1

Another fuel-sensing molecule positioned at the crossroads of nutritional status and circadian regulation is SIRT1 (silent mating type information regulation 2 homolog 1). Like AMPK, SIRT1 has emerged as a key metabolic sensor that directly links nutrient signals to metabolic homeostasis. As a member of the sirtuin protein family, SIRT1 is a class III histone deacetylase (HDAC) that, in addition to histones, deacetylates numerous transcription factors and co-regulators (Imai et al. 2000,Landry et al. 2000,Smith et al. 2000). Class III HDACS are structurally distinct from other HDACs and require the coenzyme NAD+ (nicotinamide adenine dinucleotide). The $NAD(P)^+$/NAD(P)H ratio serves as a readout for the cellular redox state, thereby enabling SIRT1 to dynamically sense cellular energy metabolism (Bordone and Guarente 2005,Haigis and Guarente 2006). The circadian transcription factor CLOCK has been reported to have histone acetyltransferase (HAT) activity, and SIRT1 was identified as the HDAC that counteracts the HAT activity of CLOCK (Asher et al. 2008,Nakahata et al. 2008) (Figure 3).

The finding that both AMPK and SIRT1 are implicated in the crosstalk of circadian and metabolic regulation is of special interest because they also regulate each other. AMPK enhances SIRT1 activity by increasing cellular NAD+ levels (Canto et al. 2009,Fulco et al. 2008) and activation of SIRT1 may cause AMPK phosphorylation via deacetylation-dependent activation of the AMPK-activating kinase liver kinase B1 (LKB1) (Hou et al. 2008,Lan et al. 2008). Therefore, AMPK and SIRT1 not only regulate each other but their metabolic actions often converge (Ruderman et al. 2010) suggesting that their close interrelationship might also play a role in circadian clock regulation.

3.2 PARP1

Recently, a second NAD+-dependent protein was identified to act as both a nutrient sensor and a modulator of clock function. Poly (ADP-ribose) polymerase 1 (PARP1) uses NAD+ to synthesize ADP-ribose polymers and adds them to itself and other proteins in a process called poly ADP-ribosylation. Like other posttranslational modifications, ADP-ribosylation affects protein function, such as ability to bind DNA (D'Amours et al. 1999). In the liver, PARP1 activity was found to be daytime dependent and probably driven by feeding rhythms rather than the local circadian clock since its cyclic activity persists in the absence of a functional liver clock. Further supporting this idea, the time of peak PARP1 activity was shifted in response to altering the time of food availability. Interestingly, PARP1 interacts with CLOCK and BMAL1, and was shown to ribosylate CLOCK in a rhythmic pattern (Asher et al. 2010) (Figure 3). PARP1−/− mice have altered circadian rhythms of locomotor activity and liver gene expression, clearly establishing a link between PARP1 and clock function. Two studies have suggested a link between PARP1 and AMPK, but they disagree about whether PARP1 lies upstream or downstream of AMPK (Shin et al. 2009,Walker et al. 2006). A functional interplay between PARP1 and SIRT1 has also been established (Bai et al. 2011a, Bai et al. 2011b, Kolthur-Seetharam et al. 2006, Pang et al. 2011) suggesting that both NAD+-dependent proteins might act in concert to link metabolic cues to circadian clock regulation.

3.3 NAMPT

Since two NAD+ sensing proteins have been linked to the circadian clock, a direct connection between the NAD+ salvage pathway and clock regulation seems likely. Indeed, *in vitro* studies have revealed that high NAD+ (and NADP+) levels decrease the binding capacity of CLOCK-BMAL1 heterodimers to their E-box targets, while high levels of NADH (and NADPH) increase binding (Rutter et al. 2001). Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in NAD+ biosynthesis and CLOCK and BMAL1 directly activate the *Nampt* promoter, leading to circadian oscillation of NAMPT activity and, ultimately, of cellular NAD+ levels (Nakahata et al. 2009,Ramsey et al. 2009). Furthermore, there seems to be a circadian feedback loop involving NAMPT and SIRT1. SIRT1 is recruited to the *Nampt* promoter thereby contributing to the circadian synthesis of its own co-factor via inhibition of CLOCK-BMAL1 transcriptional activity (Figure 3).

Thus, NAD+ levels may be both a metabolic input signal for clock regulation and a clock output signal to regulate metabolism. However, the extent to which clock-regulated *Nampt* gene expression contributes to the regulation of NAD+ sensor protein activity remains to be determined. There is compelling evidence that other mechanisms such as protein-protein interactions might underlie the circadian regulation of PARP-1 activity (Asher et al. 2010,D'Amours et al. 1999) and SIRT1 is also regulated by phosphorylation and protein-protein interactions (Schug and Li 2011). Interestingly, in myoblasts as well as muscle tissue, AMPK activation is accompanied by increased *Nampt* gene expression and consequently NAD+ levels (Costford et al. 2009,Fulco et al. 2008). Whether AMPK-mediated degradation of CRY and thus increased CLOCK-BMAL1 transcriptional activity is involved in this process remains to be determined.

4. Outstanding Questions

4.1 Diurnal regulation of AMPK

The ability of AMPK to phosphorylate cryptochromes and to stimulate their proteasome-dependent degradation suggests that AMPK may be an important mediator of metabolism-dependent clock resetting in mammalian peripheral organs. In order to serve that function, the ability of AMPK to phosphorylate cryptochromes must be stimulated by a fasting- or feeding-dependent signal at approximately the same time each day. Such a signal could involve an increase in the cellular (AMP or ADP)/ATP ratio, an increase in the expression of AMPK subunits or its upstream activating kinase LKB1 (Shaw et al. 2004), a decrease in the expression or activity of a phosphatase that removes the activating phosphorylation such as protein phosphatase 2 (PP2) a or PP2c (Davies et al. 1995), a change in the posttranslational modification of AMPK subunits (Oakhill et al. 2010), or a change in the nuclear localization of AMPK, or a combination of these mechanisms.

In rodent livers, diurnal rhythms in the phosphorylation of the AMPK substrates ACC and Raptor have been observed (Davies et al. 1992,Lamia et al. 2009) but the time of peak phosphorylation of these cytoplasmic proteins does not coincide with the time of minimum cryptochrome protein abundance. In contrast, the expression of AMPKβ2 in mouse liver exhibits a daily increase in expression at the time when nuclear cryptochrome protein is minimal (Lamia et al. 2009). While most of the transcripts encoding the seven subunits of mammalian AMPK (prkaa1, prkaa2, prkab1, prkab2, prkag1, prkag2, prkag3) as well as the upstream activating kinase LKB1 (stk11) exhibit no time-dependent variation in mouse livers, the expression of prkab2 is eight times higher in the middle of the day than it is at night. The β2 subunit of AMPK has been implicated in nuclear localization of the heterotrimeric AMPK complex (Suzuki et al. 2007) and indeed, the nuclear localization of the AMPKα1 subunit was observed to increase at the same time of day when AMPKβ2

expression peaks (Lamia et al. 2009). It remains to be determined whether a particular combination of AMPK subunits is required for catalyzing the phosphorylation of cryptochromes, but complexes containing the AMPKβ2 subunit may be preferentially involved.

At the time of the original discovery of AMPKβ2, the ratio of expression of AMPKβ2 to AMPKβ1 was found to be high in skeletal muscle and low in the liver (Thornton et al. 1998). However, those experiments were performed at a single unknown time of day so it is unclear how that ratio may depend on day-time given the strongly diurnal expression of AMPKβ2. In addition, the catalytic activity of AMPKα1 (but not AMPKα2) was approximately doubled when in complex with AMPKβ2 instead of AMPKβ1 (Thornton et al. 1998), indicating an even greater possible effect of diurnal changes in subunit availability. Because AMPK is a critical regulator of a multitude of mammalian metabolic pathways (Mihaylova and Shaw 2011), the observation that its activity and complex composition vary in a daytime-dependent manner suggests that circadian input to those pathways via diurnal regulation of AMPK may be an important and understudied mechanism by which clocks impinge on mammalian metabolism.

4.2 Central vs. peripheral clocks

Though the original motivation for studying the role of AMPK in peripheral clocks came from the observation that the timing of those clocks depends on metabolic signals (Damiola et al. 2000,Stokkan et al. 2001), AMPK could also play a role in transmitting light cues to the central SCN clock. The SCN receives light signals from the retina via neurons of the retinohypothalamic tract. However, it is unclear how the neuronal signal is converted to an intracellular cue to the core clock machinery. Diurnal rhythms in intracellular ATP concentration in SCN neurons have been observed (Womac et al. 2009); the circadian phase of SCN neurons can be shifted by glucose deprivation (Hall et al. 1997), which can activate AMPK; and light pulses acutely stimulate the expression of *Per1* and *Per2* genes (Challet et al. 2003), which would be expected downstream of cryptochrome degradation. Consistent with this possibility, genetic disruption of either AMPKα1 or AMPKα2 changed the free running period of locomotor activity in mice (Um et al. 2011). Further investigation is needed to determine whether this reflects a role of AMPK-dependent phosphorylation of clock components in the SCN.

4.3 Role of cryptochromes in AMPK-dependent metabolic pathways

The role of AMPK in the regulation of cryptochrome stability combined with several recent findings connecting cryptochromes to the regulation of glucose homeostasis (Boesgaard et al. 2010,Dupuis et al. 2010,Hu et al. 2010,Lamia et al. 2011,Zhang et al. 2010) suggests that cryptochromes may be important and previously unappreciated mediators of AMPK-dependent metabolic regulation.

As transcriptional repressors, cryptochromes are likely to mediate their effects on metabolism by modulating the activity of multiple sequence-specific DNA-binding transcription factors. Indeed, in addition to their long-established repression of CLOCK and BMAL1, they have recently been determined to modulate gluconeogenesis by repressing both CREB (cAMP response element-binding) (Zhang et al. 2010) and the glucocorticoid receptor (Lamia et al. 2011). In each case, cryptochromes were found to oppose glucose production by limiting the expression of gluconeogenic enzymes. CLOCK and BMAL1 activate glucose secretion from the liver by increasing the transcription of rate-limiting enzymes and transporters in a circadian pattern (Lamia et al. 2008). AMPK opposes glucose production by phosphorylating other transcriptional regulators including CREB regulated transcription coactivator 2 (CRTC2) (Koo et al. 2005,Shaw et al. 2005) and class II HDACs

(Mihaylova et al. 2011). Thus, AMPK-dependent degradation of cryptochromes would be expected to counteract the glucose-lowering effects of AMPK activation. Such a phenomenon may cause the glucose responsiveness to AMPK activation to vary with the time of day. It will be particularly important to determine whether the therapeutic benefit of the widely prescribed diabetes drug metformin, which lowers blood glucose at least in part by activating AMPK in the liver (Shaw et al. 2005), is altered by circadian clock function and/or day-time.

5. Conclusions

Accumulating evidence suggests that circadian clocks play an important and underappreciated role in the regulation of mammalian metabolic physiology. In humans this is supported by epidemiological studies showing that disturbance of circadian rhythms by sleep deprivation or shift work is associated with metabolic dysregulation and increased risk for obesity, type 2 diabetes and cardiovascular disease (Karlsson et al. 2005). The recent demonstrations that AMPK and other chemical energy sensors modulate clock function via posttranslational modification of core clock components suggest specific pathways that may be useful for the pharmacological control of circadian metabolic functions. The reciprocal regulation of AMPK and circadian clocks suggests that the effectiveness of widely prescribed drugs (e.g. metformin) and/or new therapies that ameliorate glucose homeostasis by activating AMPK might be improved by altering the timing of treatment. However, it remains to be determined whether AMPK acts as a metabolic sensor in human circadian clocks. Finally, future studies are needed to address whether the core clock proteins that are modified by AMPK and other enzymes with well-known roles in metabolic physiology play a role in the established physiological effects of those enzymes.

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Highlights

- Circadian clocks regulate mammalian metabolic physiology.
- AMPK contributes to clock time setting by phosphorylating clock component proteins.
- AMPK activity, subunit composition, and localization depend on clock time.

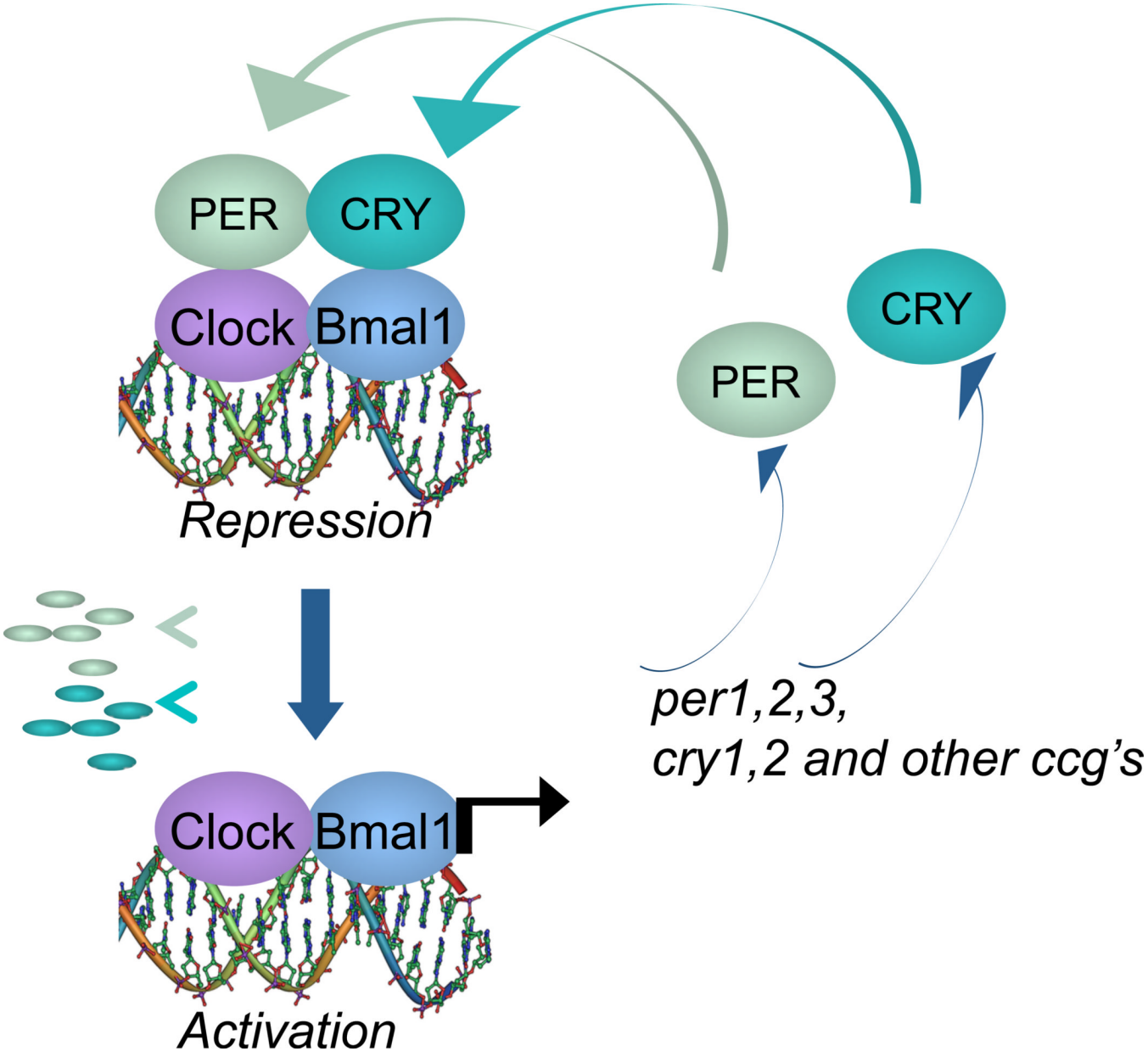

Figure 1. Core circadian clock machinery

Mammalian circadian clocks are based on a transcription and translation feedback loop in which the transcription factors CLOCK and its heterodimeric partner BMAL1 activate the transcription of genes encoding their own inhibitors, the period (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2) repressor proteins.

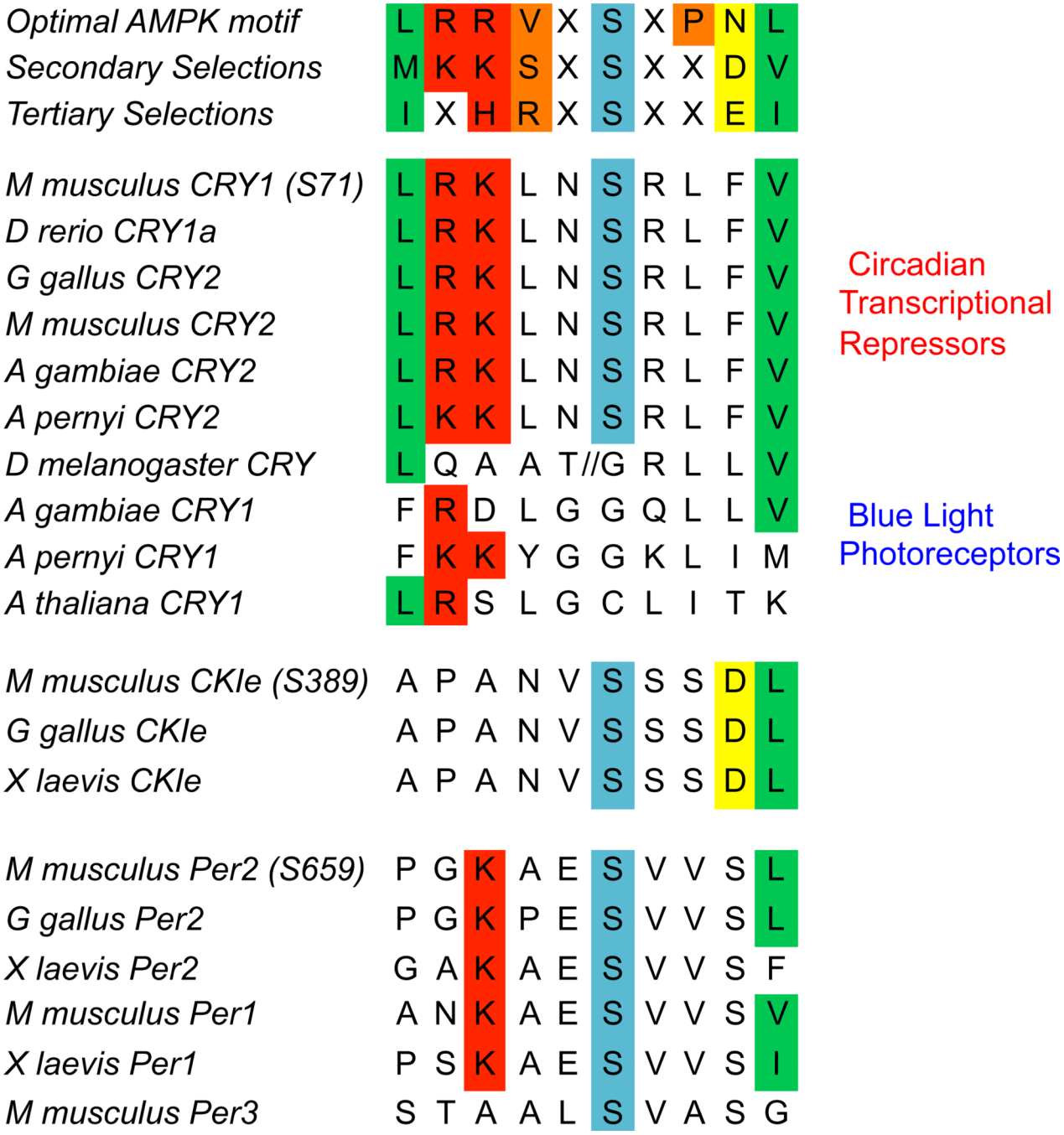

Figure 2. AMPK phosphorylation motifs in clock proteins

Several proteins that are critical for the functioning of mammalian circadian clocks contain evolutionarily conserved sequences that may be preferentially phosphorylated by AMPK. Intriguingly, the AMPK-dependent phosporylation of cryptochromes seems to represent an evolutionary switch in which cryptochromes gained the ability to sense changes in chemical energy (ATP levels) rather than electromagnetic energy (light).

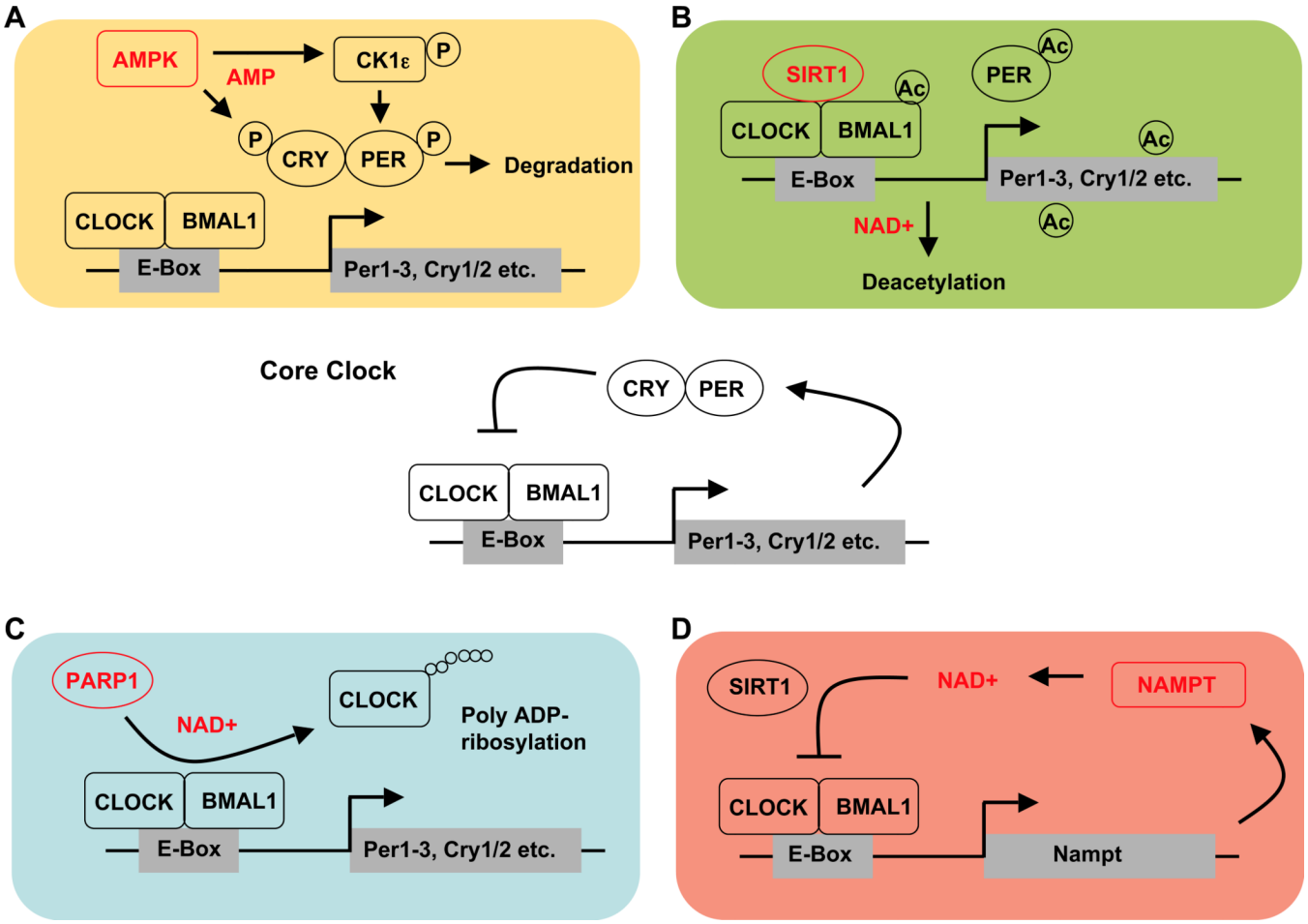

Figure 3. Metabolic inputs to the circadian core clock

A) AMPK phosphorylates CRY1 and CRY2, and targets them for degradation. AMPK has also been reported to phosphorylate CK1ε thereby increasing CK1ε activity and ultimately phosphorylation-mediated PER degradation. **B)** SIRT1 deacetylates BMAL1, PER2 and histone3 in a NAD+-dependent manner thus counteracting the histone-acetylase function of CLOCK. **C)** PARP1 poly ADP-ribosylates CLOCK in a NAD+-dependent reaction, which inhibits DNA binding by CLOCK-BMAL1. **D)** *Nampt* encodes for the rate-limiting enzyme in NAD+-synthesis and is regulated by CLOCK-BMAL1. The resulting circadian oscillations of NAD+ levels feed back on CLOCK-BMAL1 activity by affecting SIRT1 activity.