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REVIEW ARTICLE

Solving the mystery of human sleep schedules one mutation at a time

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Abstract

Sleep behavior remains one of the most enigmatic areas of life. The unanswered questions range from "why do we sleep?" to "how we can improve sleep in today's society?" Identification of mutations responsible for altered circadian regulation of human sleep lead to unique opportunities for probing these territories. In this review, we summarize causative circadian mutations found from familial genetic studies to date. We also describe how these mutations mechanistically affect circadian function and lead to altered sleep behaviors, including shifted or shortening of sleep patterns. In addition, we discuss how the investigation of mutations can not only expand our understanding of the molecular mechanisms regulating the circadian clock and sleep duration, but also bridge the pathways between clock/sleep and other human physiological conditions and ailments such as metabolic regulation and migraine headaches.

Keywords

Human genetics, mouse model, sleep behavior, sleep duration, sleep schedule

History

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Introduction

Sleep is a fundamental biological phenomenon for most organisms and humans spend, on average, a third to a fourth of their lives in the state of sleep. The lack of sleep and dysregulation of daily sleep rhythms have been linked to a number of ailments and outcomes; cancer (Sahar & Sassone-Corsi, 2009), type two diabetes (Huang *et al*., 2011), metabolic syndrome (Wolk & Somers, 2007), cellular stress (Hardeland *et al*., 2003) and most seriously, death (Montagna, 2005). Though great strides have been made in understanding sleep by utilizing model organisms, the regulation of sleep and the functional roles of sleep for humans remain mostly unclear (Frank, 2006). The molecular benefits of sleep have been linked to the pruning and reorganization of dendrites (Benington & Frank, 2003; Maret *et al*., 2011), and to the modification of gene expression for the synthesis of new biomolecules and metabolites (Tononi & Cirelli, 2006). Theories on the necessity of sleep range from the conservation of energy (Jung *et al*., 2011) to metabolic repair during sleep (Bonnet & Arand, 1996).

The Earth rotates with a 24-h (circadian) cycle and this dictates that many (if not all) of our physiological functions are subject to circadian regulation (Dunlap, 1999). What we have learnt from past studies is that our endogenous circadian clock regulates many of our body functions including sleep behavior. The most accepted model for describing sleep behavior, the ''Two Process Model'', was proposed by Borbély in 1982; Process S represents sleep homeostasis and process C represents circadian rhythm regulation (Borbély & Achermann, 1999). The ''Two Process Model'' postulates that the interaction between the sleep–wake-dependent ''process S'' and the circadian ''process C'' can explain essential aspects of sleep regulation. How both of these processes are changed by human circadian mutations will be discussed throughout this review. Research into the identification of non-circadian genes that regulate sleep homeostasis has been quite fruitful. These non-circadian genes and regulatory networks have been detailed excellently in the following reviews (Andretic *et al*., 2008; Crocker & Sehgal, 2010; Sehgal & Mignot, 2011).

Investigation of human behavioral traits is inherently challenging since we are known to modify our behaviors by social, cultural and habitual factors. Regardless, the connection between genes and behavioral traits has been established (Plomin *et al*., 2012). For humans, one of the most compelling evidence for genetic influences on behavior came from the studies done by Thomas Buchard and his colleagues at the Minnesota Center of Twin and Family Research (Bouchard *et al*., 1990). After decades of study, they concluded that there are deep-seated psychological, behavioral and emotional traits that are determined at birth. Hence, human genetics can be used as a powerful tool to investigate human behaviors. In addition, the wealth of knowledge and information that have been accumulated in the past 20–30 years from studies of model organisms, especially fruit flies (Hardin, 2011) and mice (Lowrey & Takahashi, 2011), has augmented enormously to the understanding of human sleep behaviors. Contributions from these diverse fronts have significantly improved our understanding on the regulatory mechanisms of human sleep schedule in recent years which will be discussed in this review.

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Human circadian sleep phenotypes

The majority of people go to bed from 10 pm to midnight and gets up between 6–8 O'clock in the morning. There is a small proportion of people who are either night owls (staying awake until late into the night) or morning larks (demonstrating an extreme early morning awakening) (Jones *et al*., 1999). Besides environmental influences, rare genetic variants can also directly shift sleep schedules. The first genetic form of human sleep schedule behavior was reported in 1999 (familial advanced sleep phase, FASP) (Jones *et al*., 1999). FASP individuals demonstrate a forward shift in six different measurements related to sleep (including sleep onset time, sleep offset time, dim light melatonin onset time, core body temperature, and rapid eye movement (REM) sleep onset time) compared to control subjects. Since the discovery of this rare genetic sleep trait, significant progress has been made in our understanding of human circadian rhythm disorders with the characterization of additional sleep phenotypes.

The International Classification of Sleep Disorders includes approximately 60 disorders of human sleep, among them are circadian rhythm sleep disorders (Medicine, n.d.). Circadian rhythm sleep disorders usually exhibit as a social problem in a person's sleep/wake timing, though there are many other sleep disorders demonstrating sleep dysfunction with sleep at the ''wrong times''. Circadian rhythm sleep disorders individuals suffer from difficulty initiating or ending sleep at appropriate social times.

Circadian rhythm sleep disorders include the following types according to the American Academy of Sleep Medicine (AASM) (Sack *et al*., 2007a, b). Advanced sleep phase disorder (ASPD) individuals have earlier sleep/wake times than the average population and their entire sleep–wake cycle is shifted forward several hours. ASPD people are described as ''morning larks'' that demonstrate an extreme early morning awakening before others are active. However, they also have difficulty in staying awake to satisfy domestic responsibilities in the evening, resulting in significant sleep deprivation if social responsibilities keep them from staying with their internal natural biological clock. It is worth noting, ASPD is more commonly seen in the elderly (Sack *et al*., 2007b). However, unless patients complain of a problem with waking early, ASPD can be described only as advanced sleep phase (ASP) or with a family history of familial advanced sleep phase (FASP).

In contrast to ASPD, delayed sleep phase disorder (DSPD) is marked by the opposite phenomena where the sleep–wake cycle is shifted later, and DSPD patients feel wide awake, energetic and motivated until late in the night. Although, similar to ASPD, unless patients complain of a problem with waking late, DSPD can be described only as delayed sleep phase (DSP) or with a family history of familial delayed sleep phase (FDSP). DSPD individuals often suffer from sleep deprivation since their sleep onset is delayed by the biological clock and morning waking time is dictated by the alarm clock and social responsibilities. DSPD has an estimated prevalence of 7%–16% in adolescents and young adults (Sack *et al*., 2007a, b).

Another sleep phenotype is the free-running sleep disorder (FRSD) where the sleep–wake cycle for these individuals is shifted approximately 1 h later everyday. This disorder is linked to retinal blindness and likely precipitates from the lack of light resetting of the circadian clock (Sack, 2007a, b).

Finally, irregular sleep–wake disorder (ISWD) individuals have an undefined sleep–wake cycle where sleep occurs throughout the day. This is often seen in dementia patients who are suffering from neurological dysfunction and can be seen as well in mentally retarded patients. These individuals also suffer from insomnia, as they are unable to experience consolidated sleep (Sack, 2007a, b).

Mammalian molecular clock

Circadian rhythmicity was first noted in the observation that leaf movements (opening and closing) followed a daily rhythm even in the absence of sunlight (de Mairan, 1729). It is now established that the circadian rhythms of human body functions are regulated by an endogenous molecular clock. The mammalian molecular clock is composed of a core set of transcription factors that form functional regulatory feedback loops (Brown *et al*., 2012; Mohawk *et al*., 2012; Takahashi *et al*., 2008). The main feedback loop consists of Period (PER 1/2), Cryptochrome (CRY 1/2), Brain and muscle ARNT-like 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK) (Brown *et al*., 2012; Takahashi *et al*., 2008). BMAL1 and CLOCK (or NPAS2, a paralog of CLOCK) form a heterodimer and binds to the promoter element described as an enhancer box (E-BOX) of *PER* and *CRY*. The transcription and translation of *PER* and *CRY* increase protein levels in the cytoplasm, whereby the two proteins then heterodimerize and translocate into the nucleus. In the nucleus, the CRY/PER heterodimer binds to BMAL1/CLOCK to repress transcriptional activation, leading to the termination of their own transcription (Figure 1). PER and CRY are subsequently degraded through the proteasomal pathway, allowing for the reactivation of transcription of *PER* and *CRY* by BMAL1/CLOCK (Brown *et al*., 2012; Mohawk *et al*., 2012; Takahashi *et al*., 2008) (Figure 1). This transcriptional/translational negative feedback loop takes approximately 24 h to complete, thus defining the circadian period, and denotes the proteins involved in the circadian cycle as the ''core clock'' (Takahashi, 2004). The core circadian loop is further regulated by post-translational regulation summarized later in this review. Circadian regulators that make up additional negative feedback loops or interlocking loops are: activators RAR-related orphan receptor A (RORA), D site of albumin promoter-binding protein (DBP), repressors nuclear receptor subfamily 1, group D, member 1 (NR1D1/REV-ERB) and basic helix-loop-helix family, members e40 and e41 (DEC1, 2 respectively). These activators and repressors act in a circadian manner, providing additional stability and levels of regulation (Ueda *et al*., 2005). The cycling of these factors operates without requiring extrinsic feedback, and exists in most cell types. Approximately 3%–10% of gene transcripts oscillate in a circadian manner, whose subsets vary according to cell type (Akhtar *et al*., 2002; Duffield *et al*., 2002; Hughes *et al*., 2009; Miller *et al*., 2007; Panda *et al*., 2002; Storch *et al*., 2002). The expression levels of many clock components themselves also demonstrate oscillation. Interestingly, precise rhythmicity of PER2 is necessary for driving cellular

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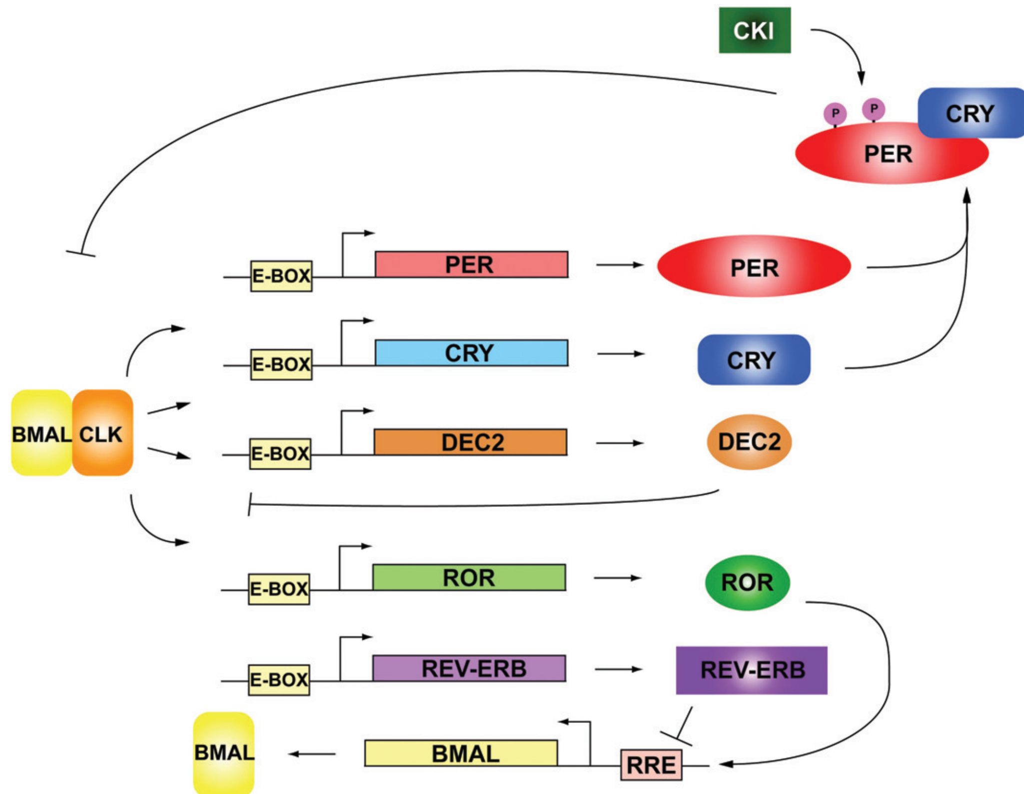

Figure 1. Circadian clockwork. The core circadian clock is made up of two interlocking transcriptional and post-translationally regulated feedback loops. BMAL1 and CLOCK heterodimerize to form an activating transcriptional complex that binds to the E-Box on the genes *PER*, *CRY*, *ROR* and *REV-ERB*. The production of PER and CRY protein builds until PER and CRY are modified post-translationally and then heterodimerize and translocate into the nuclei where they bind to CLOCK and BMAL1 and block the transcriptional activation of E-BOX specific targets. PER, CRY and BMAL1 are degraded by proteasomes. In a second loop, *BMAL1* transcription is activated and repressed by ROR and REV-ERB respectively by binding to the RRE DNA element. (see colour version of this figure at www.informahealthcare.com/bmg).

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circadian oscillations, whereas oscillations of other core clock components such as CRY1, CLOCK and BMAL1 are less critical (Chen *et al*., 2009).

Post-translational modifications in molecular clock

Post-translational regulation gives the core clock its ability to generate precisely tunable timing, since different post-translational modifications can mark the different circadian proteins for different functions at different times over the 24-h clock. Utilizing post-translational modifications the clock is directly tied to the cells' intermediary metabolism, linking the small molecule metabolites; ATP, Acetyl CoA, NAD^+, UDP-GlcNAc, to the core clock's timing. For instance, PER2 functions through heterodimerization with CRY for its translocation and the suppression of CLOCK/BMAL1 gene activation (Brown *et al*., 2012; Mohawk *et al*., 2012; Takahashi *et al*., 2008), and this heterodimerization is likely modulated by numerous post-translational modifications to ensure the correct and precise timing of transcriptional inhibition. Post-translational modifications likely regulate each of these steps during the cycle of PER2 allowing for the exact control of clock gene regulation and its fine adjustment to outside stimulus. All in all, these various regulatory pathways highlight the intricacy of post-translational regulatory mechanisms of the circadian clock.

Besides PER2, many other circadian clock proteins such as CRY, BMAL1 and CLOCK have been identified as targets of post-translational modifications (Gallego & Virshup, 2007). In fact, an understanding of the importance of complex post-translational modification in regulation of circadian rhythms emerged in recent years. Post-translational modifications are central to the hypothesis that circadian mutations affect sleep schedules through a disruption or change of normal post-translational modifications throughout the circadian period. Here, we first review the known complex network of post-translational modifications and later detail the mutations that affect circadian rhythms through post-translational alterations.

PER2 is the best example illustrating the complexity of post-translational regulation for the molecular clock (Gallego & Virshup, 2007; Vanselow & Kramer, 2007). Phosphorylation has been identified on 21 of 247 serine or threonine residues of mPER2 (Vanselow *et al*., 2006). Although the mechanistic consequences for most of these phosphorylation sites are unknown, it is hypothesized that phosphorylation localized in the N-terminus of mPER2 can lead to the stabilization and nuclear localization of the protein by CKII (Maier *et al*., 2009). Also, phosphorylation in the CKI domain increases the half-life of mPER2 (Shanware *et al*., 2011; Vanselow *et al*., 2006; Xu *et al*., 2007). In addition, as described later, the phosphorylation of PER2 at one of the key serine residues (S662) can modulate the period length (Xu *et al*., 2007). Other clock proteins such as BMAL1 and CRY are also regulated by phosphorylation. Specifically, BMAL1 has been shown to be phosphorylated by c-Jun N-

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terminal kinase (JNK), and when BMAL1 is hypophosphorylated due to JNK inhibition, the circadian period is lengthened (Yoshitane *et al*., 2012). Furthermore, BMAL1 is phosphorylated by Protein Kinase Cγ (PKCγ) in response to the food entrainable oscillation. This phosphorylation stabilizes BMAL1 protein by reducing BMAL1 ubiquitination (Zhang *et al*., 2012). CRY2 is regulated by phosphorylation in a sequential pattern initiated by dual specificity tyrosine-phosphorylation-regulated kinase 1 A (DYRK1A) at serine 557 then phosphorylation at serine 553 by glycogen synthase kinase 3β (GSK-3β) leading to CRY2 degradation (Kurabayashi *et al*., 2010). Conversely, CRY1 is regulated in the liver by adenosine monophosphate-activated protein kinase (AMPK)-mediated phosphorylation leading to CRY1 depletion from the nucleus (Lamia *et al*., 2009).

Acetylation has gained recent notoriety as another post-translational modification that regulates cellular protein functions (Haigis & Sinclair, 2010; Peng & Seto, 2011). In particular, acetylation and the subsequent deacetylation by Sirtuin-1 (SIRT1) are also implicated in the regulation and degradation of PER2 (Asher *et al*., 2008). Although the specific residues for acetylation of PER2 and how acetylation affects PER2 biophysically are unknown, SIRT1-mediated deacetylation promotes the degradation of PER2, and loss of SIRT1 increases PER2 protein stability (Asher *et al*., 2008). Similarly, BMAL1 is regulated by reversible acetylation, specifically acetylated at lysine 537 by the acetyltransferase activity of CLOCK, and deacetylated by SIRT1 (Nakahata *et al*., 2008). Acetylation of BMAL1 confers circadian timing of BMAL1 by metabolite sensing through nicotinamide adenine dinucleotide (NAD^+) (Ramsey *et al*., 2009).

Moreover, post-translational *O*-linked N-acetylglucosamine modification (*O*-GlcNAcylation) has recently been shown to exhibit regulatory effects on the circadian clock. *O*-GlcNAcylation in the heart exhibits diurnal oscillation, which is regulated by glucose, *O*-GlcNAc transferase (OGT) and *O*-GlcNAc hydrolase (OGA) levels (Durgan *et al*., 2011). OGA levels and OGT activities in turn oscillate with daily rhythms. On the other hand, *O*-GlcNAcylation levels can modulate the clock speed. Higher *O*-GlcNAcylation leads to longer circadian period and lower *O*-GlcNAcylation shortens the period (Kaasik *et al*., 2013; Kim *et al*., 2012). Like phosphorylation, *O*-GlcNAcylation regulates the molecular clock through various pathways including modification of transcriptional factor activities, nuclear translocation of clock proteins, and ubiquitin-mediated targeting for degradation of clock proteins (Kaasik *et al*., 2013; Kim *et al*., 2012; Li *et al*., 2013).

Other known post-translational modifications include ADP ribosylation on CLOCK (Asher *et al*., 2010), sumoylation on BMAL1 (Cardone *et al*., 2005; Lee *et al*., 2008), and ubiquitination on CRY (Hirano *et al*., 2013; Yoo *et al*., 2013) and BMAL1 (Zhang *et al*., 2012). Poly ADP-ribosylation of CLOCK enhances CLOCK/BMAL1 complex binding to DNA by poly(ADP-ribose) polymerase 1 (PARP-1) activity initiated in the early light phase of circadian time. Sumoylation of BMAL1 occurs in the liver and promotes the circadian transcriptional activation of the CLOCK/BMAL1 complex on DNA. This activation occurs in combination with ubiquitination of BMAL1, requiring co-modification for activation. Finally, CRY is ubiquitinated by FBXL21 and FBXL3, F-box-type ubiquitin E3 ligases. These E3 ligases work in the nucleus and cytoplasm, respectively, to activate and time CRY activity for the suppression of circadian gene activation with PER by targeting CRY for proteasomal degradation (Hirano *et al*., 2013; Yoo *et al*., 2013).

Mutations causing human circadian phenotypes

Familial advanced sleep phase

PER2-S662G

To determine the genetic basis of FASP, linkage analysis was performed in a large family segregating the FASP trait. The mutation was determined to be on chromosome 2, and further analysis identified *PER2* as the gene containing the mutation. In the h*PER2* cDNA, position 2106 was changed from A to G which causes the substitution of a serine at amino acid 662 with a glycine (S662G) (Toh *et al*., 2001). The mutation is localized in the CKI binding domain of PER2, and as the mutation substituted a glycine for a serine residue, the hypothesis that the mutation affected the phosphorylation of PER2 was tested. Initial testing suggested that the mutation of PER2 at Ser662 reduced CKIε phosphorylation (Toh *et al*., 2001); later data suggested that mutation at Ser662 disrupts the phosphorylation of CKIδ *in vitro* (Xu *et al*., 2007). Because the mutant PER2 protein demonstrated reduced phosphorylation by CKIδ, the functional consequence of a reduction of PER2 phosphorylation *in vivo* was tested (Xu *et al*., 2007). BAC transgenic mice carrying this human mutation were generated and then tested in a circadian behavioral assay. These mice recapitulate the human FASP phenotype, including both advanced sleep phase and a shortening of behavioral and cellular circadian period length (Xu *et al*., 2007). These observed phenotypes are associated with enhanced transcriptional repression, as mRNA levels of both the endogenous copy of mouse *Per2* and the introduced human copy of *PER2* decreased. The change in repressor activity is caused by a disruption of phosphorylation at Ser662, which normally results in a phosphorylation cascade of four downstream serine sites. Hence, through a still unknown mechanism, the lack of phosphorylation allows PER2 to act as a stronger repressor. Supporting this mechanism, a second transgenic mouse model with a substitution of the Ser662 to aspartic acid (which mimics constitutive phosphorylation) resulted in a lengthened circadian period and decreased repressor activity demonstrated by protein and RNA analysis. *In vitro* evidence also suggests that mutation of PER2 at S662G enhances PER2 clearance from the nuclei (Vanselow *et al*., 2006). While enhancing repressor activity would naturally decrease expression levels, another concurrent possibility for protein reduction presented by Vanselow and colleagues is that the changes in PER2 phosphorylation results in deficient nuclear retention, which then promotes PER2 degradation in the cytoplasm (Vanselow *et al*., 2006).

In the future, it will be necessary to investigate the function of phosphorylation in the CKI domain. Phosphorylation of PER2 has so far been linked to the protein half-life, the ability to interact with other proteins and subcellular localization.

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Specifically, it is important to investigate how phosphorylation sites work in tandem or in opposition of one another to unravel the mystery of circadian cycling and sleep timing.

CKIδ-T44A

Since the initial identification of a mutation that causes FASP, additional families were identified to have FASP but did not contain the previously identified mutation in *PER2*. Utilizing a candidate gene approach, a causative mutation for a second FASP family was identified in human CKIδ (Xu *et al*., 2005). The mutation occurs at Threonine 44 and is substituted with alanine (T44A). This particular amino acid was conserved in the CKI family of proteins in mouse and human as well as in the *Drosophila melanogaster* CKI homolog Doubletime (homolog of CKIδ and CKIε). Biochemical assays displayed mutation-induced decreases in phosphorylation of alpha-casein, and PER protein substrates by *in vitro* kinase assays strongly suggesting biological relevance for this genetic variant. To validate the mutated function of CKIδ, a BAC transgenic mouse model carrying *CKIδ*-T44A was generated. These mice were tested in wheel-running behavioral assays to determine the functional consequence of the mutant kinase, and their free running period (τ) was shorter than control mice by ~20 min. When the mutant transgenic mice were crossed with *CKIδ* knock-out mice, an even shorter τ was observed (22.7 h versus 23.4 h) in addition to rescuing the lethal phenotype of the *CKIδ* knock-out (the mutation does not render the kinase activity null). Intriguingly, expressing the human *CKIδ*-T44A mutation in *Drosophila* generated flies with a significantly longer period than control flies, which highlighted the underlying differences between mammals and invertebrates (Xu *et al*., 2005).

To address the mechanism of reduced kinase activity, the crystal structure of the mouse homolog CKIδ was analyzed to better understand the functional consequence of the mutating threonine to alanine (Figure 2). The mutation occurs in the loop between the beta sheets that make up the ATP binding domain in the catalytic domain of CKIδ. Specifically, the loss of threonine and replacement with a hydrophobic amino acid (alanine) may cause misfolding or loss of flexibility of the loop, which then restricts the enzyme (Figure 2). Because the mutation causes a reduction of catalytic activity, it is possible that the mutation causes a disruption in chemical catalysis of phosphate transfer from ATP, through restriction of the enzyme's flexibility.

CKIδ H46R

After identifying *CKIδ*-T44A, the gene encoding CKIδ in blood samples from over 70 FASP probands were screened, and a second mutation was identified in a single individual that predicted a histidine to arginine change at position 46 (H46R), two amino acids downstream from the T44A substitution (Brennan *et al*., 2013). Neither T44A nor H46R is present in the 1000 Genomes database and the publicly available CGI 60 Genomes database (1000 Genomes Project Consortium *et al*., 2012; Drmanac *et al*., 2010).

As previously discussed, CKIδ-T44A has reduced kinase activity in comparison to wild type (WT) enzyme. Utilizing a CKIδ-H46R isoform, the kinetics of phosphotransfer was analyzed. CKIδ-H46R similarly showed decreased kinase activity on alpha casein and the PER2 peptide (Brennan *et al*., 2013). The decrease in V_{max} suggests that the mutation causes a reduction in the catalytic rate of phosphotransfer. There may be a subtle effect on enzyme recognition of the substrate as the K_m of the mutant kinase activity is decreased with PER2 peptide but is not significantly different for casein or ATP. Utilizing molecular modeling, we hypothesize that the reduction of activity can be attributed to the loss of the hydrogen bond that forms between H46 and the glutamine residue at amino acid 48 (Figure 3). Though the function of the hydrogen bond is unknown, it may be to properly fold the loop during catalysis. It is also unclear what the structural consequence of the mutant arginine has on the tertiary structure. However, it is reasonable to hypothesize that the structural integrity in this region is important for the catalytic function of the phosphotransfer.

Further work is needed to identify the structural ramifications of the H46R and the T44A mutations, which will give

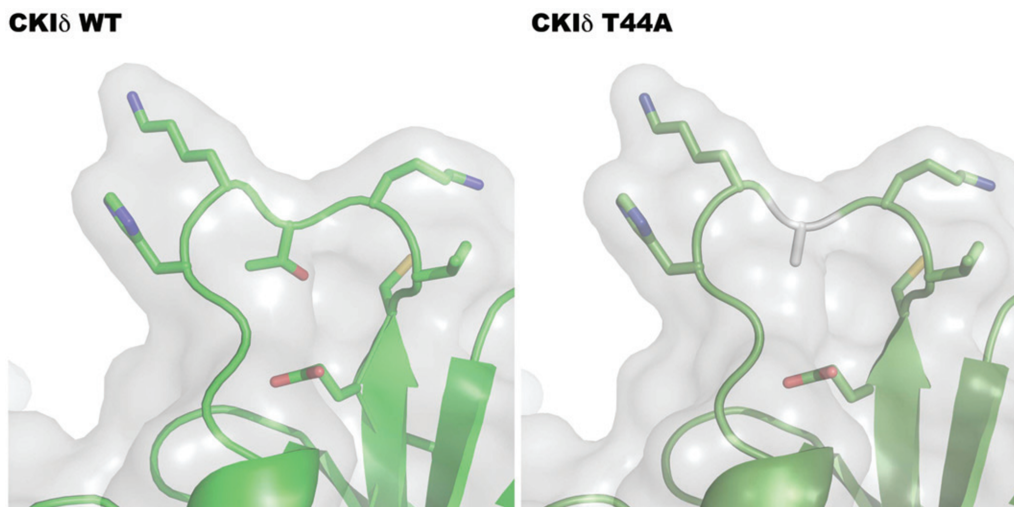

Figure 2. Molecular modeling of T44A mutation. Utilizing PyMOL, the structure of the human CKIδ with the mutation T44A was modeled from rat CKI (1CKI). The replacement of threonine with the hydrophobic amino acid alanine may cause protein misfolding or loss of flexibility of the loop, which then restricts the enzyme, likely lowering enzymatic activity. (see colour version of this figure at www.informahealthcare.com/bmg).

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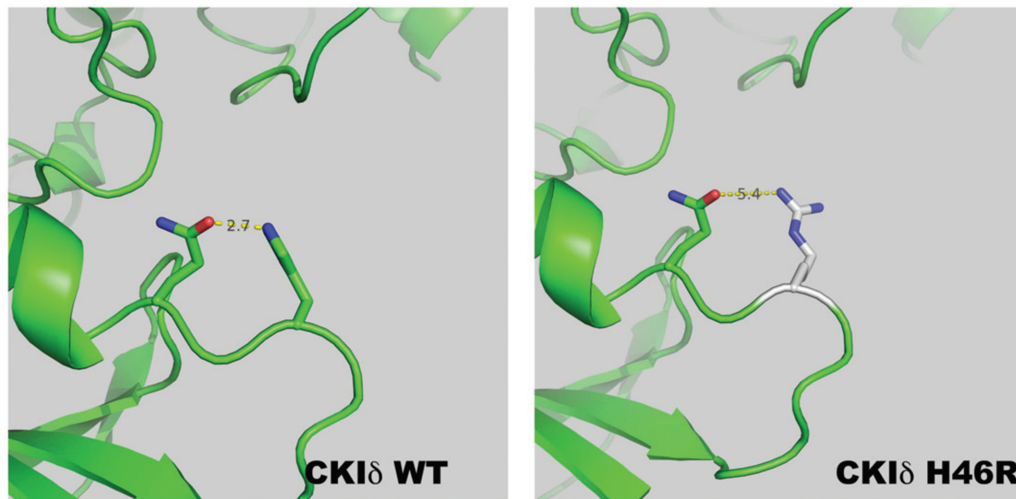

Figure 3. Molecular modeling of H46R mutation. Utilizing PyMOL, the structure of the human CKIδ with the mutation H46R was modeled from rat CKI (1CKI). The replacement of histidine with the larger guanidino containing amino acid arginine may cause protein misfolding or loss of flexibility of the loop. Specifically as shown, there is an increased distance between the hydrogen bonding of the mutant arginine with glutamine at position 48, which then restricts the enzyme, likely lowering enzymatic activity. (see colour version of this figure at www.informahealthcare.com/bmg).

insight to the structural function of the loops on CKIδ. It will also be interesting to reveal other possible cellular ramifications of mutating a kinase integral to the Wnt signaling pathway. The function and mechanism of CK1δ phosphorylation may illuminate the role phosphorylation plays in migraine headache (Brennan *et al*., 2013).

Mutation altering human sleep duration

Familial natural short sleep

A family was found to display natural short-sleep phenotype characterized by the reduction of sleep time and increased daily activity, described as familial natural short sleep (FNSS). Subsequently, a point mutation that changed the amino acid at position 384 from proline to arginine (P384R) in *DEC2* was identified (He *et al*., 2009). This mutation co-segregates with the FNSS phenotype in this family. DEC2 is a negative component of the circadian clock (Butler *et al*., 2004). It belongs to a basic helix-loop-helix (bHLH) protein family in which members can homodimerize and can affect gene transcription by direct binding to DNA (Fujimoto *et al*., 2001, 2007). P384 is localized in the C-terminal proline-rich domain and its flanking sequences are highly conserved among mammalian DEC2 orthologs. *DEC2*-P384R mutation alleviated its transcriptional repressive activity demonstrated by *in vitro* luciferase reporter assay. BAC transgenic mice carrying this P384R mutation exhibited a shorter total sleep time and longer active time per 24-h period as seen in human mutation carriers. This result was further verified by electroencephalography (EEG) and electromyography (EMG) performed on *DEC2*-P384R mutant transgenic mice and their littermates. In addition to transgenic mice, transgenic flies carrying the P384R mutation were generated to test whether *Drosophila melanogaster* would demonstrate a conserved phenomenon with humans and mice. Transgenic flies with mutant *Dec2* expressed in their mushroom bodies showed a similar lengthened active duration behavior, suggesting DEC2 plays a significant role in regulating sleep quantity.

Figure 4. DEC2 interacting domains with HDAC. DEC 2 contains most notably a basic helix-loop-helix domain that has been shown to interact with SIRT1, which may play a significant role in DNA binding regulation. By homology, there is an orange domain. HDAC1 interacts with the Ala/Gly rich region of DEC2, interestingly where the P384R mutation resides. The mutation may disrupt or promote HDAC1 interaction and may be the key to understanding the molecular mechanism of DEC2 regulation of NSS. (see colour version of this figure at www.informahealthcare.com/bmg).

The DEC2 mutation is hypothesized to vary sleep quantity by altering the structure of the protein. The mutation of DEC2 incorporates an arginine residue in place of a proline, which can incorporate a locked turn in the protein by the nature of the proline structure. The replacement of the proline with arginine introduces a charged amino acid with the guanidino group of the arginine. In addition, the amino acid substitution is in the glycine and alanine-rich region of the DEC2 protein that has been shown to interact with HDAC1, the protein deacetylase and a known regulator of transcription factors (Fujimoto *et al*., 2007) (Figure 4). Hence, the mutation may interrupt the interaction between HDAC1 and DEC2, and lead to altered gene transcription, by altering the acetylation status of DEC2 or by affecting HDAC1-dependent deacetylation of other transcription factors. Future work to elucidate the molecular mechanisms of *DEC2* on sleep length regulation includes identifying genes that are regulated by DEC2, and specifically how the FNSS mutation affects gene transcription and protein function.

Insight from murine circadian mutations

The initial insight into how circadian rhythm genes affect sleep function was revealed by the *tau* mutant Syrian hamster (Ralph & Menaker, 1988). This mutation conferred a

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free-running time in the mutant animals to that of 22 h instead of the wild type 23.5 h. Further characterization of the mutation mapped it to a single nucleotide change in the gene encoding the enzyme CKIε. This mutation has been examined *in vitro* and hypothesized to decrease enzymatic activity in the general enzyme, but has also been hypothesized to have increased specificity or gain-of-function for PER by increasing PER2 degradation (Lowrey *et al*., 2000). Interestingly, *tau* and the mutation in human CKIδ give a similar shortened period, though the mechanisms seem to differ as far as the site and the function of the mutation (gain-of-function versus loss) (Xu *et al*., 2005). Albeit the exact molecular mechanism of CKIδ regulation needs to be further explored, this reinforces the idea that phosphorylation can have many different effects on a single protein that later can result in the same phenotype.

Another mutation affecting mouse circadian function was identified using a forward genetics screen. Mice were treated with N-ethyl-N-nitrosourea (ENU), and then subjected to free-running activity measurements. Using this method, a semi-dominant mutation was identified in the *CLOCK* gene (King *et al*., 1997). This mutation deletes exon 19, which interrupts the bHLH-PAS domain and likely blocks transcriptional regulation of CLOCK. Although to date no human mutations in the *CLOCK* gene have been identified, mutations in *CLOCK* could possibly cause the ''night owl'' phenotype of FDSP by delaying the clock as seen in the mice.

Two mutations, cysteine 358 to serine (Godinho *et al*., 2007) and isoleucine 364 to threonine (Siepka *et al*., 2007), in F-box protein 3 (*FBXL3*) were found independently using ENU mutagenesis. Both mutations lead to long free-running period by affecting the expression of PERs and the stabilization of CRYs. Finally, a new mutation was recently identified in another F-box protein, FBXL21, an E3 ubiquitin ligase specific to CRY1 in the mouse (Yoo *et al*., 2013). The mutation in FBXL21 conferred a change of amino acid 149 from a glycine to a glutamine. Interestingly, this mutation leads to period shortening and antagonizes the period lengthening effect of the FBXL3 isoleucine 364 threonine mutation. FBXL21 was found to stabilize CRYs and antagonize the destabilizing action of FBXL3 on CRYs (Hirano *et al*., 2013). The *FBXL21*-G149E mutation reduces the protective effect of FBXL21. These mutations again point out the importance of post-translational modifications in the precise timing and function of the core circadian oscillators. It is not unreasonable to predict that human FBXL21/3 homologs will play similar roles in regulating human sleep behaviors, highlighting the importance of studying model organisms for understanding human circadian regulatory mechanisms.

How do human circadian mutations help us understand the regulation of human circadian clock and sleep behaviors/beyond finding the mutations?

PER2

The *PER2*-S662G mutation for FASP was found in the CKI binding region. This region of the PER2 protein is highly conserved among mammalian PERs, and there are four additional serines immediately C-terminal to the mutated serine at amino acid 662 (Xu *et al*., 2007). *In vitro* biochemical studies showed that the serine at 662 needs to be phosphorylated by a priming kinase which is followed by phosphorylation of four additional serines by CKIδ (Xu *et al*., 2007). Transgenic mice that carry the S662G mutation showed a shorter circadian period (22 h) than WT control mice (23.7 h) (Xu *et al*., 2007). Intriguingly, transgenic mice that carry a serine to aspartic acid change (to mimic a constitutively phosphorylated serine) have a longer period (24.5 h). When serine 662 is phosphorylated, it allows the four additional serines to be phosphorylated; therefore all five serines are phosphorylated. This then leads to weaker repression by PER2 (described above) and animals have a longer period (analogous to a slower clock because it will take longer to finish a cycle). However, when residue S662G cannot be phosphorylated *in vitro*, the next four serines are not phosphorylated by CKIδ. Then the PER2 protein is a stronger repressor and animals have a shorter period (similar to having a faster clock) (Xu *et al*., 2007). Studies also revealed that the hypophosphorylated PER2 is less stable and the hyperphosphorylated protein is more stable. Collectively, these results imply that PER2 acts as a circadian repressor and that when S662 phosphorylation is blocked, it adopts a conformation so that it becomes a stronger repressor and is less stable. However, when all five serines in the specific region are phosphorylated, the protein adopts a different conformation, which renders it a weaker repressor and more stable. Therefore, the suicide model for transcriptional factors (Fu, 2008) can be applied to describe PER2 (and potentially other transcription factors of the molecular clock) and offers a possible mechanism linking the transcription repressor activity to its own degradation.

Because the four serines immediately C-terminal to S662 are likely phosphorylated by CKIδ, mouse models were used to test the genetic interactions between CKIδ and PER2. The copy number of *CKIδ* does not affect the period length of WT mice. However, when *PER2*-S662G mice were crossed with *CKIδ* heterozygous knockout mice, the period was longer (22.33 h) than S662G transgenic mice. In addition, when *PER2*-S662G mice were crossed with WT *CKIδ* transgenic mice (carrying four to five copies of *CKIδ*), the period was shorter (20.83 h) than S662G mice (Xu *et al*., 2007). These results revealed that there are other sites on PER2 that can be phosphorylated by CKIδ; and when these other sites are phosphorylated, it leads to shorter period (Xu *et al*., 2007). Therefore, there are multiple sites for CKIδ action on PER2. One of them is the S662 region and when this site is fully phosphorylated, it leads to increased *PER2* mRNA and protein levels and a longer period. However, there are other sites on PER2 that when phosphorylated by CKIδ, leads to increased protein degradation, lower protein levels, and a shorter period. Under normal conditions, phosphorylation of different regions maintains a balance, leading to a stable period length. For *CKIδ* heterozygous knockout, there is lower CKIδ level but it affects both pathways to a similar extent, so period stays the same. Similarly, for WT transgenic mice, the higher CKIδ level will affect both pathways to the same extent and period therefore is unchanged (Xu *et al*., 2007). For *PER2*-S662G mutant mice, the pathway for S662 is blocked which then pushes the balance towards the other side and leads to shorter period length (Figure 5). When *PER2*-S662G

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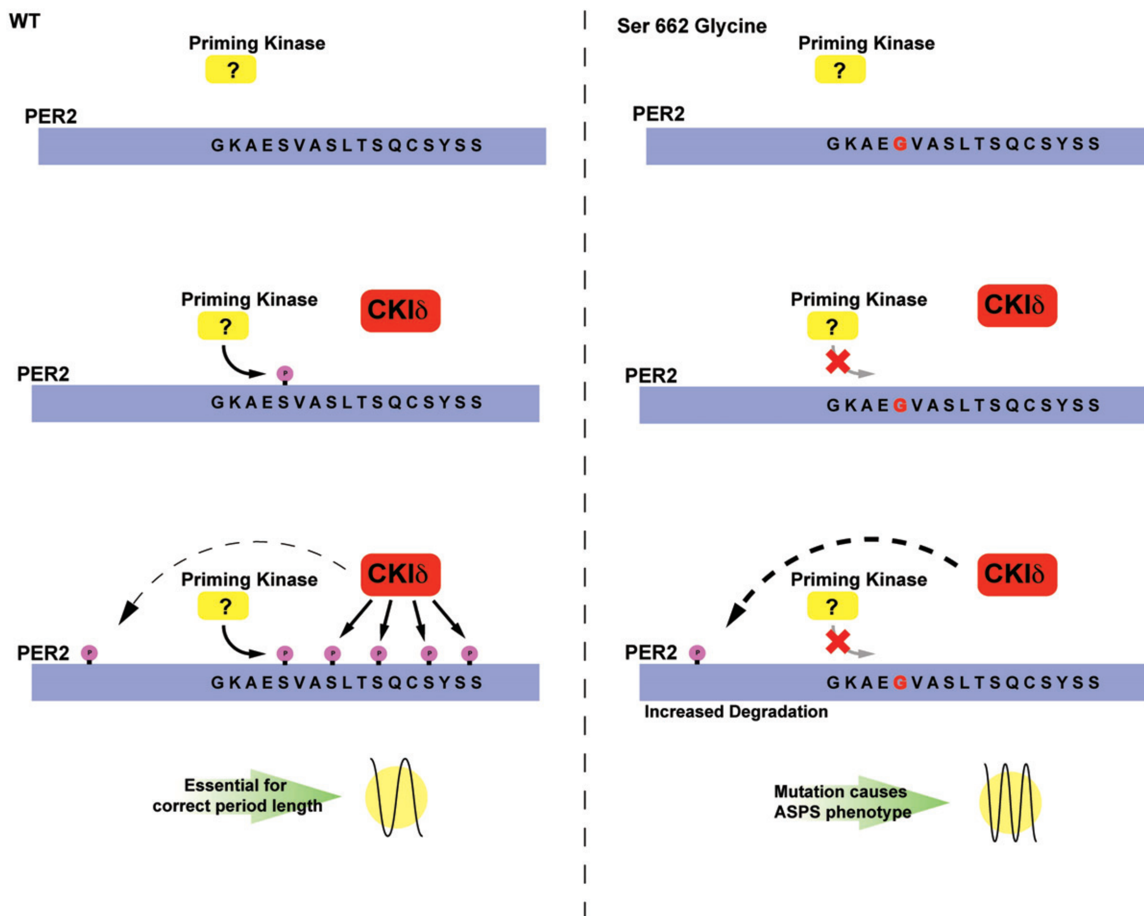

Figure 5. Mechanism of PER2 FASP. Under normal conditions, PER2 is phosphorylated by a specific kinase at the serine 662. This initial phosphorylation ''primes'' the CKI domain for the subsequent recognition by CKIδ and the phosphorylation of the remaining serine residues S665–S674. The phosphorylation of these residues renders PER2 a weaker repressor and confers a longer period length. Additional phosphorylation site(s) on PER2 by CKIδ increases PER2 degradation and leads to a shorter period. Conversely, in the mutant BAC transgenic animal carrying S662G, the priming kinase cannot recognize or phosphorylate the S662 residue, leaving the protein in the hypophosphorylated state; the lack of priming prevents CKIδ from recognizing S665–S674. However, phosphorylation by CKI on the other site(s) of PER2 is unperturbed and degradation of PER2 continues without counter balance from the S662–S674 region. This then causes a shortened period and advancement of sleep phase. (see colour version of this figure at www.informahealthcare.com/bmg).

is crossed onto *CKI*-WT transgene mice, the higher CKIδ level will push the balance towards the other side even more and produces an even shorter period (20.83 h) (Xu *et al*., 2007). When S662G mice is crossed with *CKIδ* heterozygous knockout, the reduced CKIδ level will decrease the phosphorylation unbalance and therefore gives a less short period of 22.33 h. Overall, these data point out the importance of PER2 phosphorylation in setting the speed of the clock.

Further complicating the molecular scenario, PER2 is *O*-GlcNAc modified and *O*-GlcNAcylation further enhances PER2 repressor activity (Kaasik *et al*., 2013). OGT (*O*-GlcNAc transferase) does not affect PER2-S662G repressor activity, suggesting that S662 site may be competitively regulated by phosphorylation and *O*-GlcNAcylation. Interestingly, S662, S668 and S671 of the 5-serine region in PER2 are all *O*-GlcNAcylation sites. *O*-GlcNAcylation is dependent on the level of UDP-GlcNAc substrate, and UDP-GlcNAc synthesis is dependent on nutrient fluxes including glucose (Hart, 2013). Under low glucose condition, the PER2-S662-S674 region is phosphorylated in the presence of CKIδ even with OGT and OGA (*O*-GlcNAcase). However, under conditions of high glucose, phosphorylation in this region is reduced by the presence of OGT or OGA even with the overexpression of CKIδ (Kaasik *et al*., 2013). As described above, when the first serine is phosphorylated in this 5-serine region, the four subsequent serines are phosphorylated. This then leads to a weaker repressor with longer period. But, phosphorylation in this region is also modulated by *O*-GlcNAcylation, and under high glucose condition, phosphorylation in this region is blocked by *O*-GlcNAcylation that then likely leads to a shortened period. Future *in vivo* investigation will shed light on how sugar can modulate clock speed and further our understanding of the connection between circadian clock and metabolic regulation.

CKI

One interesting observation for the *CKIδ*-T44A mutation from the very beginning was that human mutation carriers not only have the FASP phenotype but also have asthma and migraine headache (Brennan *et al*., 2013). One possible explanation for this observation is that the kinase mutation could potentially

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affect multiple substrates, therefore leading to multiple phenotypes. To gain further insight into this observation, a circadian phosphor-proteomic investigation was initiated for CKIδ (Kategaya *et al*., 2012). In addition, a parallel study was carried out for CKIε as comparison and control. Intriguingly, CKIδ and ε share some substrates/interacting partners, but also each have their own subset of unique substrates/interacting partners. One significant revelation from this circadian phosphor-proteomic study is that CKIδ and ε both demonstrate interactions with different proteins at different circadian times, pointing out the importance of considering timing while studying kinases, substrates and the pathways in which they are involved (Kategaya *et al*., 2012).

Because human mutation carriers in families with CKIδ mutations (T44A and H46R) both experience migraine headaches, the transgenic mouse model of T44A was used to investigate the possibility of migraine phenotype. *CKIδ*-T44A mice showed increased sensitivity to a migraine trigger (nitroglycerin), which induced peripheral mechanical and thermal hyperalgesia (Brennan *et al*., 2013). Migraine-related cortical excitability was examined by measuring cortical spreading depression (CSD) using optical imaging and electrophysiological recording. *CKIδ*-T44A mice had significantly lower CSD thresholds with increased number of CSD elicited by stimulation, and their cortical surface arteries were more dilated in all phases of CSD than control mice. In addition, astrocytes from *CKIδ*-T44A mice demonstrated both increased spontaneous and evoked calcium signaling than astrocytes of control mice, suggesting possible astrocytic mechanisms for CKIδ mutations to predispose carriers to migraine. Migraine is common in the general population (~12%) and both genetic and environmental factors have been implicated in contributing to this phenotype (Brennan *et al*., 2013). The association between migraine and two independent CKIδ mutations, together with the *in vitro* and *in vivo* data, suggests that these mutations contribute to the pathogenesis of migraine. Though the relationship between sleep regulation and migraine is unclear, all in all, these studies revealed that CKIδ plays a diverse yet important role in regulating brain excitability.

Model organisms

Investigating human phenotypes usually has an end goal of understanding their underlying fundamental molecular mechanisms. The approach of finding the mutation and gene in human subjects followed by generating a model animal for further investigation has the advantage of studying a single important gene, which can lead to the most relevant pathways. One critical and powerful feature for studying human conditions, whether in health or disease, is the fact that humans can communicate with researchers about their emotional and mental status. However, the major disadvantage lies in the obvious impossibility of using humans in most experimental procedures. For this reason, model organisms are necessary research tools in pursuing these investigations. On the other hand, not all mutations found in humans will give equivalent (or at times any) phenotypes in model organisms. To establish a ''good'' or ''excellent'' model for studying human conditions requires extensive characterization and comparison between the model and human subjects. This also presents challenges since not all comparisons are feasible. With behavioral phenotypes, this challenge is especially augmented and needs additional validations with extra attention from the researchers. To date, the preferred method is to generate transgenic mice that carry human mutations in order to study sleep behavior phenotypes. Mice have the advantage of having closer brain anatomy with humans, and also it is possible to measure EEG for sleep stages in mice. Hence, this model organism has recapitulated the human phenotype beautifully for many mutations as described above. However, there are mutations (or perhaps extremely rare polymorphisms) that were found in humans, but no obvious phenotype could be found in transgenic mice that carry the same mutations (Ptacek & Fu, unpublished results). In addition, by their very nature, mice are nocturnal and display a sleep–wake cycle directly opposite to that of humans. This is further compounded by the animal's natural instinct to mask to light, making sleep and activity measurements at times difficult to interpret (Mrosovsky, 1999). *Drosophila* also has been used to model human sleep behavior by generating transgenic flies carry human mutations, and in some cases the phenotypes are similar, yet other times different (He *et al*., 2009; Xu *et al*., 2005). This further highlights the complexity of choosing the ''right'' model to assist in unraveling molecular mechanisms involved in regulating human behavioral traits. Yet, *Drosophila* is a uniquely malleable model organism that researchers can utilize to investigate the possible mechanisms that may shed significant and novel insight as well.

Concluding remarks

The diverse nature of sleep dysfunction and the lack of treatments for many of these conditions accent the potential for a wealth of research and medical opportunities. The fundamental question of how our bodies integrate all information (from environment and internal cues) and then manifests them as certain sleep behaviors remains largely unclear. The search for mutations by directed genetic screens will likely identify numerous new mechanisms and pathways previously thought unrelated to sleep and further enhance our understanding of sleep. In depth studies of these mutations, genes and pathways will reveal novel mechanisms that link sleep to other phenotypes such as obesity, diabetes, mood disorders, cancer and other seemingly unrelated physiological processes. These investigations also underlie the importance of combining the fields of biochemistry, genetics, cellular/molecular biology and bioinformatics. The complex phenotype of sleep requires behavioral models in which experimentation can describe the phenotype that can be mechanistically investigated *in vitro* and in cells. Integrating these interdisciplinary approaches will exponentially increase the possibility of revealing the interwoven nature of human sleep mechanisms that will improve the lives of people in everyday society.

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Declaration of interest

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