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Authors
Zhang, Luoying
Ptáček, Louis
Fu, Ying-hui

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Diversity of Human Clock Genotypes and Consequences

Luoying Zhang[*], **Louis J. Ptáček**[*,†], and **Ying-Hui Fu**[*]

[*]Department of Neurology, University of California, San Francisco, California, USA

[†]Howard Hughes Medical Institute, University of California, San Francisco, California, USA

Abstract

The molecular clock consists of a number of genes that form transcriptional and post-transcriptional feedback loops, which function together to generate circadian oscillations that give rise to circadian rhythms of our behavioral and physiological processes. Genetic variations in these clock genes have been shown to be associated with phenotypic effects in a repertoire of biological processes, such as diurnal preference, sleep, metabolism, mood regulation, addiction, and fertility. Consistently, rodent models carrying mutations in clock genes also demonstrate similar phenotypes. Taken together, these studies suggest that human clock-gene variants contribute to the phenotypic differences observed in various behavioral and physiological processes, although to validate this requires further characterization of the molecular consequences of these polymorphisms. Investigating the diversity of human genotypes and the phenotypic effects of these genetic variations shall advance our understanding of the function of the circadian clock and how we can employ the clock to improve our overall health.

1. Introduction

The circadian clock regulates daily rhythms of behavior and physiology in organisms ranging from bacteria to human,[1] with the daily sleep and wake cycle in animals being one of the most prominent functions regulated by the clock. An intact clock enables the organism to adjust its biological processes to anticipate daily changes in the environment, whereas a disrupted clock underlies various disorders and/or diseases.[2]

Our understanding of the human molecular clock is largely based on studies in rodents and *in vitro*. The molecular clock consists of a series of transcriptional/posttranscriptional feedback loops with *Clock* and *Bmal1* at the center of the loops.[3] CLOCK/BMAL1 dimers activate the transcription of three *Period* genes (*Per1*, *2*, and *3*) and two *Cryptochrome* genes (*Cry1* and *Cry2*). PER and CRY heterodimerize and translocate into the nucleus, inhibiting the transcriptional activity of CLOCK/BMAL1. In a second loop, CLOCK/BMAL1 activates the transcription of retinoic acid-related orphan receptors, *Rev-erbα* and *Rorα*. The former inhibits, whereas the latter activates transcription of *Bmal1*. In certain tissues, neuronal PAS domain protein 2 (NPAS2) functions as a CLOCK analog.[4] CLOCK and BMAL1 are also believed to drive the expression of *Dec1* and *Dec2*, which function to repress the transactivation of CLOCK/BMAL1 at clock-gene promoters.[5,6] In addition, DBP and E4BP4 are clock-controlled positive and negative regulators, respectively, of D-boxes in the promoter regions of clock genes.[7–9] TIMELESS may also function in the clock by

associating with PER/CRY and inhibiting CLOCK/BMAL1-stimulated transcription of *Per*.[10]

Besides transcriptional control, posttranslational modifications also play a critical role in setting the speed of clock. Casein kinase 1 epsilon (CK1ε) and casein kinase 1 delta (CK1δ) impinge on the negative limb of the feedback loop by phosphorylating PERs, resulting in enhanced protein turnover and nuclear translocation, which in turn affects the transactivation by CLOCK/BMAL1.[11] Mutation in *Ck1ε* dramatically shortens the period of circadian rhythms in both hamster and mouse.[12,13] Consistently, a mutation in *CK1δ* results in familial advanced sleep phase (FASP) in humans and shorter period in a transgenic mouse model.[14] One route that phosphorylation impinges on protein turnover is to target the substrate for ubiquitylation and degradation by the 26S proteasome. CK1-mediated phosphorylation of PER leads to recruitment of Skp1-Cul1-F-box protein ubiquitin ligase and a ubiquitin ligase adaptor protein, β-transducin repeat protein (β-TrCP), leading to ubiquitylation, and degradation of PER.[15–17] Similarly, an F-box protein FBXL3 regulates the degradation of CRY.[18–20]

Genetic variations of these clock genes can contribute to physiological changes, which ultimately lead, in some cases, to alterations in disease susceptibility. In this chapter, we bring together findings from studies that examine the effects of human clock-gene variations on diverse aspects of behavior and physiology such as sleep, mood, metabolism, and cancer.

2. BMAL1

BMAL1 variants may play a causative role in type 2 diabetes (T2D) and hypertension. A genetic association study that examined 1304 individuals from 424 families primarily selected for T2D demonstrates that two *BMAL1* haplotypes are associated with T2D and hypertension.[21] Similarly in rodents, *Bmal1* is located within hypertension susceptibility loci and maps closely to a region that is genetically divergent between normotensive and spontaneously hypertensive rat.[21] Cell culture experiments revealed that a polymorphism in *Bmal1* promoter significantly affects transcriptional activation by GATA-4, which is a transcription factor known to be expressed in the cardiovascular system.[21] Therefore, this polymorphism could potentially affect *Bmal1* expression in tissues that are critical for regulating blood pressure. Moreover, *Bmal1* mutant mice show defects in glucose tolerance, reduced islet size, islet proliferation, and insulin secretion that worsen with age, consistent with genetic association studies in human.[22] Conditional knockout mice with *Bmal1* deficiency specifically in the pancreas exhibit diabetes mellitus due to impaired beta-cell function at the latest stage of stimulus-secretion coupling.[22] Notably, one of the single-nucleotide polymorphisms (SNPs) identified in the human haplotype associated with T2D is also significantly associated with susceptibility to prostate cancer.[23]

BMAL1 has been implicated in the pathogenesis of seasonal affective disorder (SAD). SNP analysis in 189 patients and 189 matched controls found an intronic variation in *BMAL1* to be associated with winter depression. Based on *in silico* studies, this site may affect the binding of transcription factors.[24] Furthermore, this variation correlates with differences in experiencing seasonal variation of energy levels.[25]

BMAL1 may contribute to fertility. An intronic polymorphism has been shown to link to the number of pregnancies and miscarriages.[25] This is in agreement with studies in *Bmal1*-null mutant mice, which demonstrates that *Bmal1* is necessary for fertility.[26,27] Loss of *Bmal1* in male mice results in reduced testosterone production,[26] while in female mice, *Bmal1* deficiency leads to impaired growth and development of the reproductive system, reduced ovulation rate, and failure of fertilized oocytes to implant.[27]

Lastly, a study investigating whether clock-gene polymorphisms predispose to alcohol use identified an intronic variant in *BMAL1* to be associated with alcohol consumption in socially drinking controls but not in individuals with alcohol dependence or abuse.[28]

3. CLOCK

The first polymorphism identified in clock genes to be associated with human phenotypes is a SNP located in the 3′-untranslated region (UTR) of *CLOCK*, rs1801260 A/G. Subjects carrying the G allele have significantly lower scores on the Horne–Österberg (HÖ) questionnaire, which assays morningness/eveningness preference and a lower score means eveningness is preferred.[29] The G allele carriers show 10- to 44-min delay in preferred timing for active and sleep phases. This finding of rs1801260 G allele associating with evening preference was further validated by independent investigations.[30–32] However, there are also several studies that were not able to observe this association between the G allele and eveningness, which may be due to differences in ethnic heritages and/or linkages to other polymorphisms (reviewed in Refs. 33 and 34).

Apart from playing a central role in the circadian clock, CLOCK is believed to participate in the regulation of sleep as well. Based on HÖ questionnaire, rs1801260 G/G homozygotes show significantly shorter sleep duration and increased daytime sleepiness compared to individuals carrying the A allele.[30,32,35] The association of rs1801260 and sleep has also been observed in patients with psychiatric disorders. Among patients with major depressive disorder (MDD) or bipolar disorder (BP), G/G homozygotes exhibit significantly increased occurrence of sleep disturbance and BP patients that are homozygous of the G allele show decreased need for sleep.[36] rs1801260 G/G homozygotes also demonstrate higher presence of insomnia during antidepressant treatment.[37] Moreover, in patients with major psychosis (mainly schizophrenia), rs1801260 G/G correlates with daytime sleepiness induced by clozapine treatment, suggesting an interaction between clozapine and *CLOCK* rs1801260 polymorphism.[38] Besides the much studied rs1801260 SNP, two variants in the intronic regions of *CLOCK* have also been shown to be associated with sleep duration based on assessment using Munich ChronoType Questionnaire.[39] Consistently, mutation in the *Clock* gene alters sleep homeostasis in mice.[40] Heterozygous and homozygous *Clock* mutant mice sleep approximately 1 and 2 h less, respectively, than wild type. The heterozygous and homozygous mutants also show 25% and 51% smaller increase of rapid eye movement (REM) sleep, respectively, during 24 h recovery sleep relative to wild-type mice.

Given the reciprocal connections between circadian rhythms/sleep and psychiatric disorders, a number of studies searched for association of *CLOCK* gene polymorphisms with mood. The much studied rs1801260 G allele exhibits significant association with BP,[41] and in

patients with over 5 years of BP history, recurrence rate for bipolar depression is significantly higher in rs1801260 G/G homozygotes.[42] Two variants downstream of the *CLOCK* gene are also significantly linked to BP.[43,44] In BP and unipolar patients undergoing a depressive episode, rs1801260 is related to neuropsychological performance and neural responses in the cingulate cortex to stimuli with moral valence.[31] In addition, the rs1801260 G allele has been shown to be associated with schizophrenia.[45] Interestingly, the rs1801260 A allele significantly correlates with attention deficit hyperactivity disorder (ADHD), implicating a protective role of the G allele in this disorder.[46,47] A synonymous polymorphism in exon 20 of the *CLOCK* gene is linked to fluvoxamine therapeutic response in MDD patients as well as remission with fluvoxamine,[48] implying interaction between the *CLOCK* polymorphism and the mechanistic actions of fluvoxamine. Again these findings are consistent with studies in *Clock* mutant mice. These mice exhibit overall behavioral profile similar to human mania, including hyperactivity, decreased sleep, reduced depression-like and anxiety-like behaviors, as well as an increase in the reward value for cocaine, sucrose, and medial forebrain bundle stimulation.[49,50] Chronic administration of the mood stabilizer lithium can bring many of these behavioral phenotypes back to wild-type levels.[49] The mutant animals exhibit increased dopaminergic activity in the ventral tegmental area, a key reward region in the brain, which could lead to the phenotypes.[49,50] Taken together, these findings in mice are in agreement with the human studies and corroborate the notion that CLOCK is involved in mood regulation.

Similar to BMAL1, CLOCK has also been suggested to play a role in metabolic processes. A number of *CLOCK* polymorphisms are related to body mass index (BMI). Two of these variants located in intron 12 (rs1554483) and the promoter region of the *CLOCK* gene (rs4864548) form a haplotype associated with BMI, while two additional variants, rs1801260 and rs3749474 (located in 3′-UTR), are individually associated with BMI.[35,51,52] Both rs1801260 and rs3749474 are significantly associated with weight, and the latter with waist circumference as well.[35] Under weight-reduction programs, rs1801260 G allele carriers display greater difficulty losing weight, higher plasma ghrelin levels, altered eating behavior, and dietary habits compared to the noncarriers.[32,35] Both rs1801260 and rs3749474, along with an additional SNP in intron 9, rs4580704, are significantly linked to changes in serum cholesterol at the end of dietary treatment.[35] In contrast to overweight/obese individuals, patients with anorexia nervosa or bulimia nervosa carrying the rs1801260 G allele have a lifetime body weight significantly lower than those carrying the A/A genotype, implying a rather complex mechanism of how this rs1801260 variant interacts with metabolism.[53] Notably, rs1801260 G carriers exhibit significantly less small dense low-density lipoprotein, an abnormal lipid metabolite and one of the risk parameters for cardiometabolic disorders, compared to individuals with rs1801260 A/A.[54] Several SNPs in the *CLOCK* gene are significantly associated with energy intake, including the aforementioned 3′-UTR SNP rs3749474, intron 9 SNP rs4580704, promoter SNP rs4864548, and an SNP in intron 11.[35] Moreover, rs3749474, rs4580704, and rs1801260 are related to plasma cytokine levels, particularly those that highly correlated with energy intake.[35] These energy intake-associated SNPs, including rs1801260, are also linked to the monounsaturated fatty acid content of red blood cell membrane, which plays a critical metabolic role.[55] rs4580704 and rs1801260 exhibit dietary fatty acid-dependent associations

with metabolic syndrome traits including glucose and insulin resistance as well as waist circumference.[55] This suggests that *CLOCK* polymorphisms interact with fatty acid to modulate metabolic processes. In addition, rs4580704 is significantly associated with the risk of hypertension.[55] A number of SNPs in the promoter and intronic regions of *CLOCK* show significant associations with susceptibility to and severity of nonalcoholic fatty liver disease, which is one of the most common abnormalities observed in obese people.[56] Two of these SNPs, promoter SNP rs4864548 and intron 12 SNP rs1554483, have been reported to be linked with BMI and energy intake as described earlier, adding further evidence suggesting a role for CLOCK in metabolic pathways. *Clock* mutant mice nicely recapitulate many of the metabolic phenotypes associated with human *CLOCK* gene polymorphisms. These animals are hyperphagic and obese and develop hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia.[57] This supports the idea that the polymorphisms in the human *CLOCK* gene are causatively linked to metabolic alterations observed in human subjects.

Lastly, *CLOCK* variants correlate with the risk and survival rate of cancer. Several SNPs located in intronic regions and 3′-UTR of *CLOCK*, including rs1801260, are significantly associated with susceptibility to prostate cancer or breast cancer.[23,58,59] Both rs1801260 and rs3749474, which have been implicated in various metabolic traits as described earlier, exhibit significant association with survival of colorectal cancer.[60]

4. NPAS2

As a paralogue of CLOCK, NPAS2 has also been implicated in circadian timing and sleep. A SNP in intron 3 of the *NPAS2* gene is associated with timing of sleep in nurses on shift-work schedule, while another SNP in intron 3 correlates with sleepiness during shift work and self-reported adaptation levels to shift-work schedule.[61] Notably, this latter SNP is also significantly linked to alcohol consumption.[61] Consistently, *Npas2*-deficient mice show reduction in sleep during the active phase and enhanced adaptability to phase advance of light–dark schedule.[62]

Like the other two circadian activators, BMAL1 and CLOCK, NPAS2 may be involved in mood regulation as well. In patients with SAD, the frequency of *NPAS2* 471 Leu/Leu genotype is significantly higher than in controls, suggesting that *NPAS2* 471 Leu/Leu contributes to disease susceptibility.[24,63] Furthermore, *NPAS2* 394 Thr correlates with lack of experiencing seasonal variation, assayed by Global Seasonal Scores which measures six items, including seasonal variation of sleep length, social activity, mood, weight, appetite, and energy level, whereas an intronic variant of *NPAS2* is associated with seasonal variation of weight.[25] Another intronic SNP is related to the number of miscarriages, implying that NPAS2 influences fertility.[25] Intronic polymorphisms in *NPAS2* have also been linked to unipolar major mood depression, autistic disorder, and chronic fatigue syndrome.[44,64,65] Notably, *NPAS2* expression is increased in patients with chronic fatigue syndrome.[65]

A missense polymorphism in *NPAS2*, 394 Ala/Thr, is linked to risks of human tumors. *NPAS2* 394 Thr is associated with reduced risk for non-Hodgkin's lymphoma[66] and prostate cancer[67] but increased risk for breast cancer.[68] In terms of effects on physiology, *NPAS2*

394 Thr correlates with lower and bioavailable testosterone, providing support for a role for NPAS2 in hormone-related cancers.[69] Another intronic SNP in *NPAS2* has been shown to be significantly associated with susceptibility to prostate cancer as well.[23]

5. PER1

PER1 may be involved in circadian timing in human. A silent polymorphism in *PER1*, 2434 T/C located in exon 18, is associated with extreme diurnal preference.[70] The C allele is more frequent in individuals with extreme morning preference than in individuals with extreme evening preference.

PER1 is believed to regulate alcohol consumption under psychosocial stress. A SNP in the promoter region of *PER1* is associated with frequency of heavy drinking in adolescents, and significant interaction is observed between this SNP and social adversity on drinking measures.[71] Consistently, this SNP is associated with alcohol dependence in adults as well. Molecular analysis revealed that cortisol-induced transcriptional activation of *PER1* is reduced in human cell lines carrying the risk allele of this SNP. Binding affinity of the transcription factor SNAIL1 to *PER1* promoter containing the risk allele is also reduced. Furthermore, *mPer1* mutant mice show increased alcohol consumption relative to wild type in response to social defeat, supporting a role for PER1 in regulating alcohol drinking induced by psychosocial stress.

Two intronic variants of *PER1* significantly correlate with susceptibility to prostate cancer.[23] One of these SNPs is also significantly associated with autistic disorder, along with a couple additional intronic SNPs.[64] *PER1* 962 Ala/Pro variant is linked to serum levels of sex steroid and insulin-like growth factor-binding protein 3, providing physiological support for a role of PER1 in hormone-related cancer.[69]

6. PER2

PER2 is the first gene found to carry mutation that causes FASP. FASP is formerly known as familial advanced sleep phase syndrome and currently referred to as familial advanced sleep phase disorder. However, according to American Academy of Sleep Medicine's classification of sleep disorders, advanced and delayed sleep phase (DSP) is only a disorder when it is problematic for the individual.[72] Therefore, in this chapter, advanced and DSP phenotypes will not be called disorders. FASP is originally identified as a highly penetrant autosomal dominant trait in three families in which affected individuals exhibit very early sleep onset and offset time.[73] HÖ questionnaire was performed on family members, and FASP subjects scored significantly higher than unaffected relatives. FASP is early onset: the youngest affected individual was 8 years old, and most FASP subjects knew they were obligate “morning larks” by 30 years of age, which is distinctly different from ASPD caused by aging.[74,75] FASP subjects from the first identified family demonstrate a 4-h phase advance of the time of sleep onset, sleep offset, first slow-wave sleep, and REM sleep compared to that of the controls, although sleep quality and quantity are not significantly different between the two groups. Narcolepsy, obstructive sleep apnea, “restless legs” syndrome, and depression were ruled out as possible causes of early sleep onset in these FASP subjects. Consistent with the sleep–wake cycle, dim-light melatonin onset, a reliable

marker of circadian phase, and core body temperature rhythms are also advanced by approximately 4 h in FASP subjects from this family. Sleep–wake and temperature rhythms of one FASP subject were monitored in time isolation and show a circadian period of 23.3 h (Fig. 3.1), which is substantially shorter than that of control subjects (24.2 h) and is consistent with the advanced phase of sleep–wake cycle.

In order to identify the mutation that leads to FASP in the subjects in this family, linkage analysis was performed, which mapped the allele to chromosome 2qter.[76] Further physical mapping was carried out and led to identification of ∼40 cDNAs localized to this region. The only coding mutation identified is in the *PER2* cDNA at position 2106 (A–G), which results in substitution of a serine at amino acid 662 with a glycine (S662G). Functional characterization was subsequently carried out to establish whether the S662G mutation causes FASP. *In vitro* study using PER2 truncation mutants demonstrates that S662 is located within CK1-binding region and the S662G mutation causes hypophosphorylation by CK1. Sequence analysis of PER2 reveals four additional serine residues that are C-terminal to S662 and each with two amino acids in between (i.e., S665, S668, S671, and S674), consistent with the CK1 recognition consensus motif. Furthermore, mutating S662 to aspartate (S662D), which mimics a phosphoserine, restores CK1-dependent phosphorylation, suggesting that S662 is a phosphorylation site on PER2. Similarly, *in vitro* phosphorylation assays using PER2 peptides that encompass residues from 660 to 674 demonstrate that PER2 peptide with a phosphate covalently linked to S662 is phosphorylated at the other residues by CK1, whereas PER2 peptide without a phosphate at S662 is not phosphorylated by CK1.[77] A quantitative assay using PER2 peptides shows that an additional 4 mol of phosphate were incorporated per mole of the PER2 peptide, corresponding to the four serine residues C-terminal to S662. Subsequent phosphoamino acid analysis revealed that the threonine and tyrosine residues on the peptide are not phosphorylated, implying that phosphorylation occurs at the serine residues. Taken together, these results suggest that phosphorylation at S662 of PER2 serves as a priming event that is critical for a cascade of phosphorylations downstream of S662 by CK1.

To investigate the functional consequences of the S662G mutation *in vivo*, transgenic mice carrying wild-type h*PER2* and h*PER2* with S662G or S662D mutations were generated using a human bacterial artificial chromosome (BAC) which carries the *cis*-acting genomic regulatory elements that can faithfully recapitulate endogenous *PER2* expression. Behavior analysis shows that the S662G transgenic mice exhibit ∼2 h shorter free-running period, whereas the S662D mice exhibit 0.5 h lengthening of period versus wild type. Under 12 h light:12 h dark (12L:12D) conditions, the S662G mice show ∼4 h phase advance of locomotor activity rhythms which is almost identical to that of human FASP subjects carrying this mutation. The S662G mutation does not significantly affect PER2 degradation or nuclear localization, but it affects *PER2* transcript levels. In the transgenic mice, both h*PER2* and the endogenous mouse *Per2* (m*Per2*) mRNA levels peak earlier for S662G and later for S662D relative to wild type, corresponding to the shorter and longer behavioral periods, respectively. Moreover, the mRNA levels are lower in S662G mice and higher in S662D mice compared to wild type. Because both mutant h*PER2* and the endogenous wild-

type m*Per2* transcript levels are reduced in the S662G mice, this argues for reduced transcriptional activity rather than reduced *PER2* mRNA stability as a result of the mutation.

Consistently, association studies have linked *PER2* to diurnal preference as well. The allele frequency of a SNP in the 5′-UTR 12 bases upstream of the translational start codon of *PER2*, -111G, is significantly higher in individuals with extreme morning preference than individuals with extreme evening preference.[78] Based on computer prediction, this polymorphism may alter the secondary structure of *PER2* mRNA. A missense variant 1244 Gly/Glu is also associated with morningness: carriers of 1244 Gly show significantly higher morning scores based on composite scale for morningness.[79] This 1244 Gly/Glu SNP is also part of a haplotype in *PER2* linked to depression vulnerability.[80] In addition, *PER2* has been implicated in sleep regulation. A synonymous SNP in *PER2*, 2229 G/A, correlates with the duration of sleep for nurses on day shift but not night shift.[61]

The *PER2* -111G allele is also linked to reduced activity in adolescents in the key neural component of the reward circuitry (medial frontal cortex).[81] Supporting the idea of a role for PER2 in reward function, m*Per2* mutant mice exhibit hypersensitized response to cocaine and strong cocaine-induced place preference.[82] Collectively, these results strongly suggest that PER2 modulates reward.

The *PER2* -111 G/C SNP correlates with metabolic and eating behavior-related phenotypes, including abdominal obesity, probability of withdrawing from weight-reduction program, extreme snacking, stress with dieting, eating when bored, and skipping breakfast.[83] Among individuals with metabolic syndromes and high levels of saturated fatty acid (SFA),-111G carriers have higher plasma lipid concentrations,[84] suggesting that the -111G/C allele interacts with plasma SFA to modify lipid levels.

PER2 participates in modulating alcohol consumption, similar to its counterpart PER1. A SNP located in intron 3 of *PER2*, 10,870 A/G, is associated with the quantity of alcohol intake.[85,86] This SNP resides in a CAT-TTT motif, which is conserved in human, chimpanzee, and rat.[85] It is also in an enhancer-like structure, which contains several transcriptional factor-binding site motifs. This SNP alters the binding motifs for Sp1, c-myb, and NF-κB, possibly resulting in altered transactivation of *PER2*. m*Per2* mutant mice exhibit increased alcohol consumption, accompanied by enhanced glutamate levels in the extracellular space in the brain. This is believed to be a result of reduced expression of the glutamate transporter gene, *Eaat1*, and thus reduced uptake of glutamate by astrocytes. Acamprosate, a drug used to prevent craving and relapse in alcoholic patients, reduced the enhanced glutamate levels and normalized the increased alcohol intake in m*Per2* mutant mice. Collectively, these data suggest that PER2 acts to suppress glutamatergic signaling, which in turn influences alcohol drinking. Besides being involved in modulating alcohol consumption, the PER2 10870 A/G SNP is also associated with SAD.[24]

PER2 is linked to the risk of cancer. An intronic SNP in *PER2* is significantly associated with susceptibility to prostate cancer,[23] whereas the aforementioned 1244 Gly/Glu associated with morningness and depression vulnerability also correlates with the risk of breast cancer in combination with an SNP in *CLOCK*.[59]

7. PER3

Several polymorphisms in *PER3* have been suggested to contribute to determination of diurnal preference and DSP.[63,87–91] The most well-studied polymorphism among these is a polymorphic repeat region with four or five copies of a 54-bp repetitive sequence (4-repeat vs. 5-repeat) in exon 18. However, this association with morningness/eveningness attenuates with age.[90,92] Human subjects homozygous for the long allele are particularly sensitive to blue-enriched light, as such light significantly suppresses evening rise of endogenous melatonin in homozygotes for the long allele but not the short allele.[93] Likewise, individuals homozygous for the long allele exhibit more pronounced response to the alerting effects of light compared to homozygotes for the short allele. Waking electroencephalographic (EEG) activity in the theta range (5–7 Hz), which is a putative correlate of sleepiness, is substantially attenuated during exposure to blue-enriched light in subjects homozygous for the long allele but not the short allele. This length polymorphism has also been shown to be one of the alleles associated with self-reported adaptation levels to shift-work schedules and sleep phase in nurses working on shifts.[61] Another SNP reported to be associated with morning–evening scores is *PER3* 647 Val/Gly.[63] A few polymorphisms in the promoter region of *PER3* are linked to DSP as well.[91] *In vitro* studies demonstrate that these promoter polymorphisms may modify the transcription of *PER3*.

PER3 may exert effects on sleep homeostasis. Individuals homozygous for the 5-repeat allele exhibit increase in markers of sleep homeostasis, including slow-wave sleep, EEG slow-wave activity (0.75–4.5 Hz) in non-REM sleep, as well as theta and alpha activity (8–12 Hz) during REM sleep and wakefulness.[94] The decrement in cognitive performance as a result of sleep deprivation is significantly larger in subjects homozygous for the long allele. Individuals of this genotype also perform worse on tests of executive function at early morning during sleep deprivation relative to homozygotes for the short allele.[95] Further study employing functional magnetic resonance imaging indicates that both genotypes recruit brain regions typically involved in working memory, but individuals homozygous for the short allele recruit supplemental anterior frontal, temporal, and subcortical regions in addition.[96] In contrast, widespread reductions of activation in prefrontal, temporal, parietal, and occipital areas were observed in homozygotes for the long allele. Accompanying increased slow-wave sleep in subjects homozygous for the long allele is an elevated sympathetic predominance and a reduction of parasympathetic predominance in the autonomic nervous system.[97] Both homozygosity for the long allele and a SNP in exon 18, 1148 Arg, are associated with reduced daytime sleepiness, and also sleepiness in nurses working during shifts.[61,98] On the other hand, homozygosity of the 4-repeat allele correlates with insomnia in alcohol-dependent patients.[99] Consistent with the idea of a role for PER3 in modulating sleep homeostasis in human, mice deficient for *Per3* exhibit altered patterns of sleep both under baseline condition and after sleep deprivation.[100]

A role for PER3 has been implicated in metabolic processes. *PER3* 639 Val is associated with T2D, while the much studied *PER3* length polymorphism modifies the effects of the timing and duration of sleep on BMI.[98,101] This is supported by *in vitro* study that demonstrates PER3 functions to inhibit adipogenesis, and *Per3* knockout mice display increased adipose tissue and decreased muscle tissue relative to wild type.[102]

An intronic SNP in *PER3* is significantly associated with susceptibility to prostate cancer.[23] At a physiological level, the aforementioned 5-repeat allele correlates with higher levels of serum insulin-like growth factor-I (IGF) and the ratio of IGF-I to IGF-binding protein 3, which may contribute to hormone-related cancer.[69] Furthermore, inflammation is an established cancer risk factor and carriers of the 5-repeat allele show elevated levels of the cytokine interleukin-6.[103]

Lastly, the 4-repeat allele of *PER3* is significantly linked to heroin dependence and postpartum onset of BP.[104,105]

8. CRY1

Two SNPs located in the promoter region of *CRY1* are associated with susceptibility to and mortality from prostate cancer, respectively.[23,106] On the other hand, a SNP within 3′-UTR of *CRY1* correlates with risk of breast cancer.[59] In addition, a SNP located 3′ downstream of *CRY1* is significantly associated with MDD.

9. CRY2

CRY2 has been suggested by various studies to act as a modulator of cancer development. Two intronic SNPs in *CRY2* are significantly associated with susceptibility to prostate cancer.[23,67] For one of these SNPs located in intron 2, rs1401417 G/C, carriers of the C allele exhibit 1.7-fold increased risk of prostate cancer.[67] This risk is increased to 4.1-fold in the C allele carriers with higher insulin resistance. This allele is also linked to breast cancer risk, along with two additional SNPs, and all three of these SNPs are significantly associated with the risk of non-Hodgkin's lymphoma.[59,107,108] Breast cancer patients have significantly higher levels of *CRY2* promoter methylation relative to controls, consistent with lower levels of *CRY2* in tumor tissues compared to adjacent normal tissues. Furthermore, *in vitro* analysis identifies alterations in the expression of breast cancer-relevant genes, immune response genes, and hematologic system development genes in response to *CRY2* knockdown.[107,108] Some of these genes are predicted to have significant effects on several disease processes, including cancer.[107] Taken together, these findings suggest that CRY2 may exert significant effects on cancer susceptibility.

Genome-wide association study identified an intronic SNP in *CRY2* to be significantly associated with fasting glucose levels in nondiabetic adults.[109] Subsequent studies reported this locus to be correlated with T2D, as well as fasting glucose in healthy children and adolescents.[110,111]

Three SNPs in *CRY2* are linked to winter depression, including one of the SNPs that have been reported to be associated with the risk of breast cancer and non-Hodgkin lymphoma.[112] Molecular analysis revealed that the levels of *CRY2* mRNA are decreased in depressed bipolar patients. While a night of total sleep deprivation results in significant upregulation of *CRY2* transcript in control subjects, it fails to do so in depressed bipolar patients. Both the genetic and molecular studies suggest that dysregulation of *CRY2* expression may be involved in vulnerability to depression.

10. REV-ERBα

REV-ERBα is primarily implicated in BP. A haplotype comprised of two SNPs located in intron 1 and 5′-UTR of *REV-ERBα*, respectively, is significantly associated with BP.[113] Furthermore, a SNP in the intronic region of *REV-ERBα*, rs2314339 C/T, is associated with long-term efficacy of lithium carbonate therapy in BP.[114] The frequency of the T allele is significantly increased in nonresponders, and patients carrying the T allele are 3.5 × more likely to show no improvement or even worsening of the illness. Consistently, another SNP located in the promoter region of *REV-ERBα* correlates with good treatment response and changes in REV-ERB*α* expression in response to lithium treatment.[115] These findings support a role for REV-ERBα in the therapeutic mechanism of lithium.

11. CK1ε

A SNP in the 3′-UTR of *CK1ε* is significantly associated with self-reported response to D-amphetamine.[116] Consistently, quantitative trait loci (QTL) analysis in mice selectively bred for high versus low sensitivity to methamphetamine identified a QTL in the *Ck1ε* gene that may cause the difference in response to methamphetamine.[117] Expression differences of *Ck1ε* is also observed in mouse lines displaying high versus low sensitivity to methamphetamine. Collectively, human and animal studies suggest that CK1ε contributes to variability in stimulant response.

An intronic SNP of *CK1ε* is linked to BP and prostate cancer.[23,43] Furthermore, another variant in *CK1ε* correlates with testosterone to dihydrotestosterone ratio in the serum, which may contribute to the pathology of prostate cancer.[69]

12. CK1δ

Exon sequencing of circadian genes for individuals that belong to amoderate-sized family with FASP led to the identification of a second mutation that causes FASP. The mutation is a threonine-to-alanine alteration at amino acid 44 of CK1δ (CK1δ-T44A), and this threonine is conserved in other mammalian CK1s and *Drosophila* CK1 (dDBT).[14] *In vitro* kinase assay demonstrates that this mutation results in decreased phosphorylation of both exogenous substrates (phosvitin and alpha-casein) and circadian substrates(PER1–3). To examine the effects of this mutation on circadian rhythms *in vivo*, BAC transgenic mice carrying either the wild type (h*CK1δ-WT*) or the mutant (h*CK1δ-T44A*) h*CK1δ* were generated. The behavioral period under free-running condition is significantly shorter in the mutant transgenic mice compared to wild type, consistent with the phase-advanced phenotype of human subjects carrying this mutation. Neither $CK1\delta^{+/-}$ norh*CK1δ-WT* transgenic mice exhibit altered period, suggesting that the period is not affected by wild-type *CK1δ* gene dosage. Thus, the shorter period observed in h*CK1δ-T44A* transgenic mice is likely due to the T44A mutation and not altered *CK1δ* gene dosage. Interestingly, expression of h*CK1δ-T44A* in *Drosophila* circadian neurons results in longer period compared to expression of h*CK1δ-WT*. This may reflect differences in the regulatory mechanism of the mammalian clock versus invertebrate clock.

The aforementioned h*PER2-S662G* and h*CK1δ-T44A* mutations indicate that phosphorylation of PER2 by CK1 is critical for circadian timing in humans. To characterize the functional relevance of the interaction between PER2 and CK1 *in vivo*, h*PER2* transgenic mice were crossed with both h*CK1δ-WT* transgenic and *CK1δ* knockout mice.[77] As described earlier in this chapter, h*PER2-S662G* transgenic mice exhibit a short period of∼22 h, whereas neither h*CK1δ-WT* transgenic nor $CK1\delta^{+/-}$ exhibits altered circadian period. However, in mice carrying both h*PER2-S662G* and h*CK1δ-WT* transgenes, the period is shorter than h*PER2-S662G* single transgenic animals by over 1 h. Consistently, expressing h*PER2-S662G* on the $CK1\delta^{+/-}$ background slightly lengthens the period compared to expressing h*PER2-S662G* on a wild-type background. On the other hand, h*PER2-S662D* transgenic mice, which show long period on wild-type background, exhibit even longer period in $CK1\delta^{+/-}$ background and a shorter period in h*CK1δ-WT* background. Therefore, decreasing *CK1δ* dosage lengthens period for both h*PER2-S662G* and h*PER2-S662D* transgenic mice. Similarly, increasing *CK1δ* dosage shortens the endogenous period of both S662 mutants but not wild type.

Taken together, these results lead to the proposal of the following model regarding how CK1 acts on PER2 to regulate circadian period: CK1 phosphorylates the serine residues downstream of S662 on PER2 after S662 is phosphorylated by a priming kinase. Phosphorylation in this region of PER2 increases *PER2* mRNA and thus protein, while CK1 likely phosphorylates some other site(s) that results in degradation of PER2. The net effect of these two opposing processes determines the level of PER2 and in turn sets circadian period. In wild-type background, the balance of these opposing effects can be maintained, thus decreasing or increasing *CK1δ* gene dosage does not change the period. In the presence of S662G mutation, the S662 residue can no longer be phosphorylated by the priming kinase, leading to hypophosphorylation of the downstream residues by CK1. Therefore, the net effect of CK1 on mutant PER2 results in reduced PER2 levels and shorter period. Decreasing *CK1δ* gene dosage partially suppresses the period shortening effect by reducing phosphorylation-mediated PER2 degradation, whereas increasing *CK1δ* gene dosage further shortens period by enhancing phosphorylation-mediated PER2 degradation.

13. CUL1

A SNP in intron 3 of *CUL1* is significantly associated with rheumatoid arthritis (RA).[118] In lymphocytic cell lines, this SNP affects transcriptional efficiency of *CUL1* promoter activity. CUL1 is highly expressed in lymphoid tissues, and suppression of CUL1 inhibits IL-8 induction, which plays an important role in migration of inflammatory cells into the affected area as seen in RA. Therefore, this SNP in intron 3 of *CUL1* could be affecting susceptibility to RA by modulating expression levels of *CUL1*. In another independent study, this SNP, along with two other, constitutes a haplotype that is significantly associated with RA and response to methotrexate treatment, a commonly prescribed drug for RA patients.[119]

14. β-TrCP

β-TrCP mutations have been implicated in cancer. Missense somatic mutations in *β-TrCP* were identified in gastric cancers.[120] In tissues carrying these mutations, β-catenin levels are increased with aberrant subcellular distribution, which may contribute to the development of gastric cancer. Further evidence came from an association study demonstrating that a 9-bp deletion polymorphism in the 3′-UTR of *β-TrCP* correlates with reduced risk of hepatocellular carcinoma (HCC).[121] Molecular analysis revealed that HCC tumor tissues with the deletion display reduced levels of β-TrCP compared to those that do not carry the deletion. Because β-TrCP is believed to be oncogenic, reduced β-TrCP levels associated with the deletion variant could explain the reduced risk of HCC. Additionally, duplication of *β-TrCP* gene is associated with split hand-split foot malformation.[122]

15. DEC1

DEC1 was identified as one of the genes located in a commonly deleted chromosomal region in a wide panel of esophageal squamous cell carcinoma.[123] *DEC1* transcript levels were significantly reduced in the majority of esophageal cancer cell lines, while introducing *DEC1* cDNA into cancer cells that lack *DEC1* expression significantly suppresses cell growth. Consistently, a polymorphism in the promoter region of *DEC1* (-249T/C) is significantly associated with the risk of squamous cell carcinoma of the head and neck (SCCHN), and human subjects homozygous for -249 C show significantly reduced susceptibility to SCCHN.[124] *In silico* analysis predicts that the -249 T to C change leads to a gain of a transcription factor-binding site. Indeed, further functional analysis demonstrated that the T–C change results in increased transcriptional activity at *DEC1* promoter and enhanced protein-DNA binding. In summary, these results suggest that DEC1 functions as a tumor suppressor and genetic variations in *DEC1* could alter susceptibility to cancer.

16. DEC2

The first human mutation identified to cause a sleep homeostasis phenotype is in *DEC2*.[125] Individuals carrying a proline to arginine mutation at amino acid position 384 (P384R) of DEC2 have approximately 2-h shorter sleep time per 24-h day compared to family members who do not carry the mutation (Fig. 3.2). Studies in cell culture demonstrated that the P384R mutation results in attenuated DEC2 repressor activity of CLOCK/BMAL1-driven transcription.

To validate that the P384R mutation is indeed causing the short-sleep phenotype and not merely associated with the phenotype, BAC transgenic mice were generated to carry wild-type h*DEC2* (h*DEC2-WT)* or h*DEC2-P384R*. h*DEC2-P384R* mice do not exhibit altered free-running period, but the duration of the activity period (alpha) is 1.2-h longer relative to *hDEC2-WT* transgenic, wild-type littermates, and *Dec2* knockout mice. This recapitulates the shorter sleep duration (i.e., inactive period) phenotype observed in humans. Moreover, when h*DEC2-P384R* is expressed in a *Dec2* knockout background, alpha is further lengthened to ∼2.5 h longer than controls.

To examine the effects of the *DEC2-P384R* mutation on sleep, EEG and electromyography were performed on mutant transgenic mice and littermate controls. h*DEC2-P384R* mice were awake for a significantly longer period of time during the light phase compared to wild type, accompanied by significant reduction of both NREM and REM sleep. Analysis of sleep architecture demonstrated decreased wake duration and an increase in the number of wake episodes in h*DEC2-P384R* mice relative to wild type. In addition, these animals exhibited significantly more NREM episodes during the light phase, but each episode is shorter in duration. These results indicate that sleep (in particular NREM sleep) is more fragmented in h*DEC2-P384R* mice than that of wild type. To better understand the role of DEC2 in sleep regulation, h*DEC2-P384R* mice and wild-type littermates were subjected to acute sleep deprivation. h*DEC2-P384R* mice showed significantly less rebound in both NREM and REM sleep, and a slower recovery of acute sleep loss. h*DEC2-P384R* mice also exhibited lower NREM delta power density change after sleep deprivation compared to wild type, which indicates that the depth of the rebound of NREM sleep is affected in h*DEC2-P384R* animals. Consistent with the mammalian data, expressing m*Dec2-P384R* in the sleep/rest center of *Drosophila* brain leads to significantly less sleep-like behavior with decreased sleep bout duration and increased sleep bout number versus flies expressing m*Dec2-WT*. In summary, these results demonstrate DEC2 as an important player in the regulation of sleep homeostasis.

17. TIMELESS

TIMELESS is associated with depression and sleep disturbances.[126] Four SNPs in or near the *TIMELESS* gene are linked to depression with fatigue in females, while two of these SNPs (rs7486220 A/G and rs1082214 C/T) are also linked to depression with early morning awakening in males. Notably, rs7486220 A and rs1082214 C correlate with depression with fatigue in females, whereas rs7486220 G and rs1082214 T correlate with depression with early morning awakening in males. In a separate set of individuals that do not have depression, rs1082214 C is correlated with higher levels of seasonal changes in mood in females, while rs1082214 T is correlated with early morning awakening and fatigue in males. Collectively, these data implicate a connection between TIMELESS and gender-dependent depression and sleep regulation.

18. Concluding Remarks

Studies of human clock-gene variants reveal that besides circadian timing, clock genes may also be involved in a number of other biological processes (Table 3.1). Most of the clock-gene polymorphisms are associated with sleep regulation, cancer development, metabolic traits, and mood disorders, implying that these processes may have particularly close connections with the circadian clock, and thus are more sensitive to alterations of the clock caused by genetic variations. In addition, *CLOCK*, *PER1–3*, and *CK1ε* polymorphisms are linked to addiction, suggesting a role for the clock in reward circuitry of the brain. *BMAL1* and *NPAS2* polymorphisms are related to fertility and seasonal variations, supporting the long-held view that circadian clock participates in seasonal adaptability. Furthermore, studies using mice deficient for clock genes verified the involvement of clock genes in a

number of the processes implicated by human genetic studies, including sleep, metabolic syndromes, mood disorders, addiction, and fertility.

It is worthwhile at this point to detail approaches to Mendelian genetics versus association studies as the distinction between the two is not well understood by many people who are interested in the current topic. Mendelian genetics deals with identification of genetic variants of strong effect and are sufficient to *cause* a phenotype. For example, genetic studies of rodents with spontaneous mutations (e.g., the *Ck1ε* mutant hamster) and forward genetic screens in model organisms where mutagenesis is performed and animals are screened for a phenotype (e.g., short/long period or arrhythmia) are focused on identifying the genes and mutations which *cause* the phenotype. Similar studies have been successful in humans where identification of FASP allowed cloning of causative genes and mutations.

In complex genetics, genetic variants are sought where there is a statistical association of the variant with a phenotype. The variant itself is only *associated* with an increased risk of the phenotype. Thus, having the variant does not mean that the carrier will have the phenotype. Neither does it mean that one without the variant cannot have the phenotype. Such a finding does not simply imply that variant is itself causative of the increased risk. Rather, it suggests that the associated variant and/or a genetic variant in the vicinity of the associated variant leads to increased risk. Consequently, we must be very careful when interpreting these data, as many such findings (positive associations) have (or will turn out to be) false positives. In some cases, a variant in a gene will be associated with the phenotype because that gene is truly linked to the biology underlying the phenotype of interest. In other cases, a genetic variant may truly be associated with the phenotype but only because it is in linkage disequilibrium with a variant in another gene. Mutations in some clock genes have been generated and result in behavioral and/or physiological phenotypes in animal models (such as the mouse studies described earlier in this chapter). These studies support the argument that the recognized clock gene associations in humans with similar or related phenotypes occur as a result of genetic variants in the respective clock genes. To validate whether these genetic variations found by the association studies lead to phenotypic changes will require generation and characterization of appropriate animal models carrying equivalent polymorphisms. Unlike Mendelian traits with very prominent phenotypes as in the cases of FASP, however, many of the behavioral and physiological phenotypes observed in association studies are relatively subtle and may exhibit complex allelic interactions, imposing great complications and challenges on studies in animal models. Nevertheless, as we learn more regarding the molecular underpinnings of the biological processes involved and as our phenotyping techniques improve, using animal models to investigate the mechanistic alterations caused by these human clock gene variants will become more effective and fruitful.

In the past few decades, much effort has been devoted into understanding *what* constitutes the circadian clock and *how* the clock functions. Thus, we currently have a handful of “clock genes” and a relatively clear picture of the molecular mechanisms regarding how these genes act together to set the phase and amplitude of the clock. One of the next big challenges in the field is answering the “*why*” question, that is, *why* is the clock built this way, or an even more fundamental question, *why* do we need a clock. At the individual

level, investigating the broad consequences of alterations in clock genes will help us understand the function of the clock and the role it plays in our overall well-being. At a population level, studying the distribution of clock genotypes and associated phenotypes across the world will facilitate unveiling the interactions between the molecular clock and environment. Insights gained from these studies shall provide answers to some of the most fundamental questions in human circadian biology. Only with such understanding can we maximize the health benefits and therapeutic values of the circadian clock.

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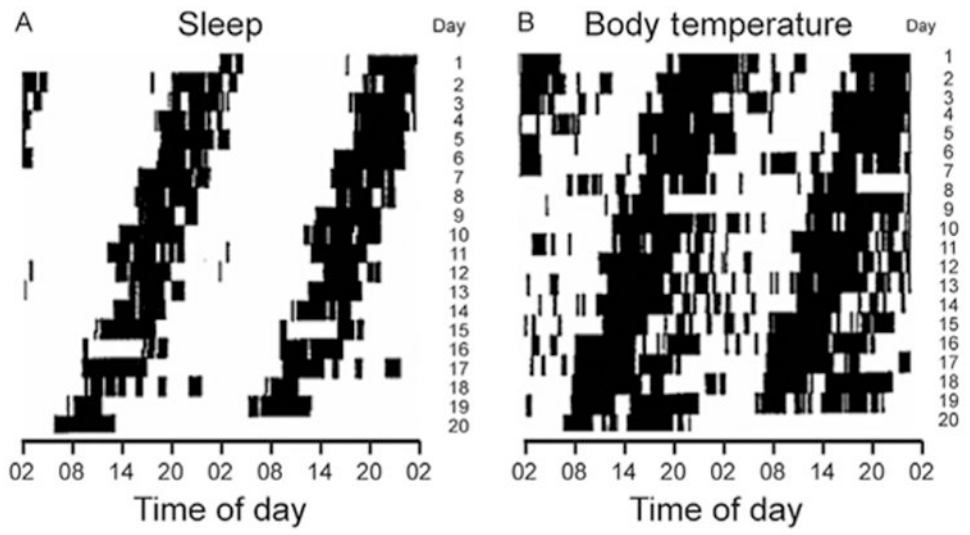

Figure 3.1.

Free-running period of sleep/wake and body temperature cycles in a FASP subject. Sleep/wake (A) and body temperature (B) rhythms of a 69-year-old female monitored in time isolation for 18 days. The data are double plotted. (A) Filled bars indicate periods of sleep derived from polygraphically-recorded sleep scored using “standard” criteria. (B) Filled bars indicate periods when body temperature is below the daily mean. The free-running period of both variables are 23.3 h based on chi-squared periodogram. *Adapted* from Ref. 73.

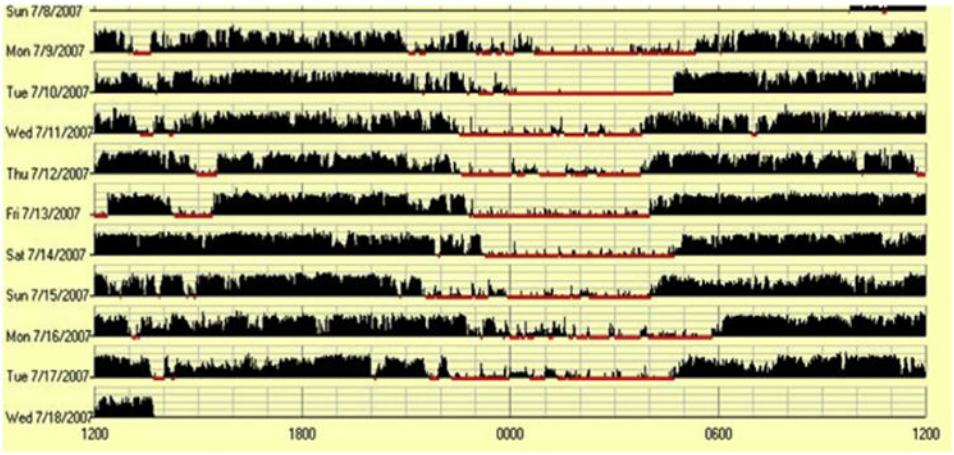

Figure 3.2.

Activity recording of a *DEC2-P384R* mutatiocarrier. Filled bars indicate periods of activity by wrist actigraphy. Extended periods of activity can be observed. *Adapted* from Ref. 125.

Table 3.1
Genetic associations between the clock genes and human phenotypes

Gene	Human phenotype associated	Phenotype of mutant mouse model
BMAL1	Type 2 diabetes and hypertension	Hypoinsulinemia and diabetes
	Prostate cancer	
	Seasonal affective disorder	
	Number of pregnancies and miscarriage	Infertility
	Alcohol consumption	
CLOCK	Eveningness preference	Circadian phenotype
	Altered sleep	Reduced sleep
	Bipolar disorder, schizophrenia, attention deficit hyperactivity disorder, and fluvoxamine response in major depressive disorder patients	Mania-like behavior
	Metabolic traits, hypertension, and nonalcoholic fatty liver disease	Obesity and metabolic syndromes
	Prostate cancer, breast cancer, and survival rate of colorectal cancer	
NPAS2	Daily timing and sleepiness	Reduced sleep and enhanced light entrainment
	Seasonal affective disorder and depression	
	Autistic disorder	
	Chronic fatigue syndrome	
	Number of miscarriages	
	Non-Hodgkin's lymphoma, prostate cancer, and breast cancer	
PER1	Extreme morning preference	Circadian phenotype
	Alcohol consumption under psychosocial stress	Increased alcohol consumption in response to social defeat
	Prostate cancer	
	Autistic disorder	
PER2	Familial advanced sleep phase	Phase advance during light/dark cycle and shorter free-running period
	Morningness preference	
	Duration of sleep (nurses on day shift)	
	Depression	
	Reduced activity in medial frontal cortex of adolescents	Hypersensitized response to cocaine and strong cocaine-induced place preference
	Metabolic traits	
	Alcohol consumption	Increased alcohol consumption
	Seasonal affective disorder	
	Prostate cancer and breast cancer	
PER3	Morningness/eveningness preference, timing of sleep phase, and sensitivity to blue-enriched light	
	Sleep homeostasis phenotypes	Altered sleep patterns
	Type 2 diabetes and body mass index	Increased adipose tissue and decreased muscle tissue
	Prostate cancer	

Gene	Human phenotype associated	Phenotype of mutant mouse model
	Heroin dependence	
	Postpartum onset of bipolar disorder	
CRY1	Prostate cancer and breast cancer	
	Major depressive disorder	
CRY2	Prostate cancer, breast cancer, and non-Hodgkin's lymphoma	
	Fasting glucose levels	
	Winter depression	
REV-ERBα	Bipolar disorder and efficacy of lithium treatment in bipolar disorder	
CK1ε	Self-reported response to D-amphetamine	A quantitative trait locus in *Ck1ε* may cause differences in response to methamphetamine
	Bipolar disorder	
	Prostate cancer	
CK1δ	Familial advanced sleep phase	Shorter free-running period
CUL1	Rheumatoid arthritis and response to methotrexate treatment	
B-TrCP	Gastric cancers and hepatocellular carcinoma	
	Split hand-split foot malformation	
DEC1	Squamous cell carcinoma of the head and neck	
DEC2	Shorter sleep duration	Reduced sleep amount
TIMELESS	Depression and sleep disturbances	