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Genetics of Circadian Rhythms in Mammalian Model Organisms

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Abstract

The mammalian circadian system is a complex hierarchical temporal network which is organized around an ensemble of uniquely coupled cells comprising the principal circadian pacemaker in the suprachiasmatic nucleus of the hypothalamus. This central pacemaker is entrained each day by the environmental light/dark cycle and transmits synchronizing cues to cell-autonomous oscillators in tissues throughout the body. Within cells of the central pacemaker and the peripheral tissues, the underlying molecular mechanism by which oscillations in gene expression occur involves interconnected feedback loops of transcription and translation. Over the past 10 years we have learned much regarding the genetics of this system, including how it is particularly resilient when challenged by single-gene mutations, how accessory transcriptional loops enhance the robustness of oscillations, how epigenetic mechanisms contribute to the control of circadian gene expression, and how, from coupled neuronal networks, emergent clock properties arise. Here we will explore the genetics of the mammalian circadian system from cell-autonomous molecular oscillations, to interactions among central and peripheral oscillators and ultimately, to the daily rhythms of behavior observed in the animal.

I. INTRODUCTION

The rising and setting of the sun each day causes predictable environmental changes to which most organisms on earth have adapted by evolving endogenous biological timing systems with a period of approximately 24 hours (Young and Kay, 2001). These circadian (~24 hr) clocks anticipate environmental cycles and control daily rhythms in biochemistry, physiology and behavior. Across phyla, all circadian clocks share several fundamental properties: they are synchronized (entrained) each day to external cues, they are self-sustained and produce oscillations that persist in the absence of any external cues, they are temperature compensated such that temperature changes in the physiological range do not alter their endogenous period, and of particular relevance to this review, they are cell-autonomous and genetically-determined. In all of the major model organisms in which circadian rhythms have been studied there has emerged a central organizing principle of the molecular clockwork: within cells a set of clock genes and their protein products together participate in autoregulatory feedback loops of transcription and translation to produce an oscillation with a period of about 24 hr (Lowrey and Takahashi, 2004; Takahashi *et al.*, 2008).

Recent work, however, has prompted a reappraisal of the transcription/translation model as the sole generative mechanism of the molecular circadian oscillator in mammals. For example, it is now clear that oscillations of some mammalian core clock components are dispensable for circadian function (Fan *et al.*, 2007; Liu *et al.*, 2008), and there is some evidence, albeit preliminary, for circadian rhythms in the absence of transcription in some mammalian cells (O'Neill and Reddy, 2011). Perhaps more importantly, however,

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limitations of the conventional perturbation analysis methods that helped elucidate the transcription/translation model have become apparent. No longer is it sufficient to knock out a clock gene in a mouse and then assess the consequences on behavior (locomotor activity) or gene expression (changes in RNA and protein levels in cells) alone. We now appreciate that the mammalian circadian clock is a more complex hierarchical system than originally imagined, and thus understanding it requires analysis at many levels.

New technologies and clock models have revealed higher-order genetic properties of the mammalian clock system in which the elimination of one component may be compensated for by other components in ways that are more complex than simple redundancy, and they have demonstrated the important roles of accessory feedback loops and gene networks in conferring stability and robustness on the system (Ueda *et al.*, 2005; Ukai-Tadenuma *et al.*, 2008; Baggs *et al.*, 2009). Further, novel approaches have elucidated the importance of networks of coupled cells from which emergent circadian clock properties arise and even buffer the system against the effects of mutations (Liu *et al.*, 2007b; Abraham *et al.*, 2010; Buhr *et al.*, 2010; Ko *et al.*, 2010). These, and other advances, are making clearer the fundamental properties of each level of organization of the mammalian circadian system from cell-autonomous molecular oscillations, to tissue-specific properties, to the interaction of central and peripheral oscillators, and ultimately to the overt daily rhythms of behavior observed in the animal.

Here we present some of the key findings in the field of mammalian circadian biology over the past 10 years and introduce many of the new technologies that are revolutionizing our understanding of the clock system. Our emphasis will be primarily on work from the principal model organism used to study mammalian biology—the mouse. Indeed, for no other mammalian model is there the extensive repertoire of experimental resources and techniques as for the mouse (Silver, 1995; Nagy *et al.*, 2003; Hedrich and Bullock, 2004; Fox *et al.*, 2007; Adams and van der Weyden, 2008; Blake *et al.*, 2010). We will not, however, explore in depth the intriguing link between the mammalian circadian clock and metabolism, first proposed by McKnight and colleagues a decade ago (Rutter *et al.*, 2002), and now well established, as it is beyond the scope of this review. Instead, we refer the reader to several recent comprehensive treatments of this specific topic (Green *et al.*, 2008; Bass and Takahashi, 2010; Maury *et al.*, 2010; Asher and Schibler, 2011).

II. THE BEGINNING OF MAMMALIAN CLOCK GENETICS

A. Serendipitous discovery of the Syrian hamster *tau* mutant

Before discussing the current state of mammalian clock genetics and the details of the molecular clockwork in mammals, we would first like to reflect back briefly on the period from approximately 1985 to 2000 when the study of mammalian clock genetics began. Indeed, it was in 1985 that Martin Ralph, at the time a graduate student in the laboratory of Michael Menaker (then at the University of Oregon), identified a single outbred Syrian hamster (*Mesocricetus auratus*) with an unusually early onset of locomotor activity. Following transfer from a light/dark (LD) cycle to constant darkness (DD), this animal exhibited an endogenous free-running period of 22 hr compared to 23.5 hr, the shortest previously reported circadian period for this species. Recognizing the implications of possibly discovering the first mammalian circadian mutant, Ralph had the foresight to cross this animal to wild-type hamsters and analyze the behavioral rhythms in the offspring. The free-running periods for the resulting F_1 progeny (1:1 ratio; 22 hr : 24 hr) confirmed that the aberrant phenotype was heritable. Intercrosses produced an F_2 generation with a 1:2:1 Mendelian ratio of 20 hr : 22 hr : 24 hr periods. Thus, this spontaneous mutation designated *tau* (after the circadian symbol for period length), segregated in a semidominant manner and seemed to involve a single autosomal locus (Table 1) (Ralph and Menaker, 1988).

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As the first mammalian circadian mutation, *tau* figured prominently in many studies addressing behavioral and physiological aspects of mammalian circadian biology. Perhaps the most important result obtained from the *tau* model was the definitive demonstration through transplantation experiments that the suprachiasmatic nucleus (SCN) of the hypothalamus harbors the central circadian pacemaker in mammals. Adult hamsters rendered behaviorally arrhythmic by SCN lesioning exhibited restored rhythmicity following transplantation of donor SCN tissue into the third ventricle. Not only was host rhythmicity rescued, but the restored rhythms had periods reflecting the genotype of the donor animal (Ralph *et al.*, 1990). Further, when firing rate rhythms of individual SCN neurons on fixed microelectrode plates were recorded, cells from *tau* animals helped show that the circadian period of the whole tissue/animal is determined by averaging widely dispersed periods of individual SCN clock cells. This was the first demonstration that the *tau* mutation affects circadian function in a cell-autonomous manner (Liu *et al.*, 1997b). Two additional seminal findings using the *tau* model include the first report that a diffusible signal can drive circadian rhythms in a mammal (Silver *et al.*, 1996), and that SCN-independent circadian oscillators reside in the mammalian retina (Tosini and Menaker, 1996; Tosini and Menaker, 1998).

Despite the importance to the field of mammalian circadian biology of the *tau* model, from a genetic standpoint it is unfortunate that the mutation occurred in the Syrian hamster rather than in the mouse, a mammalian model for which even in 1985 more comprehensive genetic resources were available. Efforts to develop dense genetic maps and physical mapping reagents for the mouse were well underway at the inception of the Human Genome Project and its inclusion of the mouse as a model genome sequencing project (Muller and Grossniklaus, 2010). The Syrian hamster, however, became one of many “orphan genomes” not included in the publically-funded mapping effort (Jacob, 1996). And so the quest to clone and characterize the *tau* mutation would not come to fruition until twelve years after Ralph and Menaker's 1988 report of the mutant. Our laboratory, using a comparative genomics approach called positional syntenic cloning, demonstrated that the *tau* mutation results from a single nucleotide change in the gene encoding casein kinase I epsilon (*CK1ε*) (Lowrey *et al.*, 2000).

B. Forward genetics and the *Clock* mutation

With the realization that the *tau* mutant hamster, while advantageous for physiological studies, was not immediately a genetically tractable model, our laboratory, in collaboration with William F. Dove, Lawrence H. Pinto, and Fred W. Turek, initiated a dominant circadian behavioral screen of first-generation offspring of male C57BL/6J mice mutagenized with the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU). We adopted this forward genetics approach in mice encouraged by the successful mutagenesis screens in *Drosophila* for circadian defects some 20 years earlier by Seymour Benzer's group (Konopka and Benzer, 1971). Of the 304 animals tested, we recovered a mouse with a single-gene, semidominant mutation, *Clock* (for circadian locomotor output cycles kaput), that significantly lengthened its free-running period (24.8 hr) compared to wild-type C57BL/6J mice (23.3–23.8 hr). Homozygous *Clock* mutant animals exhibited an extremely long initial free-running period in DD of 26–29 hr, followed by a complete loss of rhythmicity after two weeks in DD (Table 1). We mapped the mutation to mouse chromosome 5 (Vitaterna *et al.*, 1994) and subsequently identified the gene through a combination of positional cloning and transgenic rescue of the mutant phenotype (Antoch *et al.*, 1997; King *et al.*, 1997b). Sequence analysis revealed that the *Clock* mutation is caused by an A→T transversion in a splice donor site in the intron between exons 18 and 19 of the gene, resulting in a transcript missing exon 19 ($Clock^{\Delta 19}$). This deletion disrupts the transactivation domain of the basic helix-loop-helix (bHLH)-PAS (*Period-Arnt-Single-*

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minded) transcription factor encoded by *Clock*. Additional genetic approaches revealed that *Clock* is an antimorph—a specific type of dominant negative mutation (King *et al.*, 1997a).

Identification of the *Clock* mutation was proof of principle that, as in *Drosophila*, forward genetic screens for behavioral defects in mice are feasible (Takahashi *et al.*, 1994; Bacon *et al.*, 2004; Clark *et al.*, 2004; Siepka and Takahashi, 2005). Identifying mammalian clock genes by recovering mutants was, however, not the only approach during this "birth" of mammalian clock genetics in the 1990s. Several of what proved to be mammalian core clock genes were cloned by homology to known genes in other organisms, or by identification of paralogs in the same organism. These include three mouse orthologs of the *Drosophila period* gene (*Per1, Per2*, and *Per3*), two mouse *Cryptochrome* orthologs (*Cry1* and *Cry2*), and brain and muscle ARNT-like protein 1 (*Bmal1* or *Mop3*), another bHLH-PAS protein, all reviewed comprehensively elsewhere (Lowrey and Takahashi, 2000; Young and Kay, 2001; Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). Indeed, the 1990s witnessed the description of a molecular model of the mammalian core circadian oscillator based on a transcription/translation feedback loop with striking similarity to models proposed for other, phylogenetically divergent organisms, including *Drosophila, Arabidopsis*, and *Neurospora* (Dunlap, 1999). This rapid elucidation of the basic mechanism by which mammalian cells keep time was aptly characterized by one colleague as a "clockwork explosion" (Reppert, 1998).

III. OVERVIEW OF THE MAMMALIAN CLOCK SYSTEM

The mammalian circadian system is organized around three major physiological components: an input pathway by which environmental cues (most importantly light) are transmitted to the central or 'master' pacemaker, the central pacemaker itself, and finally a set of output pathways by which the central pacemaker regulates circadian rhythms throughout the body (Takahashi *et al.*, 2001; Quintero *et al.*, 2003; Lowrey and Takahashi, 2004). Light entrainment of the circadian system relies on the eye (Nelson and Zucker, 1981; Foster *et al.*, 1991) where, within the retina, the rods and cones and a recently discovered subset (~1%) of intrinsically-photosensitive retinal ganglion cells (ipRGCs) reside (Do and Yau, 2010). The ipRGCs respond to light stimulation independently of the rod-cone system (Berson *et al.*, 2002; Hattar *et al.*, 2002), and are directly photosensitive owing to their expression of the photopigment melanopsin (Provencio *et al.*, 1998; Gooley *et al.*, 2001; Dacey *et al.*, 2005; Fu *et al.*, 2005; Melyan *et al.*, 2005; Panda *et al.*, 2005; Qiu *et al.*, 2005). Mice lacking either the rod-cone system (Freedman *et al.*, 1999), or melanopsin ($Opn4^{-/-}$) (Panda *et al.*, 2002b; Ruby *et al.*, 2002; Lucas *et al.*, 2003), exhibit normal entrainment to light. Loss of both the rod-cone system and melanopsin, however, render mice unable to entrain to photic stimuli (Hattar *et al.*, 2003; Panda *et al.*, 2003). Because the ipRGCs, via which all retinal input to the SCN is transmitted, receive synaptic input containing non-visual information from the rods and cones, selective destruction of the ipRGCs in mice also prevents circadian photoentrainment (Goz *et al.*, 2008; Hatori *et al.*, 2008).

Photic information received by the retina is transmitted via the retinohypothalamic tract (RHT) which is formed from the axons of the ipRGCs, to the bilaterally-paired suprachiasmatic nuclei of the hypothalamus, the location of the central pacemaker in mammals (Moore and Eichler, 1972; Moore and Lenn, 1972; Stephan and Zucker, 1972; Moore *et al.*, 1995; Gooley *et al.*, 2001). Light stimulation of the ipRGCs causes their axon terminals to release glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) onto postsynaptic SCN neurons (Ebling, 1996; Hannibal, 2002; Hannibal *et al.*, 2004; Michel *et al.*, 2006; Morin and Allen, 2006). Glutamate-induced calcium influx activates several protein kinase pathways in SCN neurons which ultimately lead to

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phosphorylation of Ca^{2+}/cAMP-response element binding protein (CREB) (Golombek and Rosenstein, 2010). Within the promoters of many core clock genes, reside Ca^{2+}/cAMP-response elements (CREs) to which phospho-CREB homodimers bind to activate transcription (Zhang *et al.*, 2005). Two particularly important CREB clock targets with respect to photic entrainment are the *Per1* and *Per2* genes, both of which contain CREs in their promoters (Travnickova-Bendova *et al.*, 2002) and are rapidly induced in SCN neurons by nocturnal light exposure (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997). Circadian rhythms of locomotor activity in mice are phase advanced or phase delayed depending on the time at night during which a photic stimulus occurs (Golombek and Rosenstein, 2010). The resulting increase in PER protein presumably affects the molecular clock in SCN neurons by opposing the action of the positive effectors of the core clock feedback loop discussed later (Yan and Silver, 2004).

Each SCN of the mouse contains approximately 10,000 neurons (Abrahamson and Moore, 2001). When dissociated from SCN tissue (Welsh *et al.*, 1995; Liu *et al.*, 1997b; Herzog *et al.*, 1998; Honma *et al.*, 1998) or when grown as immortalized cells (Earnest *et al.*, 1999), these neurons can independently generate self-sustained circadian rhythms. Intact SCN neurons, however, couple to form a network that expresses synchronized rhythms (Welsh *et al.*, 2010). Ongoing work seeks to clarify the nature of the coupling mechanisms that give rise to the unique SCN network, yet it is clear that neurotransmitters, neuropeptides, gap junctions, and chemical synaptic mechanisms are involved (Welsh *et al.*, 2010). For example, the presence of vasoactive intestinal polypeptide (VIP) and its G-protein coupled receptor, $VPAC_2$, are important for maintaining circadian rhythmicity of gene expression in SCN cells, and for normal expression of rhythmic behavior in mice. Disrupting the genes for VIP ($Vip^{-/-}$) (Colwell *et al.*, 2003) or its receptor $VPAC_2$ ($Vipr2^{-/-}$) (Harmar *et al.*, 2002; Cutler *et al.*, 2003), leads to severely compromised circadian rhythms in behavior, neuronal firing, and gene expression, owing to intercellular desynchronization among SCN neurons (Table 1) (Aton *et al.*, 2005; Maywood *et al.*, 2006; Brown *et al.*, 2007; Hughes *et al.*, 2008). In normal mice, circadian rhythms generated within the SCN network are much more robust than those produced by individual neurons (Yamaguchi *et al.*, 2003), even in the presence of clock gene mutations (Liu *et al.*, 2007b). Indeed, recent work has shown that when the autonomous circadian oscillation of individual SCN neurons is eliminated by core clock gene mutation, molecular noise and intercellular coupling are sufficient to elicit stochastic, quasi-circadian oscillations as an emergent property of the SCN network (Ko *et al.*, 2010).

Photic input to the SCN via the RHT is transduced into neural and humoral output signals that synchronize other rhythms in the body, including those of temperature, hormone secretion, and rest/wake (Aston-Jones *et al.*, 2001; Buijs and Kalsbeek, 2001; Brown *et al.*, 2002). Synchronizing signals reach peripheral tissues by both autonomic neural connections (Buijs and Kalsbeek, 2001; Vujovic *et al.*, 2008), and through the release of hormones such as glucocorticoids (Balsalobre *et al.*, 2000; Le Minh *et al.*, 2001). In the absence of the SCN, circadian rhythms in most peripheral tissues damp out after a few days from desynchronization among the cells in the tissue, yet at the single cell level circadian rhythms persist (Balsalobre *et al.*, 1998; Yamazaki *et al.*, 2000; Nagoshi *et al.*, 2004; Welsh *et al.*, 2004; Yoo *et al.*, 2004). The circadian rhythm of locomotor activity commonly monitored in mice and other rodents to determine endogenous circadian period relies on diffusible signals released from the SCN (Silver *et al.*, 1996), several candidate molecules for which have been identified including transforming growth factor α (TGFα) (Kramer *et al.*, 2001; Kramer *et al.*, 2005), cardiotrophin-like cytokine (Kraves and Weitz, 2006), prokineticin 2 (PK2) (Cheng *et al.*, 2002; Li *et al.*, 2006) and potentially others (Hatcher *et al.*, 2008).

It is important to note that there are oscillators in some mammalian brain regions and tissues that, in the absence of the SCN, can drive local physiological rhythms. Two such well-

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characterized regions include the retina (Tosini and Menaker, 1996) and the olfactory bulb (Granados-Fuentes *et al.*, 2004a; Granados-Fuentes *et al.*, 2004b; Granados-Fuentes *et al.*, 2006). Further, two extra-SCN pacemakers, the food-entrainable oscillator (FEO) and the methamphetamine-sensitive circadian oscillator (MASCO), can drive circadian behavioral and endocrine rhythms in the absence of the SCN or functional canonical clock genes (Honma *et al.*, 2008; Honma and Honma, 2009; Mohawk *et al.*, 2009; Storch and Weitz, 2009; Pezuk *et al.*, 2010).

IV. THE MAMMALIAN CIRCADIAN MOLECULAR OSCILLATOR

The mammalian circadian molecular oscillator model proposed following the discovery of the core clock genes described earlier encompasses our current understanding of the circadian control of gene expression in cells throughout the body. Core circadian clock genes are genes whose protein products are necessary components for the generation and regulation of circadian rhythms; that is, proteins which form the primary molecular circadian oscillatory mechanism within individual cells. In this model, positive and negative core clock components form a feedback loop with a time constant of about 24 hr per cycle (Figure 1). This loop begins during the day when two bHLH-PAS transcription factors, CLOCK and BMAL1 heterodimerize, translocate to the nucleus, and initiate transcription from genes containing E-box (5′-CACGTG-3′) or E′-box (5′-CACGTT-3′) *cis*-regulatory elements, including the *Per* and *Cry* genes (King *et al.*, 1997b; Gekakis *et al.*, 1998; Kume *et al.*, 1999; Bunger *et al.*, 2000; Zheng *et al.*, 2001; Yoo *et al.*, 2005). PER and CRY proteins heterodimerize and, along with other proteins such as CK1ε, form a complex in the cytoplasm that translocates to the nucleus where they accumulate and subsequently represses transcription of their own (and other) genes by directly inhibiting CLOCK/BMAL1 (Griffin *et al.*, 1999; Kume *et al.*, 1999; Lee *et al.*, 2001; Sato *et al.*, 2006). Thus, the CLOCK/BMAL1 heterodimer forms the positive, or transactivating component in this loop, while the PER/CRY complex acts as the negative, or transinhibiting component (Figure 1). Following several posttranscriptional and posttranslational steps discussed later, the PER/CRY complex is targeted for degradation via the proteasomal pathway, thereby relieving inhibition such that CLOCK/BMAL1 can initiate a new cycle of transcription. This relatively straightforward feedback loop forms what has become known as the mammalian "core" oscillator mechanism.

The general molecular mechanism just described governs circadian output rhythms in all cells throughout the body, although there are tissue-specific differences. For example, in the forebrain, neuronal PAS domain protein 2 (NPAS2) appears to be the more relevant BMAL1 partner (Reick *et al.*, 2001). Thus, the CLOCK(NPAS2)/BMAL1 complex initiates the rhythmic transcription of clock-controlled genes in tissues throughout the body. Microarray studies have shown that approximately 10–15% of all mammalian transcripts exhibit a circadian oscillation from one cell type/tissue to another (Akhtar *et al.*, 2002; Duffield *et al.*, 2002; Panda *et al.*, 2002a; Storch *et al.*, 2002; Oishi *et al.*, 2003; Miller *et al.*, 2007). These studies, however, may underrepresent the true number of genes under circadian control. Powerful statistical tests are required to identify cycling transcripts from noisy microarray data sets (Doherty and Kay, 2010). Development of new nonparametric statistical algorithms promises to provide more accurate measurements of period, phase and amplitude than traditional analysis methods (Hughes *et al.*, 2010). Hence, continued improvements in data analysis methods should allow the identification of rhythmic transcripts in noisy, low-amplitude data and provide a more precise estimate of the number of genes under circadian control in various mammalian tissues.

Following the discovery of several other bona fide clock genes, it soon became evident that accessory regulatory loops interconnect with the core loop just described and add not only

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robustness and stability to the clock mechanism, but also provide additional layers of control and link to a myriad of other pathways in the cell. The first of these accessory loops involves members of the large nuclear receptor family. The mouse *Bmal1* promoter contains two cognate RevErbA/ROR-binding elements (REREs) via which the nuclear receptors RORα (retinoic acid receptor-related orphan receptor α), RORβ, or RORγ activate (Sato *et al.*, 2004; Akashi and Takumi, 2005; Guillaumond *et al.*, 2005), and REV-ERBα (reverse orientation c-erbA α) or REV-ERBβ repress (Preitner *et al.*, 2002; Triqueneaux *et al.*, 2004; Guillaumond *et al.*, 2005), *Bmal1* expression, although the REV-ERBs are likely more important in this process (Figure 1) (Liu *et al.*, 2008). Interestingly, the expression of the aforementioned nuclear receptors is circadian and relies on CLOCK/BMAL1-mediated activation through E-boxes in their promoters, although the *Ror* and *Rev-erb* transcripts cycle antiphase to each other (Yang *et al.*, 2006). The RORα-mediated activation of *Bmal1* transcription is enhanced by peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1 α (PGC1α), the expression of which cycles in liver and muscle (Liu *et al.*, 2007c). Members of the PAR bZIP transcription factor family, including the activators DBP (D-box binding protein), TEF (thyrotroph embryonic factor), HLF (hepatic leukemia factor), and the repressor E4BP4 (E4 promoter-binding protein 4), act via D-box elements in target genes to form a second accessory feedback loop (Mitsui *et al.*, 2001; Ueda *et al.*, 2005; Ohno *et al.*, 2007).

V. BEHAVIORAL, MOLECULAR, AND CELL/TISSUE EFFECTS OF CIRCADIAN CLOCK GENE MUTATIONS

Naturally-occurring, chemically-induced, or targeted mutations exist for all of the core clock genes (Table 1). These mutations have helped define the role of each component of the molecular oscillator (Lowrey and Takahashi, 2004; Ko and Takahashi, 2006; Takahashi *et al.*, 2008). At times, however, results from disruption of clock components have been unexpected.

A. Behavioral and molecular effects

One of these surprises occurred with the generation of a mouse *Clock* knockout model. Interestingly, unlike $Clock^{\Delta 19}$ animals, $Clock^{-/-}$ mice continue to express circadian rhythms of locomotor activity in DD, albeit with a slightly shorter period compared to wild-type animals, and experience only modest alterations in circadian gene expression in the SCN (Table 1) (Debruyne *et al.*, 2006). Subsequent work has revealed that in the SCN, NPAS2 can compensate for CLOCK by heterodimerizing with BMAL1 to activate transcription from E-box-containing target genes (DeBruyne *et al.*, 2007a), but that molecular circadian rhythms in peripheral tissues are dependent on the presence of CLOCK (DeBruyne *et al.*, 2007b). $Npas2^{-/-}$ animals, however, experience only subtle changes in circadian locomotor activity and gene expression (Dudley *et al.*, 2003; DeBruyne *et al.*, 2007a), suggesting that CLOCK has a more important role in the molecular oscillator. Hence, although there is partial functional redundancy between CLOCK and NPAS2, it is clearly tissue specific (Reick *et al.*, 2001; DeBruyne *et al.*, 2007b). As expected, CLOCK/NPAS2 double knockout animals are completely arrhythmic in DD (DeBruyne *et al.*, 2007a).

Knockout of *Bmal1* in mice results in behavioral arrhythmicity in DD and disrupted molecular rhythms of gene expression even though its paralog, BMAL2 (MOP9), is also expressed in the SCN and can form a transcriptionally-competent complex with CLOCK (Table 1) (Bunger *et al.*, 2000; Hogenesch *et al.*, 2000; Dardente *et al.*, 2007). Indeed, *Bmal1* is the only core clock gene for which loss of function causes an immediate loss of circadian locomotor behavior in DD. Constitutive expression of *Bmal1* in $Bmal1^{-/-}$ mice or cells restores circadian rhythmicity (McDearmon *et al.*, 2006; Liu *et al.*, 2008), demonstrating that

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cycling *Bmal1* mRNA is not necessary for circadian rhythm generation. That BMAL2 cannot rescue the $Bmal1^{-/-}$ phenotype most likely results from the dependence of *Bmal2* expression on CLOCK/BMAL1-mediated activation. Hence, disrupting *Bmal1* is likely functionally equivalent to creating a double *Bmal1/Bmal2* null animal (Shi *et al.*, 2010).

Per1 null mutations have been independently generated by three groups (Table 1). The mutant progeny from these lines exhibit subtle differences in circadian behavior. Homozygous $Per1^{ldc}$ mice have about a 0.5 hr shorter free-running period in DD than wild-type controls and experience a gradual loss of rhythmicity after two weeks in DD (Bae *et al.*, 2001). $Per1^{Brdm1}$ mice express a free-running period approximately 1 hr shorter than wild-type animals and maintain rhythmicity (Zheng *et al.*, 2001). This result is consistent with the circadian behavior of the $Per1^{-/-}$ mutant line generated by a third group which exhibits a 0.7 hr shorter free-running period with no loss of rhythmicity (Cermakian *et al.*, 2001). The phenotypic disparities observed among the three *Per1* null studies may result from differences in targeting approaches or genetic backgrounds. Two independent null mutations in *Per2* have been reported (Table 1). Both mutant lines exhibit a loss of behavioral rhythmicity in DD, yet the $Per2^{Brdm1}$ line expresses a 1.5 hr shorter period for several days before experiencing arrhythmicity (Zheng *et al.*, 1999). Most animals of the $Per2^{ldc}$ line exhibit immediate arrhythmicity upon exposure to DD (Bae *et al.*, 2001). As expected from the results just presented, double *Per1/Per2* knockout animals experience behavioral and molecular arrhythmicity in constant conditions (Bae *et al.*, 2001; Zheng *et al.*, 2001). Because loss of *Per3* has no effect on circadian rhythms either in *Per1/Per3* or *Per2/Per3* double mutant mice, *Per3* is not an essential component of the circadian core clock mechanism (Shearman *et al.*, 2000a; Bae *et al.*, 2001).

Targeted disruption of either of the two *Cry* genes results in opposite effects on circadian behavior—$Cry1^{-/-}$ animals have a 1 hr shorter and $Cry2^{-/-}$ animals have a 1 hr longer free-running period in DD compared to wild-type animals (Thresher *et al.*, 1998; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999) (Table 1). Similar to *Per1/Per2* double knockouts, *Cry1/Cry2* double knockout animals experience a complete loss of behavioral and molecular rhythmicity when transferred to DD (Thresher *et al.*, 1998; Okamura *et al.*, 1999; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). *Per/Cry* compound knockouts also exhibit interesting behavioral phenotypes (Table 1). $Per1^{Brdm1}/Cry1^{-/-}$ mice have normal circadian rhythms of behavior and gene expression (Oster *et al.*, 2003). Deletion of *Per1* rescues the short period phenotype observed in $Cry1^{-/-}$ mutants, revealing that *Per1* is a nonallelic suppressor of *Cry1*. $Per1^{Brdm1}/Cry2^{-/-}$ mice have more complex phenotypes. Mutants up to six months of age express behavioral rhythms 1.5 hr longer than wild-type controls and have normal rhythms of *Per2* expression. After approximately 6 months of age, $Per1^{Brdm1}/Cry2^{-/-}$ animals exhibit disrupted entrainment to LD cycles, and subsequently experience arrhythmicity upon release into DD (Oster *et al.*, 2003). In addition, the older animals have blunted *Per2* rhythms in the SCN revealing an age-sensitive effect in this compound mutant. $Per2^{Brdm1}/Cry1^{-/-}$ mutants experience immediate behavioral and molecular arrhythmicity in DD (Oster *et al.*, 2002). $Per2^{Brdm1}/Cry2^{-/-}$ animals, however, maintain behavioral and molecular circadian rhythmicity in DD with a slightly shorter free-running period compared to wild-type controls. Thus, inactivation of *Cry2* in *Per2* null animals restores circadian rhythmicity. As a result, *Cry2* is a nonallelic suppressor of *Per2* (Oster *et al.*, 2002).

The double *Per1/Per2* and *Cry1/Cry2* knockout results make sense given that the PER/CRY complex is necessary to directly inhibit CLOCK/BMAL1-mediated transcription (Griffin *et al.*, 1999; Kume *et al.*, 1999; Shearman *et al.*, 2000b; Lee *et al.*, 2001; Sato *et al.*, 2006). Until recently, transient transfection assays pointed to a more prominent role for CRY in inhibiting CLOCK/BMAL1 (Dardente *et al.*, 2007), yet new evidence suggests that PER may be the more important of the negative effectors as its constitutive expression disrupts

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the circadian clock in fibroblasts and hepatocytes. Furthermore, constitutive PER2 expression in the SCN of transgenic mice results in the loss of circadian rhythms of locomotor behavior in a conditional and reversible manner (Chen *et al.*, 2009). Finally, biochemical evidence demonstrates that PER2 directly binds to the CLOCK/BMAL1 complex in a rhythmic way, and that it brings CRY into contact with CLOCK/BMAL1. Rhythmic expression of PER in turn drives the rhythmic inhibition of CLOCK/BMAL1, and it is PER that is the rate-limiting component of the inhibitor complex (Chen *et al.*, 2009). This is substantiated by independent work demonstrating that PER2 is also a more potent inhibitor of CLOCK/BMAL2-mediated transactivation than is CRY (Sasaki *et al.*, 2009).

B. Cell/tissue effects

The analysis of behavioral (locomotor activity) and molecular (RNA/protein) rhythms in mice with mutations in core circadian clock genes just described is insufficient to provide a comprehensive view of molecular clock function. For example, most of the above studies do not take into account differences in central versus peripheral oscillators, potential intercellular interactions in producing the observed phenotypes, or reveal unique properties of individual cellular oscillators. New methods allowing continuous monitoring of circadian rhythms in cultured tissues and individual cells in real time for periods of 20 days or more via bioluminescent technology have revealed many clock properties not evident from behavioral and molecular analyses alone.

By crossing clock gene knockout mice to the $Per2^{Luciferase}$ ($Per2^{Luc}$) knockin reporter mouse line in which a luciferase gene is fused to the 3'-end of the endogenous *Per2* gene (Yoo *et al.*, 2004), one group has measured the effects of clock gene perturbations at the level of tissues, populations of cultured cells, and single dissociated cells from both the SCN and peripheral tissues (Liu *et al.*, 2007b). In SCN tissue explants, disruption of *Per1, Per3, Cry1* or *Cry2* individually has no effect on the maintenance of circadian rhythmicity, and the observed period for each mutant SCN tissue reflects the free-running behavioral period of the corresponding mutant animal model (Table 1 and Table 2). In peripheral tissue explants (e.g., liver, lung, cornea), unlike SCN explants, $Cry1^{-/-}$ and $Per1^{-/-}$ are required for robust, sustained circadian rhythmicity (Table 2), a property of peripheral tissues not evident from the previously described behavioral and molecular studies (Table 1). $Cry2^{-/-}$ and $Per3^{-/-}$ mutant peripheral tissues maintain rhythmicity with slightly longer and shorter periods, respectively, compared to wild-type controls, again consistent with behavioral results (Table 1). In dissociated fibroblast cells in culture, *Per1, Per2* and *Cry1* are required to maintain circadian oscillations. Thus it seems that, in fibroblast cultures at least, *Per1* and *Per2* are not functionally redundant (Liu *et al.*, 2007b).

When single fibroblast cells are imaged for circadian rhythms of bioluminescence, again *Per1* and *Cry1* prove necessary to sustain circadian oscillations, confirming the results observed in fibroblast cultures (Liu *et al.*, 2007b). Single $Cry2^{-/-}$ fibroblast cells are rhythmic with a slightly longer period compared to individual wild-type cells, consistent with the behavioral phenotype of *Cry2* null mice (Table 1 and Table 2). To measure rhythms of bioluminescence from single SCN neurons, they must first be uncoupled by mechanical dissociation into single cells (Welsh *et al.*, 1995; Herzog *et al.*, 1998). Similar to the result in single fibroblasts, single $Cry2^{-/-}$ SCN neurons are rhythmic with a period longer than wild-type SCN neurons (Table 2). Single $Cry1^{-/-}$ and $Per1^{-/-}$ SCN neurons, in contrast to single $Cry2^{-/-}$ SCN neurons or $Cry1^{-/-}$ and $Per1^{-/-}$ SCN tissue explants, exhibit arrhythmicity (Table 2). This is an important result not apparent in earlier behavioral studies of *Per1* and *Cry1* null mutants and demonstrates that the robustness of circadian oscillations observed in $Cry1^{-/-}$ and $Per1^{-/-}$ SCN explant tissue is not a cell-autonomous property. Instead, the ability of $Cry1^{-/-}$ and $Per1^{-/-}$ SCN tissue to maintain circadian rhythmicity despite mutations in core clock components that, at the single-cell level cause arrhythmicity,

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depends on intercellular coupling among SCN neurons (Liu *et al.*, 2007b). This property of intercellular coupling, and not unique intracellular molecular mechanisms, is what distinguishes SCN neurons from cells of peripheral tissues.

As mentioned previously, *Bmal1* null mutant animals experience an immediate loss of circadian behavior upon transfer to constant conditions (Bunger *et al.*, 2000). Using bioluminescence monitoring methods with $Bmal1^{-/-}$ SCN and peripheral tissues similar to those just described, a recent study has elucidated interesting properties of the SCN network not discovered in previous behavioral and molecular investigations (Ko *et al.*, 2010). SCN explants from $Bmal1^{-/-}$ mice crossed to the $Per2^{Luc}$ reporter line exhibit rhythmic but highly variable (noisy) oscillations of PER2::LUC bioluminescence for more than 35 days in culture (Table 2). This is an unexpected result given that the behavioral and molecular rhythms of gene expression in $Bmal1^{-/-}$ animals are arrhythmic (Table 1). The authors of this study refer to these quasi-circadian rhythms generated by the $Bmal1^{-/-}$ SCN explants as stochastic. As expected, bioluminescence monitoring demonstrates that all peripheral tissues from $Bmal1^{-/-}$ animals are arrhythmic (Table 2). Analysis of single dispersed $Bmal1^{-/-}$ SCN neurons, however, reveals that they are arrhythmic and do not exhibit the stochastic rhythms of bioluminescence observed in $Bmal1^{-/-}$ SCN explants. This result seems not to depend from what subtype of SCN neuron recordings are made or from what region of the SCN the neurons are obtained—all dispersed $Bmal1^{-/-}$ SCN neurons exhibit arrhythmicity. Further, $Bmal1^{-/-}$ SCN slices treated with tetrodotoxin (TTX) experience an immediate cessation of stochastic rhythmicity, a result of loss of rhythmicity at the single-cell level. Upon removal of TTX from SCN slices, stochastic rhythmicity is restored thereby confirming the importance of intercellular coupling in generating the observed PER2::LUC rhythms in the SCN slices. Taken together, these bioluminescence results demonstrate that the quasi-circadian rhythms in $Bmal1^{-/-}$ SCN explants is not a cell-autonomous property, but rather an emergent rhythmic property of the SCN intercellular network (Ko *et al.*, 2010).

VI. POSTTRANSLATIONAL MODIFICATION OF CLOCK PROTEINS

Posttranslational modifications of the core clock components play a crucial role in generating the delays necessary to establish the ~24 hr rhythm of the mammalian circadian clock. Some of these modifications are absolutely essential to clock function while others simply fine-tune the rhythm. Phosphorylation of clock proteins was the first posttranslational process observed in the mammalian molecular clock, and we understand more about this mechanism than any other. The list of identified posttranslational modifications of mammalian clock proteins has grown rapidly and now, in addition to phosphorylation, includes dephosphorylation, ubiquitination, sumoylation, and acetylation.

A. Phosphorylation

1. Casein kinase 1 (CK1)—Posttranslational modification as an important clock-related process in higher eukaryotes became apparent with the identification in *Drosophila* of *doubletime*, a gene encoding a fly casein kinase 1 ortholog that phosphorylates PER (Kloss *et al.*, 1998; Price *et al.*, 1998). Subsequently, we identified casein kinase 1 epsilon (*CK1ε*) as the gene affected by the Syrian hamster *tau* mutation, and showed that in vitro $CK1\varepsilon^{tau}$ is hypomorphic toward various substrates, including mammalian PER1 and PER2 proteins (Lowrey *et al.*, 2000). Others demonstrated that, in vitro and in cultured cells, CK1ε and the closely-related family member, CK1δ, can phosphorylate PER (Keesler *et al.*, 2000; Vielhaber *et al.*, 2000; Camacho *et al.*, 2001; Akashi *et al.*, 2002). Further work revealed that CK1δ/ε-mediated phosphorylation regulates PER subcellular localization and its ability to repress CLOCK/BMAL1-mediated transcription, and promotes its ubiquitin-degradation via the 26S proteasome (Vielhaber *et al.*, 2000; Eide *et al.*, 2005b; Shirogane *et al.*, 2005; Vanselow *et al.*, 2006; Ohsaki *et al.*, 2008).

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Although our work showed that CK1ε^{tau} was a hypomorph in in vitro assays, a study using mathematical modeling and in vivo analyses has reported that the *CK1ε*^{tau} anion binding site mutation causes loss of enzyme function toward canonical acidic CK1ε substrates, but gain of function toward the non-canonical β-transducin repeat-containing protein (βTrCP) binding site on PER2 (Gallego *et al.*, 2006a). Indeed, biochemical evidence substantiates the model's prediction as do behavioral, neurophysiological and cellular studies from a mouse model of the hamster *tau* mutation (Gallego *et al.*, 2006a; Meng *et al.*, 2008). Another group, however, has published findings that contradict this interpretation. They report that *CK1ε*^{tau} is actually a partial loss of function mutation as the mutant kinase is unable to phosphorylate sites that promote nuclear localization of PER2, but that it can phosphorylate amino acids required for PER2 proteasomal degradation (Vanselow *et al.*, 2006). By mapping all of the CK1ε phosphorylation sites on PER2, they opine that the different sites differentially target PER2 to two cellular locations—nucleus or proteasome. Both interpretations agree, however, that the *tau* allele is a particularly interesting mutation biochemically as it differentially affects CK1ε activity toward specific substrates.

The *tau* mutation focused much attention on the role of CK1ε in the mammalian molecular clock, yet as mentioned above, CK1δ also phosphorylates PER proteins and targets them for degradation (Camacho *et al.*, 2001; Xu *et al.*, 2005), and both kinases associate with PER/CRY repressor complexes in vivo (Figure 1) (Lee *et al.*, 2001). Thus, to better define the role of these two CK1 enzymes, null mutants of both have been generated independently by different laboratories. The free-running period of the locomotor activity rhythm of *CK1ε*$^{-/-}$ mice is slightly, but significantly, longer than wild-type controls (Table 1) (Meng *et al.*, 2008; Etchegaray *et al.*, 2009). Two groups have reported that *CK1δ*$^{-/-}$ mice die during the perinatal period, thus the free-running behavioral period has been studied in *CK1δ*$^{+/-}$ heterozygous animals. In one case, one copy of the *CK1δ* null allele results in no difference in free-running period compared to controls (Xu *et al.*, 2005), while another group's heterozygous null animal exhibits a slight increase in circadian period (Etchegaray *et al.*, 2009). In addition, compared to *CK1ε*-deficient tissue, mouse embryonic fibroblasts (MEFs) and liver tissue deficient in *CK1δ* have about a one to two hour longer circadian period in vitro (Etchegaray *et al.*, 2009; Lee *et al.*, 2009). When monitored from neonatal SCN explants, PER2::LUC bioluminescence rhythms from *CK1δ*$^{-/-}$ mice are longer compared to wild-type controls, yet there is no significant difference in PER2::LUC rhythms from *CK1ε*$^{-/-}$ SCN compared to controls (Etchegaray *et al.*, 2010).

Pharmacological approaches have also been used to study the roles of CK1ε and CK1δ in the mammalian clock with the general CK1δ/ε inhibitors CKI-7, IC261 and D4476, all of which lengthen circadian period in cultured cells (Eide *et al.*, 2005a; Vanselow *et al.*, 2006; Reischl *et al.*, 2007; Hirota *et al.*, 2008). An inhibitor specific for CK1ε (PF-4800567) has only a slight effect on the period of oscillating rat-1 fibroblasts stably transfected with a *Per2::luc* reporter compared to the dual CK1δ/ε inhibitor (PF-670462) which causes an increase in fibroblast circadian period (Walton *et al.*, 2009). Single injections into rats of the dual CK1δ/ε inhibitor induce large phase delays in circadian locomotor rhythms under free-running and entrained conditions (Badura *et al.*, 2007; Sprouse *et al.*, 2010). Daily treatment with PF-670462 significantly lengthens locomotor behavioral rhythms in a dose-dependent manner in wild-type, *CK1ε*^{tau}, and *CK1ε*$^{-/-}$ mice (Meng *et al.*, 2010). Selective inhibition of CK1ε with PF-4800567 has no significant effect on behavioral rhythms in wild-type or *CK1ε*$^{-/-}$ mice, yet it lengthens the free-running locomotor activity rhythm of *CK1ε*^{tau} animals. How does inhibition of the CK1 enzymes affect molecular clock function? PF-670462 seems to work by stabilizing PER2 nuclear localization in SCN neurons and peripheral tissues. This prolongs PER2-mediated negative feedback, thereby lengthening circadian period (Meng *et al.*, 2010). Together with the *CK1* knockout experiments, these results suggest that CK1δ has a more prominent role compared to CK1ε in the mammalian

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clockwork; that is, the two kinases seem not to be equally redundant. It is clear, however, that loss of both CK1ε and CK1δ causes arrhythmicity in MEFs (Lee *et al.*, 2009), and that knockdown of both kinases in human U2OS (osteosarcoma) cells additively lengthens circadian period to more than 30 hr (Isojima *et al.*, 2009). It remains to be determined if this partial functional redundancy derives from unequal expression levels of the two kinases in cells throughout the body. Some evidence suggests that, at least in MEFs, CK1δ is twice as abundant as CK1ε (Lee *et al.*, 2009).

One group has reported a surprising result in their work with CK1δ/ε in which a novel, non-catalytic clock-related role for these kinases is revealed (Lee *et al.*, 2009). Overexpression of the CK1δ/ε-binding domain of PER2 (CKBD-P2) in MEFs severely disrupts PER2::LUC rhythms of bioluminescence, presumably because the CKBD-P2 fragment interferes (competes) with the interaction between CK1δ/ε and PER. In addition, PER1 and PER2::LUC levels are lower in the CKBD-P2-expressing MEFs, while CK1δ/ε levels are higher than normal. This suggests that the CKBD-P2 enhances the stability of CK1δ/ε via physical interaction. Furthermore, the low levels of PER observed in the CKBD-P2-expressing MEFs may result from the inhibition of CK1δ/ε-specific phosphorylation of PER, and from the reduced physical interaction between CK1δ/ε and PER which, under normal circumstances, may confer stability to PER (Lee *et al.*, 2009). Overexpression of the CKBD region of PER3 in MEFs does not have an effect on the circadian rhythms in these cells, mirroring previous reports showing that PER3 does not interact with CK1δ/ε. That the stabilizing role of CK1δ/ε toward PER is non-catalytic is supported by experiments in which dominant-negative CK1δ/ε (K38R)-expressing MEFs do not experience reduced PER levels (Lee *et al.*, 2009). Interestingly, a non-catalytic circadian role has also been reported recently for the *Drosophila* CK1 family member, DBT (Yu *et al.*, 2009).

Finally, in a high-throughput screen of approximately 120,000 compounds using U2OS cells expressing luciferase under the control of the mouse *Bmal1* promoter, another CK1 family member, CK1α, has been identified as a mammalian clock regulatory kinase (Hirota *et al.*, 2010). A purine derivative identified in this screen, longdaysin, inhibits CK1δ, CK1α, and ERK2 (MAPK1) and prevents them from phosphorylating PER1, causing a dramatic 13 hr lengthening of period in U2OS cells. Knockdown by siRNA of one of the aforementioned kinases alone is insufficient to recapitulate the 13 hr period lengthening effect, yet combinatorial knockdown of all three kinases additively increases period and closely mirrors the effect of longdaysin treatment (Hirota *et al.*, 2010). Results from this interesting study suggest that multiple kinases participate in a network to enhance robustness of the molecular clock mechanism.

2. Glycogen synthase kinase-3β (GSK-3β)—Glycogen synthase kinase-3 (GSK-3) is a serine-threonine, phosphate-directed protein kinase of which there are two isoforms in mammals: GSK-3α and GSK-3β (Ali *et al.*, 2001). GSK-3 was initially characterized as a kinase involved in metabolism and energy storage, yet it has since been shown to play a role in many intracellular pathways (Doble and Woodgett, 2003). Knockout models for both isoforms have been generated, but *Gsk-3β*$^{-/-}$ mice experience embryonic lethality (Hoeflich *et al.*, 2000; MacAulay *et al.*, 2007). Interestingly, GSK-3 is sensitive to lithium. Presumably, Li^{+} competes directly for binding to GSK-3 with Mg^{2+}, a required cofactor for GSK-3 function (Klein and Melton, 1996; Stambolic *et al.*, 1996; Ryves and Harwood, 2001). Several studies have documented the effects of lithium treatment on circadian rhythms in mammals, including a consistent effect of lengthening the free-running period of behavioral rhythms, notably those of locomotor activity and drinking (Seggie *et al.*, 1982; LeSauter and Silver, 1993; Iwahana *et al.*, 2004). This period-lengthening effect of lithium is also observed for the firing rate rhythms in isolated mouse SCN neurons (Abe *et al.*, 2000). Other work has shown that GSK-3β is rhythmically expressed in the SCN and liver of mice,

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and that it undergoes a daily cycle in phosphorylation in vivo as well as in serum-shocked NIH3T3 mouse fibroblasts (Harada *et al.*, 2005; Iitaka *et al.*, 2005). Lithium chloride treatment phase delays, while overexpression of GSK-3β, phase advances clock gene expression in fibroblasts (Iitaka *et al.*, 2005). In addition, *Gsk-3α* RNAi knockdown in *Gsk-3β*$^{-/-}$ MEFs, induces a phase delay in the *Per2* RNA rhythm, as does treatment of MEFs with kenpaullone, a GSK-3 antagonist (Kaladchibachi *et al.*, 2007). Surprisingly, however, a recent high-throughput screen has identified small molecule inhibitors of GSK-3β that shorten circadian period, a result confirmed by siRNA knockdown of GSK-3β (Hirota *et al.*, 2008). These effects of inhibition of GSK-3β on the molecular clock in cells and on clock-controlled behavior in mammals have prompted further investigation into the potential clock-related targets of this enzyme. In addition, work in *Drosophila* has shown that *shaggy*, the fly ortholog of mammalian GSK-3β, is an important component in determining circadian period length in that organism (Martinek *et al.*, 2001).

Mammalian targets of GSK-3β phosphorylation include the positive and negative components of the core circadian transcriptional/translational feedback loop, CLOCK/BMAL1 and PER/CRY, respectively. Phosphorylation by GSK-3β of PER2 promotes its nuclear localization (Iitaka *et al.*, 2005). GSK-3β-mediated phosphorylation at Ser553 of CRY2 leads to its degradation by the proteasome (Harada *et al.*, 2005), yet GSK-3β phosphorylation promotes the stabilization of REV-ERBα (Yin *et al.*, 2006). Lithium treatment of cultured cells results in the rapid proteasomal degradation of REV-ERBα and concomitant derepression of *Bmal1* transcription owing to inhibition of GSK-3β (Yin *et al.*, 2006). Others have identified a phosho-degron region on the CLOCK protein that is phosphorylated by GSK-3β, and which promotes degradation of CLOCK (Spengler *et al.*, 2009). Finally, BMAL1, the major CLOCK dimerization partner, is phosphorylated by GSK-3β on Ser17 and Thr21. This modification targets BMAL1 for ubiquitination and subsequent degradation by the proteasome (Sahar *et al.*, 2010).

3. Casein kinase 2 (CK2)—Casein kinase 2, a serine/threonine kinase, is a tetramer composed of two α catalytic and two β regulatory subunits, and, similar to CK1 prefers acidic substrates (Meggio and Pinna, 2003). A role for this kinase in the regulation of circadian rhythms was first reported in *Arabidopsis, Drosophila*, and *Neurospora* (Gallego and Virshup, 2007). Recently, participation of CK2 in the mammalian molecular clock mechanism has been reported by three groups. First, BMAL1 is a substrate for CK2α at Ser90, a residue that undergoes rhythmic phosphorylation (Tamaru *et al.*, 2009). Nuclear localization of BMAL1 seems to depend on CK2α phosphorylation, as knockdown of CK2α results in cytoplasmic BMAL1 accumulation and a concomitant disruption of *Per2* mRNA rhythms (Tamaru *et al.*, 2009).

PER2 is also a CK2α substrate. One study has shown that CK2α both phosphorylates PER2 at Ser53 and enhances CK1ε-mediated PER2 destabilization (Tsuchiya *et al.*, 2009). These two actions of CK2α on PER2 are independent, as the CK2α-mediated potentiation of CK1ε-dependent degradation of PER2 does not require phosphorylation of Ser53. Although it has not been demonstrated, it may be that CK2α acts to phosphorylate CK1ε thereby upregulating its activity toward PER2, given that a catalytically inactive form of CK2α (CK2α-K68A) fails to enhance CK1ε-dependent PER2 degradation (Tsuchiya *et al.*, 2009). Finally, in a recent RNAi screen for clock-related components, downregulation of either CK2α or CK2β lengthens circadian period, while knockdown of both subunits leads to arrhythmicity (Maier *et al.*, 2009). Furthermore, overexpression of CK2α has a period-shortening effect. This same study showed that CK2α binds PER2 and promotes phosphorylation near the N-terminus, although the proposed sites do not correspond to Ser53 mentioned before. Also, unlike the previous study, CK2α phosphorylation is proposed to stabilize PER2 rather than enhance its degradation. Inhibition of CK2α was

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shown to delay PER2 nuclear accumulation, suggesting that CK2α phosphorylation of PER2 may provide a signal for nuclear entry (Maier *et al.*, 2009). These, and other questions, need to be addressed to better understand the role of CK2 in the mammalian clock mechanism.

4. Other kinases—BMAL1 has been identified as a substrate for several kinases including CK1ε and GSK-3β as described above. In addition, mitogen-activated protein kinase (MAPK) phosphorylates BMAL1 on several residues, including Thr534 which negatively regulates the transactivation potential of the CLOCK/BMAL1 complex at E-boxes (Sanada *et al.*, 2002). Another study has shown recently that RACK1 (receptor for activated C kinase-1) recruits activated protein kinase Cα (PKCα) to the CLOCK/BMAL1 complex during the negative feedback phase of the circadian cycle in the nucleus where it phosphorylates BMAL1 and suppresses CLOCK/BMAL1 transcriptional activity (Robles *et al.*, 2010). Knockdown of either RACK1 or PKCα in fibroblast cultures shortens circadian period. *Prkca*$^{-/-}$ mice have a normal circadian period, yet they experience impaired photic resetting of behavioral rhythms (Jakubcakova *et al.*, 2007). This difference between the knockout phenotype and cell culture results highlights the need for further work.

CLOCK has been shown to be phosphorylated throughout the circadian cycle, yet it is hyperphosphorylated during the negative feedback phase (Yoshitane *et al.*, 2009). Specific residues have been identified as phosphorylation sites on CLOCK, including Ser38, Ser42, and Ser427. The kinase or kinases responsible for phosphorylating these sites, however, remain undetermined. CLOCKΔ19, which lacks the binding site for CLOCK-interacting protein circadian (CIPC), a PER/CRY-independent negative regulator of CLOCK/BMAL1-mediated transcription, is less phosphorylated and more stable than wild-type CLOCK (Zhao *et al.*, 2007). Others have shown that CLOCK is a substrate for phosphorylation by protein kinase G II (PKG-II) and by two protein kinase C isoforms (PKCα and PKCγ) (Tischkau *et al.*, 2004; Shim *et al.*, 2007).

Finally, it will be important to assess the contribution of sequential phosphorylation events on clock proteins. For example, some kinases such as CK1δ/ε require priming phosphorylation of their target sites (Knippschild *et al.*, 2005). Phosphorylation of human PER2 at the Ser662 familial advanced sleep phase syndrome (FASPS) site occurs by an unidentified kinase and is necessary for subsequent CK1δ/ε phosphorylation (Xu *et al.*, 2007). Similarly, GSK-3β phosphorylation of CRY2 requires priming phosphorylation by DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) (Kurabayashi *et al.*, 2010). In many cases, the priming kinases for these so-called “phosphate directed” kinases such as CK1δ/ε and GSK-3 remain to be identified for particular circadian substrates.

B. Dephosphorylation

Protein phosphatases, although fewer in number in the mammalian genome relative to kinases, also play an important role in the molecular clock (Gallego and Virshup, 2007). Reversible phosphorylation of clock-relevant substrates presumably confers on clock the flexibility to respond appropriately to various stimuli. Few studies, however, have explored the role of phosphatases on the mammalian clock mechanism. The major clock kinases, CK1ε and CK1δ, both undergo autophosphorylation which downregulates their activity. At least eight autophosphorylation sites must be dephosphorylated by phosphatases to activate CK1δ/ε (Gietzen and Virshup, 1999). Additional evidence suggests that CK1δ/ε participates in a futile autophosphorylation/dephosphorylation cycle in vivo which acts to regulate kinase activity (Rivers *et al.*, 1998). One group has shown that it is protein phosphatase 5 (PP5) that dephosphorylates CK1δ/ε. Furthermore, the same study demonstrated that the

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CRY proteins interact with and inhibit noncompetitively, PP5. As a result, the CRY proteins seem to indirectly regulate the activity of CK1δ/ε by inhibiting the phosphatase activity of PP5 (Partch *et al.*, 2006). Another study has found a role for PP1 in the clock mechanism (Gallego *et al.*, 2006b). PP1 can bind to and dephosphorylate CK1δ/ε-phosphorylated PER2 thereby negatively regulating its degradation by the proteasome. Overexpression of a dominant negative form of PP1, or use of PP1 inhibitors, results in accelerated degradation of PER2 (Gallego *et al.*, 2006b).

C. Ubiquitination

One of the major pathways for protein degradation in cells is the ubiquitin-dependent proteasomal mechanism. This system requires the attachment of multiple ubiquitin molecules to lysine residues on the target protein (Ciechanover *et al.*, 2000; Nandi *et al.*, 2006). Polyubiquitinated proteins are directed to the 26S proteasome, a large multicatalytic protease, where they are then degraded to small peptides (Nandi *et al.*, 2006). Attachment of ubiquitin to a protein is a three-step process. First, ubiquitin is adenylated by an activating enzyme (E1), then transferred to a conjugating enzyme (E2), and finally linked to the target protein by a ligase (E3) (Wilkinson, 1999; Ciechanover *et al.*, 2000). The specificity of this system is determined by the E3 ligases which can be categorized into at least six subtypes. With respect to the mammalian clock mechanism, the SCF E3 subtype is the most relevant. SCF complexes are multimers and are named for their constituent protein components, Skp1, Cdc53 or Cullin, and any one of a number of proteins containing an F-box motif, each of which recognizes a particular target protein (Nandi *et al.*, 2006). It is the F-box protein that confers specificity to each SCF complex (Cardozo and Pagano, 2004). The SCF complexes are constitutively active enzymes that recognize and ubiquitinate only phosphorylated substrates (Kornitzer and Ciechanover, 2000). Consequently, this system links protein phosphorylation to proteolytic degradation by the 26S proteasome (Cardozo and Pagano, 2004).

Turnover of the PER/CRY repressor complex is an important event in relieving inhibition of CLOCK/BMAL1 such that a new circadian day can begin. Insight into how this repressor complex is cleared has come from several studies. Recently, our laboratory and another independently recovered long period behavioral mutants, *Overtime* (Siepka *et al.*, 2007) and *After-hours* (Godinho *et al.*, 2007), respectively, through ENU mutagenesis screens of mice. Both mutations affect the same gene, *Fbxl3*, which encodes the F-box and leucine-rich repeat protein 3 (FBXL3). FBXL3 is involved in ubiquitination of the CRY proteins, which targets them for degradation (Busino *et al.*, 2007). The proteasomal-mediated degradation of CRY1 and CRY2, however, appears to be differentially regulated. CRY1 is phosphorylated by AMPK (AMP-activated protein kinase) which promotes its FBXL13-dependent degradation (Figure 1) (Lamia *et al.*, 2009). CRY2, however, is phosphorylated by GSK-3β on Ser553 (Harada *et al.*, 2005), which first requires priming phosphorylation by DYRK1A on Ser557, both of which are at the C-terminal tail of CRY2 (Kurabayashi *et al.*, 2010). This Ser557/Ser553 phosphorylation mechanism promotes proteasomal degradation of CRY2 by what is likely an FBXL13-independent mechanism. Indeed, it seems that Ser557/Ser553 phosphorylation and subsequent turnover of CRY2 slows its cytosolic accumulation rate and allows its timely nuclear translocation (Kurabayashi *et al.*, 2010). The FBXL13-dependent degradation mechanism acts during the declining phase of negative feedback when CRY2 nuclear clearance occurs (Godinho *et al.*, 2007). Taken together, these two mechanisms of CRY2 degradation suggest a model by which negative feedback is controlled.

As discussed earlier, phosphorylation of the PER proteins by CK1δ/ε promotes their polyubiquitination (Eide *et al.*, 2005a; Shirogane *et al.*, 2005; Reischl *et al.*, 2007; Ohsaki *et al.*, 2008). Ubiquitination of the PERs is mediated by the F-box proteins βTrCP1 and/or βTrCP2 (Figure 1) (Vielhaber *et al.*, 2000; Eide *et al.*, 2005b; Shirogane *et al.*, 2005;

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Vanselow *et al.*, 2006; Ohsaki *et al.*, 2008). Following ubiquitination, the PER proteins are degraded by the 26S proteasome (Gallego and Virshup, 2007).

D. Sumoylation

The BMAL1 protein undergoes extensive posttranslational modification in cells, including phosphorylation, acetylation, ubiquitination, and sumoylation. Small ubiquitin-like modifier (SUMO) proteins are covalently attached at lysine residues of target proteins to modify their function (Wilkinson and Henley, 2010). Sumoylation has been shown to affect nuclear/cytosolic localization of proteins, progression through the cell cycle, protein stability, and transcriptional regulation (Gareau and Lima, 2010). In mice and humans, there are three *Sumo* paralogs (*Sumo1–3*). SUMO2 and SUMO3 are 95% identical and are often referred to as SUMO2/3. Two groups have independently demonstrated that BMAL1 is rhythmically polysumoylated at a conserved lysine residue (K259) in the PAS domain linker region by all three SUMO proteins, and that this process is dependent on CLOCK, the BMAL1 dimerization partner (Cardone *et al.*, 2005; Lee *et al.*, 2008). Furthermore, sumoylation localizes BMAL1 to the promyelocytic leukemia nuclear body, potentiates CLOCK/BMAL1 transactivation of clock-controlled genes, and promotes BMAL1 ubiquitin-dependent proteasomal degradation (Lee *et al.*, 2008).

E. Acetylation, deacetylation and chromatin remodeling

Rhythmic changes in chromatin architecture participate in the activation and repression of transcription via posttranslational modifications at histone N-terminal tail regions (Imhof and Becker, 2001). Acetylation of lysine residues or phosphorylation of serine residues in histone tails induces a transcription-permissive nucleosome conformation, whereas deacetylation, dephosphorylation, or methylation of histone lysine residues promotes a transcription-inhibitory nucleosome conformation. These, and other covalent modifications that occur at histones to alter the degree of chromatin condensation, have become known as the “histone code” (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Chromatin remodeling as a possible circadian regulatory mechanism was first suggested by an experiment demonstrating that in mice, light pulses during the subjective night promote phosphorylation of serine 10 of histone H3 (Crosio *et al.*, 2000). Subsequent work has shown that at the *Per1* and *Per2* promoters, lysine 9 of histone H3 is rhythmically acetylated (Etchegaray *et al.*, 2003) and that CLOCK and NPAS2 may act to recruit histone acetyltransferases (HATs) to the *Per1* promoter (Etchegaray *et al.*, 2003; Curtis *et al.*, 2004).

Interestingly, CLOCK has been shown to exhibit intrinsic HAT activity toward lysine residues of histones H3 and H4 (Doi *et al.*, 2006). This suggests that CLOCK, while activating transcription with its partner, BMAL1, may rhythmically acetylate histones at clock-controlled genes and thereby participate in chromatin remodeling. CLOCK also acetylates non-histone substrates, including BMAL1 at lysine 537 (Hirayama *et al.*, 2007), as well as the glucocorticoid receptor (Nader *et al.*, 2009). Additional work is needed to clarify the relationship of CLOCK HAT activity to the circadian control of gene expression. Recently, a role for CLOCK HAT activity in facilitating herpes simplex virus gene expression in infected mammalian cells has been reported (Kalamvoki and Roizman, 2010), hence this activity of CLOCK may not be restricted solely to circadian regulation.

Histone deacetylases (HDACs) act to remove acetyl groups from histone tails to promote transcriptional repression (Finkel *et al.*, 2009). The NAD^+-dependent HDAC Sirtuin 1 (SIRT1) has been shown recently to be involved in the mammalian molecular clock mechanism. SIRT1 acts to deacetylate lysines 9 and 14 of histone H3 as well as lysine 16 of histone H4 leading to chromatin condensation and transcriptional repression (Vaquero *et al.*, 2004; Nakahata *et al.*, 2008). Furthermore, SIRT1 seems to bind CLOCK-BMAL1 to form a

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complex that is recruited in a circadian manner to promoters of clock-controlled genes. Through its interaction with CLOCK-BMAL1, SIRT1 participates in the circadian expression of *Bmal1, Rorc, Per2* and *Cry1* (Asher *et al.*, 2008). Interestingly, SIRT1 also acts directly on two core clock components—it reverses the CLOCK-mediated acetylation of lysine 537 of BMAL1 (Nakahata *et al.*, 2008), and deacetylates and promotes the degradation of PER2 (Asher *et al.*, 2008).

It is interesting to note here that because SIRT1 is controlled by the cellular NAD^+:NADH ratio, its activity is intimately tied to a cell's redox state. This finding fits nicely with earlier work showing that the transcriptional activity of CLOCK and NPAS2 is regulated by cellular redox state (Rutter *et al.*, 2001). Expression of the gene encoding the rate-limiting enzyme in the NAD^+ salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT), is circadian owing to E-box-mediated CLOCK/BMAL1 activation. The resulting daily oscillation in NAMPT activity produces rhythmic levels of NAD^+ in cells (Nakahata *et al.*, 2009; Ramsey *et al.*, 2009). Hence, a novel feedback loop connecting cellular metabolism and the circadian clock has been uncovered whereby CLOCK/BMAL1 positively regulates NAD^+ levels, and thus SIRT1 activity through the circadian control of *Nampt* expression. SIRT1, in turn, negatively regulates CLOCK/BMAL1 activity by promoting transcriptional repression and participates in the oscillation of its own coenzyme, NAD^+. As mentioned earlier, this and several other connections between the circadian system and metabolic pathways represent an emerging area of investigation as reviewed comprehensively elsewhere (Green *et al.*, 2008; Bass and Takahashi, 2010; Bellet and Sassone-Corsi, 2010; Maury *et al.*, 2010; Asher and Schibler, 2011).

VII. POSTTRANSCRIPTIONAL CLOCK MECHANISMS

Despite significant progress in elucidating the role of posttranslational regulation of the molecular clock in mammals, only recently have the contributions of posttranscriptional regulatory processes to clock function been explored (Kojima *et al.*, 2011; Staiger and Koster, 2011). Because many of the core clock genes, as well as clock-controlled genes, exhibit circadian oscillations in their transcript levels it is important to determine what processes mediate daily mRNA turnover in mammalian cells. Furthermore, in mice only between 33–50% of the genes that encode rhythmic proteins also manifest rhythmic transcript levels, indicating that the other mRNAs are regulated at the posttranscriptional level (Reddy *et al.*, 2006; Deery *et al.*, 2009).

A. MicroRNAs

Of particular relevance to the elucidation of posttranscriptional clock mechanisms, is recent work revealing circadian control of the expression of microRNAs (miRNAs) and their role in the cycling of clock gene transcripts in mammals. Transcribed from non-coding genomic regions, miRNAs are short, single-stranded RNA molecules 19–25 nucleotides in length that interact with the 3′ untranslated regions (3′UTRs) of target transcripts to induce the cleavage/destabilization of, or to repress translation of, the target mRNA (Bushati and Cohen, 2007; Rana, 2007; Bartel, 2009; Guo *et al.*, 2010). If one report is correct in positing that most mammalian mRNAs are conserved targets of miRNAs (Friedman *et al.*, 2008), it will be important to understand how this regulatory mechanism affects the molecular clock.

Results from a genome-wide screen to identify CREB-regulated miRNAs (Impey *et al.*, 2004) have encouraged analysis of miRNA expression specifically in the SCN of mice (Cheng *et al.*, 2007). Two miRNAs, miR-219 and miR-132, exhibit circadian expression in the SCN and harbor cAMP-reponse elements (CREs) in their promoters, yet only in miR-219 has an E-box element (noncanonical) been identified. Coexpression of CLOCK and BMAL1 in PC12 cells induces expression of miR-219, but not of miR-132. Under

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constant conditions, both miRNAs exhibit a circadian oscillation solely in the SCN, peaking during the mid-subjective day. This rhythm is abolished in *Cry1/Cry2* double mutant mice. Evidence that miR-132 downregulates translation of *Per2* comes from treatment of mice with an antagomir to this miRNA. Antagomirs, modified oligoribonucleotides complementary to a specific miRNA, act to block miRNA function (Krutzfeldt *et al.*, 2005). The miR-132 antagomir results in increased PER2 expression in mice. Finally, circadian period length and light-dependent clock resetting are altered by the antagomir-mediated silencing of miR-219 and miR-132, respectively (Cheng *et al.*, 2007).

To further investigate the role of miR-132 in photic entrainment of the SCN clock, one group has generated a transgenic mouse model that conditionally expresses miR-132 in the SCN and forebrain (Alvarez-Saavedra *et al.*, 2011). Following photic stimulation, the miR-123 transgenic animals experience attenuation in light-induced resetting of behavioral rhythms, and a concomitant decline in PER1 and PER2 levels. Analysis of putative miR-132 targets in the SCN has revealed several that are involved in chromatin remodeling and translational control. Through posttranscriptional regulation of these genes, it is proposed that miR-132 is able to control chromatin remodeling and translation within SCN neurons, thus mediating clock entrainment by regulating *Per* expression. Indeed, the *Per1* and *Per2* promoters are bound to, and activated by, methyl CpG island binding protein 2 (MeCP2), whereas PABP-interacting protein 2A (PAIP2A) and B-cell translocation gene 2 (BTG2) have the opposite effect—they suppress PER translation by promoting *Per* mRNA decay (Alvarez-Saavedra *et al.*, 2011).

Another group has identified a role for the miR-192/194 cluster in the regulation of the *Per* family in HeLa and NIH3T3 cells (Nagel *et al.*, 2009). The 3′UTRs of all three *Per* genes contain putative target sites for miR-192, miR-194, or both. Evidence shows that miR-192/194 downregulates the *Per* genes by acting on mRNA. This downregulation causes a shortening of the circadian period (Nagel *et al.*, 2009).

In liver, the most abundant microRNA, miR-122, is transcribed in a rhythmic manner with pri-mir-122 peaking at zeitgeber time 0 (ZT0) and reaching a nadir at ZT12, although the levels of miR-122 remain constant throughout the day (Gatfield *et al.*, 2009). The expression of miR-122 is most likely regulated by REV-ERBα/β through two conserved ROREs in this gene's promoter as *Rev-erbα*$^{-/-}$ mice experience attenuation in pri-mir-122 accumulation. Microarray analyses of gene expression in liver tissue of mice treated with a miR-122 antisense oligonucleotide have demonstrated several mRNAs that are under circadian regulation including peroxisome proliferator-activated receptor β/δ (PPARβ/δ) and the PPARα coactivator SMARCD1/BAF60a (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1), both circadian regulators of metabolism (Yang *et al.*, 2006; Li *et al.*, 2008). Indeed, the 3′UTR of *Pparβ/δ* contains target sites for miR-122, and this protein is upregulated by two-to-threefold upon inactivation of miR-122 (Gatfield *et al.*, 2009). A similar mechanism of miR-122 regulation at the 3′UTR of *Smarcd1/Baf60a* is apparent. The identification of the circadian regulation of miR-122 expression in liver and its role in the circadian control of downstream targets involved in metabolism provides further insight into tissue-specific links between the circadian system and metabolic function.

B. RNA binding proteins

In addition to the miRNA mechanisms just described, several RNA binding proteins have been identified that regulate clock-related RNA transcripts by different mechanisms. Most RNAs contain *cis*-acting elements in their 3′UTRs to which *trans*-acting protein factors can bind and regulate splicing, transport, stability and translation (Moore, 2005; Gratacós and Brewer, 2010). The first RNA binding protein with a demonstrated effect on the mouse

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molecular clockwork is LARK, for which there are two forms, LARK1 (RBM4) and LARK2 (RBM4B) (Kojima *et al.*, 2007). Although transcripts for both *Lark* isoforms are found in the SCN, only the protein levels oscillate. Interestingly, the phase of the LARK protein oscillation is similar to that for PER1. Investigation of the *Per1* 3′UTR has revealed a specific *cis* element to which LARK binds and promotes translation. Knockdown of *Lark* transcript causes a shorter circadian period, while overexpression of LARK protein increases circadian period length. Thus, LARK seems to act on *Per1* posttranscriptionally by enhancing translation and conferring robustness on the PER1 protein oscillation (Kojima *et al.*, 2007).

One of the more common *cis*-acting elements in mammalian 3′UTRs is the AU-rich element (ARE) (Gratacós and Brewer, 2010). A protein which binds these AREs and is abundant in many tissues is polypyrimidine tract-binding protein 1 (PTBP1; also known as hnRNP I). Recently, circadian oscillation of the mouse *Per2* mRNA has been shown to be regulated by PTBP1 (Woo *et al.*, 2009). PTBP1 binds to the 3′UTR of the *Per2* transcript and promotes its decay. Cytoplasmic PTBP1 levels increase at the time at which there is a concomitant rapid decline in *Per2* RNA levels. Upon knockdown of PTBP1 expression with siRNA, the peak amplitude of *Per2* expression increased. Because several other RNA binding proteins have been detected at the *Per2* 3′UTR, it may be that the effect of PTBP1 on *Per2* RNA decay relies on additional proteins (Woo *et al.*, 2009).

Another RNA binding protein implicated in the regulation of a circadian gene is heterogeneous nuclear ribonucleoprotein D (HNRNPD; also known as AUF1). HNRNPD binds to specific sequences in target mRNA 3′UTRs and regulates transcript stability or the promotion of translation (Gratacós and Brewer, 2010). One group's examination of the *Cry1* transcript has revealed a 610-bp 3′UTR which contains an ARE that, when deleted, results in *Cry1* mRNA stability (Woo *et al.*, 2010). HNRNPD has been identified as the protein responsible for binding to the *Cry1* ARE and promoting mRNA turnover. Similar to the findings for PTPB1 and *Per2*, the cytoplasmic levels of HNRNPD levels exhibit a maximum as *Cry1* mRNA levels are in decline. Knockdown of HNRNPD results in stabilized *Cry1* transcript and enhanced oscillation amplitude (Woo *et al.*, 2010). Thus, cytoplasmic destabilization of both the *Per2* and *Cry1* transcripts share many similarities. It will be interesting to determine what posttranscriptional processes act on the other core clock gene transcripts.

Decay pathways for mRNAs in mammalian cells are either deadenylation-dependent or deadenylation-independent (Gratacós and Brewer, 2010). Both PTBP1 and HNRNPD are involved in the deadenylation-dependent pathway. Deadenylation involves the removal of a transcript's poly(A) tail followed by breakdown of the RNA. It is interesting to note that a circadian deadenylase gene, *Nocturnin* (*Ccrn4l*), has been characterized in mammals where it is rhythmically expressed in multiple tissues, including the SCN (Wang *et al.*, 2001). *Noc* circadian expression is damped in the liver of *Clock* mutant mice, and is constitutively elevated in *Cry* double mutant animals (Oishi *et al.*, 2003). Although $Noc^{-/-}$ mice exhibit normal locomotor activity rhythms and clock gene expression, they do have several aberrant metabolic phenotypes (Green *et al.*, 2007). Thus, *Noc* is a clock-controlled gene—a clock output. Interestingly, it has recently been shown that circadian expression of *Noc* in mouse liver is controlled by miR-122 (Kojima *et al.*, 2010).

VIII. EFFECTS OF TEMPERATURE ON THE MAMMALIAN CLOCK

A. Temperature as an entraining agent

Temperature is an important environmental entraining agent for many organisms, yet in homoeothermic vertebrates, including mammals, changes in ambient temperature either do

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not entrain circadian rhythms of locomotor activity, or do so poorly (Hoffmann, 1969; Aschoff and Tokura, 1986; Francis and Coleman, 1997; Palkova *et al.*, 1999). Mammals do, however, experience circadian rhythms in core body temperature with a fluctuation of 1–4°C that are regulated by the SCN (Refinetti and Menaker, 1992). As mentioned previously, cells of most peripheral tissues throughout the mammalian body harbor cell-autonomous circadian oscillators (Welsh *et al.*, 1995; Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004; Welsh *et al.*, 2004; Yoo *et al.*, 2004) which are synchronized to external cues by rhythmic signals from the SCN (Silver *et al.*, 1996; Earnest *et al.*, 1999; Buijs and Kalsbeek, 2001). These observations raise the intriguing question as to what effect, if any, the normal circadian variation in core body temperature may have upon the cell-autonomous oscillators in peripheral tissues. Could body temperature entrain peripheral oscillators in mammals and, perhaps just as interesting, what properties of the SCN prevent it from being synchronized by environmental temperature cycles?

Studies have demonstrated that circadian rhythms of gene expression in cultures of rat-1 fibroblasts (Brown *et al.*, 2002) and rat astrocytes (Prolo *et al.*, 2005) can be entrained to temperature fluctuations of 4°C and 1.5°C, respectively. Although rhythmicity in both cell types damps within a few cycles upon cessation of the temperature rhythms, damping can be delayed by exposing fibroblasts to natural body temperature oscillations (Brown *et al.*, 2002) or by co-culturing astrocytes with SCN explants (Prolo *et al.*, 2005). Furthermore, when mice are exposed to inverted environmental temperature cycles of 37°C during the day and 24°C during the night, circadian rhythms of gene expression in the liver are reversed without affecting the central clock in the SCN (Brown *et al.*, 2002). It should be mentioned that this phenomenon of decoupling peripheral circadian rhythms from the SCN has also been observed under paradigms of restricted feeding in mammals, and that restricted feeding can alter body temperature rhythms (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). Indeed, it has been proposed that food availability and environmental temperature are related, but independent, entraining cues (Brown *et al.*, 2002). Thus, it is clear that in cultured nonneuronal mammalian cells, and in peripheral tissues in vivo in mammals, temperature cycles can entrain circadian rhythms of gene expression.

In contrast to peripheral oscillators, SCN rhythms in vivo are unaffected by environmental temperature changes that phase shift circadian rhythms in other brain regions (Brown *et al.*, 2002). Recent work in our lab has extended this finding by demonstrating that in mice, the resistance of the SCN to entrainment by ambient temperature is not a cell-autonomous property of individual SCN neurons. Rather, this phenomenon is an emergent property of the SCN network and is therefore dependent upon intercellular coupling among neurons (Buhr *et al.*, 2010). Compelling evidence for the role of intercellular coupling comes from experiments showing that blocking either voltage-gated Na^+ channels with TTX or L-type, but not T-type, Ca^{2+} channels with nimodipine, renders the SCN susceptible to resetting by temperature pulses. Furthermore, communication between the neurochemically-distinct ventral and dorsal regions of the SCN is necessary as cultures of either of these regions alone are shifted in response to temperature changes (Buhr *et al.*, 2010).

Work implying a role for heat shock factor 1 (HSF1) and other components of the heat shock response pathway in circadian gene expression in mammalian liver (Kornmann *et al.*, 2007; Reinke *et al.*, 2008) has encouraged investigation of the possible involvement of this pathway in temperature resetting in mammals. When SCN cultures are pulsed for 1 hr with the heat shock pathway antagonist KNK437, no phase shifts occur. In contrast, the same treatment induces strong phase shifts in pituitary and lung cultures, indicating that in non-SCN tissues, KNK437 mimics the effect of 1-hr cool (33.5°C) pulses in reducing HSF1-mediated transcription (Buhr *et al.*, 2010). Phase shifts to warm (38.6°C) pulses observed in lung and pituitary are blocked both by KNK437 and quercetin, another HSF1 inhibitor.

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Continuous inhibition of HSF1-mediated transcription via chronic KNK437 administration to SCN, lung and pituitary cultures causes an increase in circadian period. This effect is consistent with period lengthening of the circadian rhythm of locomotor activity observed in *Hsf1*$^{-/-}$ mice (Reinke *et al.*, 2008). Taken together, these results suggest a molecular mechanism that involves HSF1 in temperature resetting in mammalian peripheral tissues (Buhr *et al.*, 2010).

It is important to mention here that two reports, in contrast to the work just presented, provide evidence that rat SCN slice cultures do exhibit entrainment to temperature cycles (Ruby *et al.*, 1999; Herzog and Huckfeldt, 2003). Several differences in these studies may account for this discrepancy. First, the work of Ruby *et al.* (1999) relied on extracellular recordings from single neurons as glass electrodes advanced along tracks through SCN slices maintained in culture for up to 60 hr. Herzog and Huckfeldt (2003) measured PER1::LUC rhythms from neonatal and juvenile rat SCN slices in cultures for up to two weeks and observed that these slice preparations were more sensitive to temperature pulses than adult tissues. In contrast, our studies used organotypic cultures of SCN and peripheral tissues from *Per2*^{Luc} reporter mice and measured in real time the PER2::LUC bioluminescence rhythm continuously for several days (Yoo *et al.*, 2004; Buhr *et al.*, 2010). The in vivo study of Brown et al. (2002) examined mice exposed to environmental temperature changes over a several day period and measured *Dbp* expression in SCN sections by in situ hybridization. Thus, the SCN culture techniques and output rhythms measured in each study differed. A final possibility is that species-specific differences between mice and rats account for the differences observed in some of these studies. Further work is necessary to clarify these issues.

B. Temperature compensation

One of the hallmarks of circadian rhythms in all organisms, from cyanobacteria to mammals, is that they are temperature compensated—daily rhythms remain constant as temperature increases or decreases across a physiologically viable range (Pittendrigh, 1993). This clock property can be expressed quantitatively as the Q_{10}, or temperature coefficient—the change in the rate of a biological rhythm or biochemical reaction as a result of increasing the ambient temperature by 10°C. The Q_{10} for most biochemical reactions is 2 to 3, yet circadian rhythms usually have a Q_{10} between 0.8–1.2 (Sweeney and Hastings, 1960). Efforts to elucidate a molecular mechanism underlying the temperature compensation property of circadian clocks have been unsuccessful until recently. The striking demonstration that the circadian rhythm of phosphorylation of the cyanobacterial clock protein, KaiC, can be reconstituted in vitro with just three Kai proteins and ATP, has led to experiments revealing that this in vitro oscillator is also temperature compensated (Nakajima *et al.*, 2005; Tomita *et al.*, 2005). Hence, as the cyanobacterial example aptly illustrates, temperature compensation must be an inherent property of at least some of the biochemical reactions comprising the molecular mechanism of the circadian clock of any given species.

In mammals, temperature compensation has been demonstrated in cell culture for rat-1 (Izumo *et al.*, 2003) and mouse (Tsuchiya *et al.*, 2003; Dibner *et al.*, 2009) fibroblasts, and for neonatal rat SCN neurons (Herzog and Huckfeldt, 2003). As for whole neural tissues, hamster retina (Tosini and Menaker, 1998), and rat (Ruby *et al.*, 1999), mouse (Buhr *et al.*, 2010), and ground squirrel (Ruby and Heller, 1996) SCN are temperature compensated. Several mammalian peripheral tissues also exhibit this property (Reyes *et al.*, 2008), as do human red blood cells (O'Neill and Reddy, 2011). Work by our group has also shown that the temperature compensation observed in SCN tissue is a cell-autonomous property and does not rely on intercellular coupling, unlike the resistance to temperature resetting of the SCN which is an emergent property of the SCN network (Buhr *et al.*, 2010).

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Genetic mutations affecting the period of the circadian clock in mammals can also affect temperature compensation as shown for isolated *tau* mutant hamster retinal cultures (Tosini and Menaker, 1998). This is particularly intriguing in light of recent work demonstrating both in cell culture and in cell-free in vitro reactions, that the enzymatic activity of CK1δ/ε toward circadian substrates such as PER2 is temperature-insensitive, but temperature-sensitive toward non-circadian substrates (Isojima *et al.*, 2009). Both wild-type and $CK1\varepsilon^{tau}$ exhibit temperature-insensitive phosphorylation of a peptide containing the PER2 βTrCP binding region, yet when a peptide substrate derived from the FASPS region of PER2 is tested, an increase in the temperature sensitivity of the $CK1\varepsilon^{tau}$ mutant enzyme is revealed (Isojima *et al.*, 2009). An effect of autophosphorylation state on CK1δ/ε activity toward circadian-relevant substrates in vitro is also apparent. Thus, similar to the cyanobacterial system mentioned previously, the temperature-insensitive phosphorylation of mammalian circadian substrates by CK1δ/ε can be reconstituted in vitro, and this property of CK1δ/ε is dependent both on the substrate and on the phosphorylation state of the enzyme (Isojima *et al.*, 2009). Additional work will be necessary to replicate these results and to elucidate the biochemical mechanism underlying CK1δ/ε circadian-specific temperature compensation.

IX. UNRESOLVED ISSUES AND FUTURE DIRECTIONS

As mentioned at the beginning of this review, extensive work has shown that across phyla, the primary molecular mechanism underlying cell-autonomous circadian oscillators is composed of autoregulatory feedback loops of transcription and translation. Hence, the existence of transcription-independent oscillations and a potential role for such oscillations in the function of the cellular clock in mammals and other organisms seems surprising. Indeed, it was the cell-free recapitulation in a test tube of the circadian rhythm of KaiC phosphorylation by colleagues working on the cyanobacterial circadian clock (Nakajima *et al.*, 2005; Tomita *et al.*, 2005) that generated recent interest in transcription-independent clock processes. Further work in cyanobacteria has shown that both circadian transcriptional/translational mechanisms and transcription-independent posttranslational mechanisms are necessary for a competent circadian clock in this organism (Kitayama *et al.*, 2008).

Hints that transcription-independent processes in the mammalian circadian clock exist come from several studies. The surprising discovery that rhythmic transcription of the core clock genes *Bmal1, Cry1*, and *Cry2* (Fan *et al.*, 2007; Liu *et al.*, 2008) is not necessary for circadian clock function in mammalian cells, and that the mammalian cellular clock is particularly resilient to attenuation of transcription (Dibner *et al.*, 2009), suggests that mechanisms other than the transcriptional/translational feedback loop are involved in the generation of oscillations. Perhaps the most striking demonstration of a transcription-independent circadian rhythm in mammals is the recent report of a daily oscillation in human red blood cells of the oxidation and subsequent monomer-to-dimer transition of peroxiredoxin (PRX), a protein that inactivates reactive oxygen species (O'Neill and Reddy, 2011). In liver cells, expression of PRX is circadian (Reddy *et al.*, 2006), but this is not possible in mature erythrocytes which have no nucleus. Inhibitors of transcription and translation have no effect on the circadian rhythm of PRX oxidation in human red blood cells further suggesting that the observed PRX oxidation rhythm in erythrocytes is a transcription-independent process. Moreover, detection of another circadian rhythm in erythrocytes—the transition of hemoglobin between dimer and tetramer states—seems also to be transcription-independent. A circadian rhythm of PRX oxidation has also been demonstrated by the same group in the green algae *Ostreococcus tauri*, a primitive eukaryote, even when gene expression is halted by exposing these cells to DD (O'Neill *et al.*, 2011). Although these findings must be repeated and validated in other model organisms, they suggest an intriguing avenue for further work.

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A major goal toward understanding any biological system is to successfully model that system mathematically. Accomplishing this requires that biologists and modelers work together to incorporate experimental results into models such that they may be used to make testable predictions. The ongoing development of models of the mammalian circadian clock has been particularly helpful recently as they take into account the "combinatorial complexity" of clock component interactions as well as stochastic properties (Yamada and Forger, 2010). Model-based predictions regarding the effects of mutations in core clock genes (Liu *et al.*, 2007b), the role of posttranslational processes on clock components (Gallego *et al.*, 2006a), and the electrical properties of the SCN (Belle *et al.*, 2009), have been validated empirically. The necessity for both molecular noise and intercellular coupling to induce rhythms in a population of SCN neurons has also been predicted via modeling and subsequently confirmed by experiment (Ko *et al.*, 2010). A future challenge to clock modelers will be to address the complex connections and interactions between the mammalian circadian clock and other systems, including the many emerging links between the clock and metabolism.

While there are many potentially fruitful paths of pursuit in the study of mammalian circadian clock genetics, we will conclude by mentioning a few here. Large-scale screening of small-molecule libraries may yield promising targets for therapeutic intervention of circadian-related genetic disorders in humans including FASPS and seasonal affective disorder (Liu *et al.*, 2007a). New approaches in synthetic biology in which artificial transcriptional circuits are used to define networks of oscillating genes or to interrogate the function of circadian-related *cis*-acting elements promise to further our understanding of clock transcriptional pathways (Ueda *et al.*, 2005; Kumaki *et al.*, 2008; Ukai-Tadenuma *et al.*, 2008). Understanding circadian phenotypes that occur in mammals in the absence of underlying changes in DNA sequence–via epigenetic processes–will require ongoing work (Bellet and Sassone-Corsi, 2010; Ripperger and Merrow, 2011). Elucidation of the molecular and biochemical mechanisms of temperature-insensitive phenomena in mammalian clock systems remains an important goal (Brown *et al.*, 2002; Isojima *et al.*, 2009; Buhr *et al.*, 2010). In all of these endeavors, the mouse will remain the mammalian genetic model of choice.

X. CONCLUSION

Over the past 10 years remarkable progress has been made in our understanding of the genetics of the mammalian circadian clock. The transcriptional/translational feedback loop model of the molecular oscillator within cells, for which there is evidence across phyla, has formed the foundation of our understanding of the molecular clockwork. This model, however, must be modified given the new levels of hierarchy and complexity evident from recent work. It is necessary to study the mammalian clock at all levels from single cells, to cell-cell interactions within a tissue, to tissue-level properties, and finally, at the level of behavior. Emergent clock properties arise from the interactions among cells—properties that cannot be studied at the single-cell level. Likewise, focusing on behavior or tissue-specific processes alone will overlook cell-autonomous clock properties. New advances in reporter technology, microarrays, mathematical modeling, perturbation analysis methods, and systems biology will continue to elucidate properties of the molecular clock. Hence, a lesson learned from the work presented herein is that with respect to the mammalian circadian clock, a systems-wide approach must be advocated. In the near future we should look forward to a better understanding of how the mammalian clockwork is integrated with the other physiological systems of the body, and perhaps be better able to develop therapies for human clock-related disorders.

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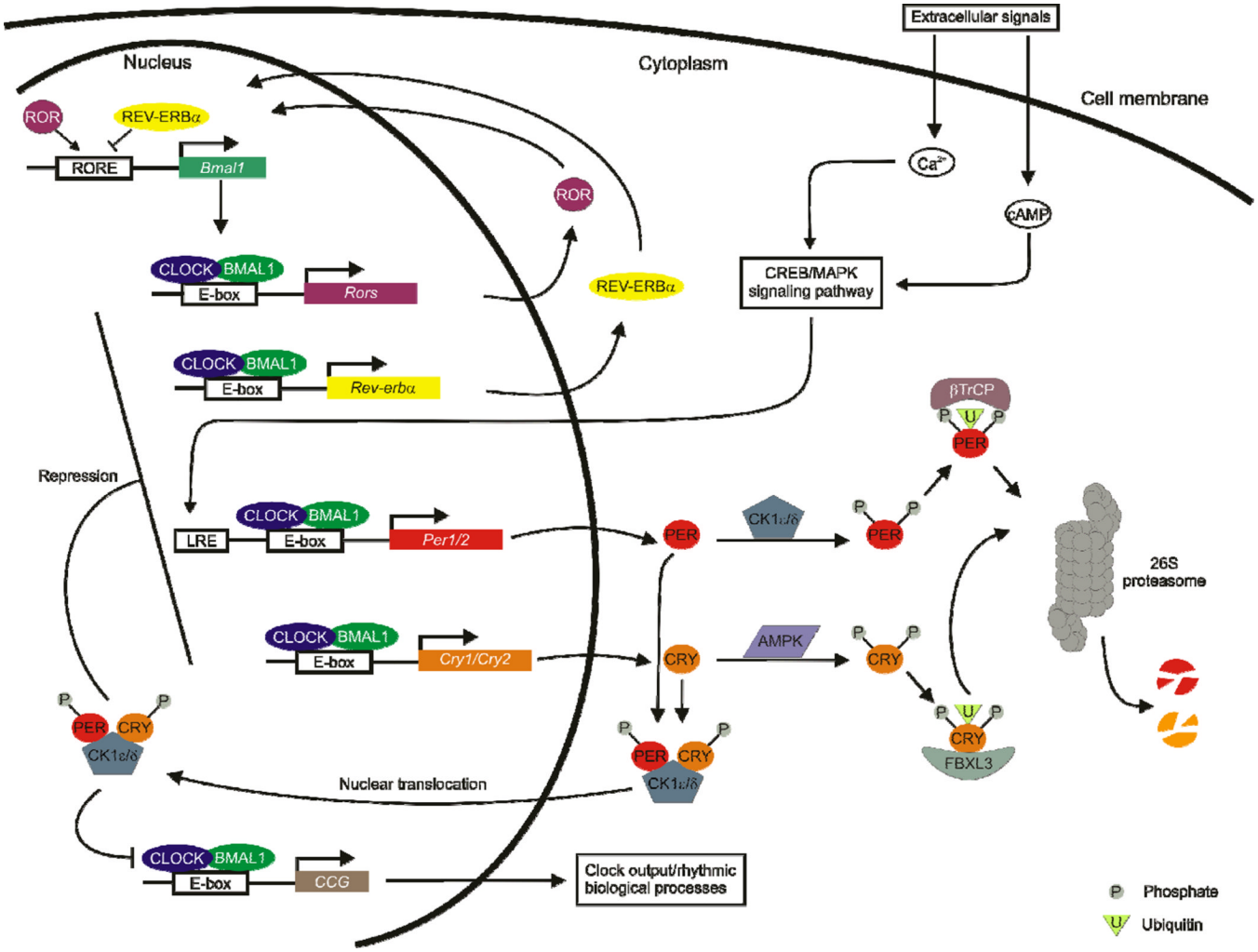

Figure 1.

Model of the mammalian cell-autonomous oscillator as described in the text. Abbreviations: CCG, clock-controlled gene; P, phosphate; U, ubiquitin.

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Table 1

Behavioral phenotypes of mutations in mouse clock and clock-related genes

Gene(s)	Protein product(s)	Mutant allele(s)	Mutant phenotype(s)	References
Clock	bHLH-PAS transcription factor	$Clock^{\Delta 19/\Delta 19}$ $Clock^{-/-}$	4 hr longer pd/arrhythmic 0.4 hr shorter pd	(Vitaterna *et al.*, 1994) (Debruyne *et al.*, 2006)
Npas2 (*Mop4*)	bHLH-PAS transcription factor	$Npas2^{-/-}$	0.2 hr shorter pd	(Dudley *et al.*, 2003)
Clock/Npas2	bHLH-PAS transcription factors	$Clock^{-/-}/Npas2^{-/-}$	Arrhythmic	(DeBruyne *et al.*, 2007a)
Bmal1 (*Arntl*, *Mop3*)	bHLH-PAS transcription factor	$Bmal1^{-/-}$	Arrhythmic	(Bunger *et al.*, 2000)
Cry1	flavoprotein	$Cry1^{-/-}$	1 hr shorter pd	(van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999)
Cry2	flavoprotein	$Cry2^{-/-}$	1 hr longer pd	(Thresher *et al.*, 1998; van der Horst *et al.*, 1999)
Cry1/Cry2	flavoproteins	$Cry1^{-/-}/Cry2^{-/-}$	Arrhythmic	(van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999)
Per1	PAS protein	$Per1^{-/-}$	0.7 hr shorter pd	(Cermakian *et al.*, 2001)
		$Per1^{brdml}$	1 hr shorter pd	(Zheng *et al.*, 2001)
		$Per1^{ldc}$	0.5 hr shorter pd/arrhythmic	(Bae *et al.*, 2001)
Per2	PAS protein	$Per2^{brdml}$	1.5 hr shorter pd/arrhythmic	(Zheng *et al.*, 1999)
		$Per2^{ldc}$	Arrhythmic	(Bae *et al.*, 2001)
Per3	PAS protein	$Per3^{-/-}$	0–0.5 hr shorter pd	(Shearman *et al.*, 2000a)
Per1/Per2	PAS proteins	$Per1^{brdml}/Per2^{brdml}$	Arrhythmic	(Zheng *et al.*, 2001)
		$Per1^{brdml}/Per2^{brdml}$	Arrhythmic	(Bae *et al.*, 2001)
Per1/Cry1	PAS protein/flavoprotein	$Per1^{brdml}/Cry1^{-/-}$	Normal behavior	(Oster *et al.*, 2003)
Per1/Cry2	PAS protein/flavoprotein	$Per1^{brdm1}/Cry2^{-/-}$	<6 months, 1.5 hr longer pd; >6 months, arrhythmic	(Oster *et al.*, 2003)
Per2/Cry1	PAS protein/flavoprotein	$Per2^{brdm1}/Cry1^{-/-}$	Arrhythmic	(Oster *et al.*, 2002)
Per2/Cry2	PAS protein/flavoprotein	$Per2^{brdm1}/Cry2^{-/-}$	0–0.4 hr shorter pd	(Oster *et al.*, 2002)
Rev-erbα (*Nr1d1*)	nuclear receptor	$Rev\text{-}erb\alpha^{-/-}$	0.5 hr shorter pd; disrupted entrainment	(Preitner *et al.*, 2002)
Rev-erbβ (*Nr1d2*)	nuclear receptor	—	—	—
Rorα (*Rora*)	nuclear receptor	$Ror\alpha^{-/-}$ (*staggerer*)	0.5 hr shorter pd; disrupted entrainment	(Sato *et al.*, 2004)
Rorβ (*Rorb*)	nuclear receptor	$Ror\beta^{-/-}$	0.5 hr longer pd	(Masana *et al.*, 2007)
Rorγ (*Rorc*)	nuclear receptor	$Ror\gamma^{-/-}$	Normal behavior	(Liu *et al.*, 2008)
Dec1 (*Bhlhe40*, *Stra13*, *Sharp-2*)	bHLH transcription factor	$Stra13^{-/-}$	0.15 hr longer pd	(Nakashima *et al.*, 2008)
Dec2 (*Bhlhe41*, *Sharp-1*)	bHLH transcription factor	$Sharp\text{-}1^{-/-}$	Delayed resetting	(Rossner *et al.*, 2008)
CK1δ (*Csnk1d*)	casein kinase 1	$CK1\delta^{+/-}$	0–0.5 hr longer pd	(Xu *et al.*, 2005; Etchegaray *et al.*, 2009)
CK1ε (*Csnk1e*)	casein kinase 1	$CK1\varepsilon^{-/-}$	0.2–0.4 hr longer pd	(Meng *et al.*, 2008;

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Gene(s)	Protein product(s)	Mutant allele(s)	Mutant phenotype(s)	References
				Etchegaray *et al.*, 2009)
		*CK1ε^{tau}	4 hr shorter pd	(Lowrey *et al.*, 2000; Meng *et al.*, 2008)
CK1α (*Csnk1a1*)	casein kinase 1	—	—	—
Fbxl3	F-box protein	*Fbxl3^{Ovtm}*	2 hr longer pd	(Siepka *et al.*, 2007)
		Fbxl3^{Afh}	3 hr longer pd	(Godinho *et al.*, 2007)
Bmal2 (*Arntl2*, *Mop9*, *Clif*)	bHLH-PAS transcription factor	—	—	—
Pgc1a (*Ppargc1a*)	transcriptional coactivator	*Pgc1a$^{-/-}$*	0.3 hr longer pd	(Liu *et al.*, 2007c)
Mtnr1a (*Mel1a*)	G protein-coupled receptor	*Mtnr1a$^{-/-}$*	Normal behavior	(Liu *et al.*, 1997a)
Mtnr1b (*Mel1b*)	G protein-coupled receptor	*Mtnr1b$^{-/-}$*	Normal behavior	(Jin *et al.*, 2003)
Opn4	melanopsin; opsin 4	*Opn4$^{-/-}$*	Attenuated photic responses	(Panda *et al.*, 2002b; Ruby *et al.*, 2002)
Dbp	PAR bZIP transcription factor	*Dbp$^{-/-}$*	0.5 hr shorter pd	(Lopez-Molina *et al.*, 1997)
Vipr2	G protein-coupled receptor	*Vipr2$^{-/-}$*	Disrupted locomotor rhythm	(Harmar *et al.*, 2002; Cutler *et al.*, 2003)
Vip	peptide hormone	*Vip$^{-/-}$*	1 hr shorter pd/arrhythmic	(Colwell *et al.*, 2003)
Prok2 (*PK2*)	secreted protein	*Prok2$^{-/-}$*	Reduced locomotor activity	(Li *et al.*, 2006)
Nocturnin (*Ccrn4l*)	deadenylase	*Noc$^{-/-}$*	Normal behavior	(Green *et al.*, 2007)

Gene symbols listed here are the predominate forms used in the scientific literature; alternate forms are given in parentheses. When the predominant symbol differs from standard mouse gene nomenclature, the standard form is given in parentheses. For genes with more than two symbols in parentheses, the form adhering to standard mouse gene nomenclature is underlined.

* First identified as a mutation in Syrian hamster (*Mesocricetus auratus*).

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Table 2

Cell and tissue phenotypes of mutations in mouse clock and clock-related genes

Gene(s)	Mutant allele(s)	Cellular phenotype(s)	Tissue phenotype(s)	References
Clock	*Clock*$^{-/-}$	—	SCN: WT; lung, liver: AR	(DeBruyne *et al.*, 2007a; DeBruyne *et al.*, 2007b)
Npas2 (*Mop4*)	*Npas2*$^{-/-}$	—	SCN, lung, liver: WT	(DeBruyne *et al.*, 2007b)
Bmal1 (*Arntl*, *Mop3*)	*Bmal1*$^{-/-}$	Fibroblasts, SCN neurons: AR	SCN: variable/stochastic; pituitary, liver, lung, cornea: AR	(Liu *et al.*, 2008; Ko *etal.*, 2010)
Cry1	*Cry1*$^{-/-}$	Fibroblasts, SCN neurons: AR	SCN: short; lung, liver, cornea: AR	(Liu *et al.*, 2007b)
Cry2	*Cry2*$^{-/-}$	Fibroblasts, SCN neurons: long	SCN, lung, liver, cornea: long	(Liu *et al.*, 2007b)
Cry1/Cry2	*Cry1*$^{-/-}$/*Cry2*$^{-/-}$	Fibroblasts: AR	SCN, lung, liver, cornea: AR	(Yagita *et al.*, 2001; Liu *et al.*, 2007b)
Per1	*Per1*^{ldc}	Fibroblasts, SCN neurons: AR	SCN: WT; lung: AR	(Liu *et al.*, 2007b)
Per2	*Per2*^{ldc}	Fibroblasts: AR	—	(Liu *et al.*, 2007b)
Per3	*Per3*$^{-/-}$	Fibroblasts: short	SCN, lung: short	(Liu *et al.*, 2007b)
Rev-erbα (*Nr1d1*)	*Rev-erbα*$^{-/-}$	Fibroblasts: WT	—	(Liu *et al.*, 2008)
Rorα (*Rora*)	*Rorα*$^{-/-}$ (*staggerer*)	Fibroblasts: WT	—	(Liu *et al.*, 2008)
Rorγ (*Rorc*)	*Rorγ*$^{-/-}$	Fibroblasts: WT	Lung, liver: WT	(Liu *et al.*, 2008)
CK1δ (*Csnk1d*)	*CK1δ*$^{-/-}$	Fibroblasts: long	*SCN, liver: long	(Etchegaray *et al.*, 2009; Lee *et al.*, 2009; Etchegaray *et al.*, 2010)
CK1ε (*Csnk1e*)	*CK1ε*$^{-/-}$	Fibroblasts: WT	*SCN, liver: WT	(Etchegaray *et al.*, 2009; Etchegaray *et al.*, 2010)
	CK1ε^{tau}	Fibroblasts: short	SCN, pituitary, lung, kidney: short	(Meng *et al.*, 2008)

Gene symbols are as in Table 1. Abbreviations: AR, arrhythmic; WT, wild type;

*neonatal tissue. Table modified from (Baggs *et al.*, 2009).