DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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CIRCADIAN CLOCK GENES IN MAMMALIAN CLOCKWORK, METABOLISM AND BEHAVIOUR

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This thesis is based of the following publications and referred to by relevant Roman numeral in the text:

I Zheng B, Albrecht U, **Kaasik K**, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC (2001). Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. Cell. Jun 1; 105 (5): 683–94.

II **Kaasik K**, Lee CC (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature. Jul 22; 430 (6998): 467–71.

III Walz K, Spencer C, **Kaasik K**, Lee CC, Lupski JR, Paylor R (2004). Behavioral characterization of mouse models for Smith-Magenis syndrome and dup (17)(p11.2p11.2). Hum Mol Genet. Feb 15; 13 (4): 367–78.

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LIST OF ABBREVIATIONS

ALA	aminolevulinate acid
bHLH	basic helix-loop-helix
^{o}C	degrees Celsius
CCG	clock-controlled gene
CGS	contiguous gene syndrome
CLD	cytoplasmic localization domain
CO	carbon monoxide
CT	circadian time in hours after onset of subjective day
DD	constant darkness
del	deletion
DM	double mutant
dup	duplication
ECL	enhanced chemiluminescence
EMS	ethyl methanesulfonate
FASPS	familial advanced sleep phase syndrome
Hprt	hypoxanthine-guanine phosphoribosyltransferase
hr	hour
kDa	kilo Dalton
LRE	light-response element
Mb	mega base
LD	light (12h): dark (12h) cycle
min	minute
ND	not done
PAR bZIP	proline and acidic amino acid-rich basic leucine zipper
SCN	suprachiasmatic nucleus
SMS	Smith-Magenis syndrome
wt	wild type
ZT	zeitgeber time (time in hours in an entrainment cycle, ZT0 is onset of light in a LD cycle and ZT12 is offset of light in a LD cycle).
PAS	*P*er-*A*rnt-*S*ingle-minded (PER, ARNT and SIM are founder proteins for the PAS domain homology)

INTRODUCTION

The circadian clock is a widespread cellular mechanism that underlies diverse rhythmic functions from bacteria to mammals. In mammals the clock influences sleep-wake cycle, cardiovascular activity, endocrinology, body temperature, renal activity, physiology of gastro-intestinal tract, hepatic metabolism and many other metabolic and behavioral processes. Genetic analysis in the past ten years has uncovered several key components and the basic molecular mechanisms governing circadian clocks. Anatomically, in mammals, the circadian system represents a complex multi-oscillatory temporal network of coupled neurons of the suprachiasmatic nucleus (SCN) in the hypothalamus, that is entrained to the daily light-dark cycle. The SCN transmits environmental synchronizing signals to independent circadian oscillators in peripheral tissues. In addition, the peripheral tissues themselves have their own circadian clock function. The SCN acts as the principal circadian pacemaker synchronizing the peripheral clocks into appropriate phase. It is thought that a core set of circadian clock genes common to all cells encode for proteins that autoregulate their own expression and that of clock output genes.

In the present study, the central clock components in mouse were studied with genetic and molecular tools. The aim of this study was to investigate clock genes dependent transcriptional profiles and how circadian clock genes autoregulate their own expression.

The studies presented in this thesis show that central clock components have different functions with respect to transcriptional activation of downstream genes. Differential expression studies of genes in circadian clock mutant mice indicate that key metabolic and behavioral pathways are controlled by the endogenous clock mechanism. The expression of *aminolevulinate synthase* mRNAs encoding for rate-limiting enzymes of haem biosynthesis was shown to be under circadian clock control. The studies presented here show that haem biosynthesis and circadian clock are reciprocally regulated. Porphyrin derivatives are proposed as potential therapeutics for human circadian disorders. In the final part of this thesis, a circadian phenotype of mouse models of one such disorder, Smith-Magenis syndrome, is described.

REVIEW OF LITERATURE

1. Biological timing and its ecological advantage

Most organisms live in a continuously changing environment that varies in a rhythmic fashion related to the Earth's rotation around its axis. This planetary movement produces a daily solar cycle with a periodicity of approximately 24-hr. Alternating change in exposure to sunlight generates dramatic and predictable changes in the environment. Organisms have internal timekeeping mechanisms that respond to the daily solar cycle resulting in cycling of many physiological and behavioral responses with a period of about 24-hr or circadian.

In 1729, French astronomer Jean Jacques d'Ortous de Marian put the mimosa plants in the dark for several days. He found that even in the absence of sunlight, the plants continued to open their leaves during the day and close them at night. Based on this experiment, he concluded that the observed cycle was not a result of external forces (Sun) but for instead was an innate property of the organism. Here, the science of biological clocks began in a cellar.

Circadian rhythms are evident in a vast number of behaviors exhibited by organisms in all branches of the evolutionary tree from bacteria to humans. The discovery that cyanobacteria, which represent one the most ancient life forms on Earth, have a clock mechanism, indicates that this is indeed a very ancient biological system (Kondo *et al.*, 1994). Animals have been long observed to display daily activity patterns. Many mammals are night-active or nocturnal (e.g. mice and bats) initiating their activity at twilight. Majority of birds however are day-active or diurnal. Many species of plants exhibit daily leaf movements and time their flowering to maximize species-specific cross-pollination by insects. Honeybees in turn maximize their foraging activity to coincide with the flowering time. Such symbiotic activity rhythms persist in constant conditions even without timing cues indicating that an internal clock is responsible.

It is argued that an internal pacemaker mechanism that couples the organism to external cycles provides evolutionary advantages over passive responses to changing environmental conditions. Innate time keeping allows anticipation and appropriate response to cyclic environmental events. For example, developmental events in many species are gated to a particular time of day maximizing the survival of offspring. For instance, fruit flies eclose from their pupal cases near dawn at the time of maximal humidity. Such timing is not dependent on the humidity in the environment as temporal control of eclosion is evident in constant laboratory conditions suggesting again an internal clock regulation.

Continuous monitoring of ongoing time in the daily cycle by an internal clock is exploited to control an internal temporal program of physiological processes. Animals and plants that are kept in artificial light-dark cycles that substantially differ from 24-hr show impaired biological performance (Pittendrigh, 1993).

The ubiquitous presence of circadian rhythms in various life forms strongly suggests adaptive value. Few experimentally controlled examples are available that demonstrate direct requirement for clock function in survival of populations over several generations in a competitive environment. One of the best examples involves co-culturing experiments of mutant cyanobacterial strains that have equivalent growth rates but differ in their clock free-running rhythms (Ouyang *et al.*, 1998). A wild type strain with 25-hr rhythm was used along with two strains that either had long rhythm (30-hr) or short rhythm (23-hr). Pairs of the three strains were initially mixed at equal concentrations and cultured at different light-dark (LD) cycles of either a 22-hr day of 11-hr light and 11-hr darkness or a 30-hr day of 15-hr light and 15-hr darkness. Twenty-seven days later, the ratio of the three strains was determined in the grown cultures. Invariably, the strain that had its endogenous rhythm periodicity closest (in resonance) to the imposed light-dark cycles had taken over the culture. The competitive advantage is however only evident in a cycling environment. Co-cultures grown in a stable environmental condition such as constant light showed no preferential selection of rhythmic strains over strains with a disrupted clock (Woelfle *et al.*, 2004).

2. Anatomical basis of circadian rhythms

Neuronal centers required for persistent physiological and behavioral rhythms have been identified in several model organisms. The anatomical basis of circadian pacemakers in marine mollusks *Aplysia* and *Bulla* has been extensively studied. In these organisms, the eyes contain a circadian pacemaker that is capable of producing self-sustained circadian rhythms in neuronal firing even when cultured in isolation from the rest of the organism (Block and McMahon, 1984). Surgical lesion studies and tissue transplantation experiments have found circadian pacemakers in either optic lobes of crickets and cockroaches or in cerebral lobes in flies and moths (Helfrich-Forster, 2004). In higher plants, no central circadian pacemaker appears to be present despite quite complex tissue organization (Thain *et al.*, 2000).

In mammals, the circadian pacemaker resides in neurons in the hypothalamus above the optic chiasm in the suprachiasmatic nucleus (SCN). The SCN is a small, paired nucleus each consists of ~10,000 neurons. Surgical ablation of the SCN causes arrhythmia in wheel-running behavior (Ralph *et al.*, 1990). Ablation and transplantation studies demonstrated that the SCN determines the period of the behavioral rhythms. Experiments with short period hamsters with 20-hr behavioral rhythms showed that donor SCNs obtained from animals could restore behavioral rhythms in wild type SCN-lesioned hamsters and with the period of the donor (Ralph *et al.*, 1990). In addition to neurons, many other cell types and tissues have functional circadian oscillators.

Circadian clock can be restored in cell culture (Balsalobre *et al.*, 2000). Monitoring of circadian gene activity in ex-vivo organ cultures shows the widespread presence of circadian clocks in animals (Plautz *et al.*, 1997; Yamazaki *et al.*, 2000; Yoo *et al.*, 2004). Experiments with SCN-lesioned mice carrying a knock-in of a luciferase reporter in the *mPer*2 locus showed that peripheral organs are likely to be "free-running" in the absence of the SCN. However in order to keep the proper phase in individual cells within an organ and among multiple organs, the SCN is essential. Nagoshi and colleagues revealed by real-time cellular imaging of gene expression that fibroblasts contain a robust, self-sustained and cell-autonomous circadian oscillator, with a range of properties that both overlap and contrast with those of the neural clock of the suprachiasmatic nuclei (Nagoshi *et al.*, 2004). A major question that remains is the exact nature of the signaling mechanisms that are needed to maintain synchrony within each tissue and between the tissues.

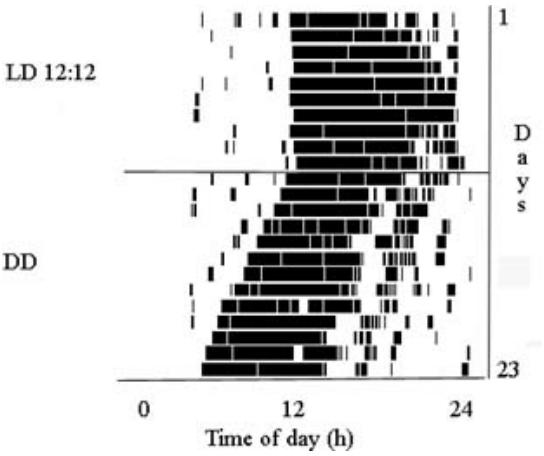
3. Defining features of circadian rhythms

Several biological criteria have been postulated as formal properties of circadian oscillator clocks. Firstly, the observed output rhythms of an organism must be persistent in constant condition independent of environmental time cues. Secondly, the period length of the oscillation is stable over a wide range of temperatures (temperature compensation). Thirdly, the clock mechanism has to be entrainable by cycling environmental cues.

Under constant conditions the periodicity of overt rhythms observed in many organisms slightly deviates from 24-hr depending on the species. The period length of such free-running rhythms is remarkably precise from one day to the next. This is illustrated by the locomotor activity of *Mus musculus* shown in Figure 1. As illustrated in the wheel running behavior, in the absence of any exogenous time cue, the animal maintains a rhythmic behavior, but with a period that is shorter than 24-hr. This feature led to the formulation of the word "circadian" which is derived from the Latin words circa (about) and dia (day). The average length of an endogenous rhythm is referred as "period" or "tau". The biological clock sets a circadian rhythm, or cycle, which influences organisms physiological functions and behaviors. The molecular basis for the persistence of the near 24-hr oscillations is poorly understood. In multicellular organisms, intercellular interactions contribute to the stability of circadian clocks. Dissociated neurons from the mammalian circadian pacemaker SCN show a distribution of period lengths in their firing rhythms with a mean period length that correlates with overall output rhythms (Liu *et al.*, 1997). Neurons in the intact SCN fire in synchrony with a near 24-hr rhythm that reflects the overt rhythm of the animal. Neuronal activity is required to establish synchrony between individual SCN neurons (Yamaguchi *et al.*, 2003). In unicellular

organisms such as cyanobacteria, the intracellular clockwork produces stable 24-hr rhythmicity with no apparent interaction between neighboring individual cells (Mihalcescu *et al.*, 2004).

A



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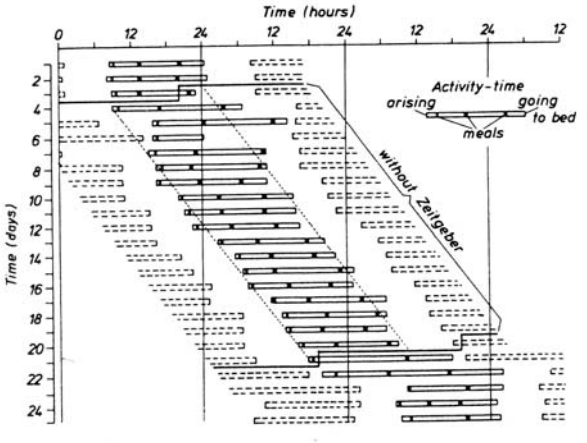

Figure 1. Mouse and human activity rhythms. **A**. Locomotor activity rhythms of a house mouse in the 12-hr light/12-hr dark (LD) and constant darkness (DD). Black area indicates activity recordings. When the animal is left in constant darkness, the rhythm is shorter than 24-hr, resulting in a progressive shift from the original phase. **B.** In a human experiment, the behavioral rhythm is also seen to free-run with a long period of about 25-hr. These small and opposite differences from 24-hr in the two cases strongly support the rhythm being endogenously generated. Fig. 1A is the experimental data of the author. Fig. 1B is adapted from (Wever, 1979).

The second feature of circadian rhythms is temperature compensation. The rates of most biochemical reactions double with every 10ºC increase in temperature while the free-running period length of an organism is constant at different ambient temperatures. The advantage of temperature compensation is apparent for organisms that do not control their body temperature but it also occurs in homeothermic animals. Many warm-blooded animals hibernate or show daily bouts of torpor that can lead to dramatic changes in body temperature. Temperature compensation is critical to maintain a near 24-hr period length of their internal clock. The mechanism of temperature compensation in circadian clock is unknown. A hypothesis postulated in the early days of circadian research suggests temperature compensation to be a function of two counter imposed reactions. While the rate of both reactions increases with temperature, the product of one reaction inhibits the second reaction that determines the period length of the clock. Thus, a rise in temperature would increase the inhibitor levels that would keep the rate of the second reaction constant or temperature compensated (Sweeney and Hastings, 1960). Despite the apparent resistance of the period length to temperature changes, the phase of the oscillation is sensitive to temperature pulses and cycles.

The third property of circadian rhythms is their ability to be entrained by cycling environmental factors or “Zeitgebers” (German Zeitgeber “timegiver”). Light and temperature transitions are the major entrainment cues that reset the internal clock. Food availability, steroid injections and other stimuli can act as entrainment cues. When an entrained rhythm is being studied, phase is referred to in hours of Zeitgeber time (ZT). Melatonin as a Zeitgeber is shown in Figure 2. When Zeitgeber is removed from the system, the phase is referred as circadian time (CT). Light pulse can shift the phase of locomotor activity rhythm under free-running condition. The ability of light to reset a clock allows it to maintain temporal alignment with external time cues. For example, synchronization of the mammalian clock to local time (as occurs following travel across time zones) occurs largely through light-induced phase shifting and resetting of the core oscillator. Therefore, light and temperature pulses can change the phase of the onset of a biological rhythm. The magnitude and direction of this time shift is dependent on the circadian phase in which the stimulus is given. Phase response curves, plots of phase shifts in response to stimulus as a function of circadian time, describe such phase dependent responses. For example in *Drosophila melanogaster*, light pulses administered in the early subjective day have minimal effect on the phase of the rhythm. In contrast, light pulses produce phase delays when given during early night and phase advances when stimulus is presented in late night (e.g. Curtin *et al.*, 1995; Martinek *et al.*, 2001). True entrainment is displayed when the reset phase of the rhythm is maintained after removal of the entraining agent under constant conditions. In mammals, light signaling occurs via the retinohypothalamic tract that connects the retina with the SCN. Outer-retinal cells with rod and cone photoreceptors as well as melanopsin-expressing retinal ganglion cells located

at inner layers of the retina are required for photic entrainment (Berson *et al.*, 2002; Hattar *et al.*, 2002; Panda *et al.*, 2003). Multiple photoreceptor systems are required for entrainment in other organisms as well. In *Drosophila,* mutations that eliminate the compound eyes and extra-retinal structures affect photic entrainment synergistically. Flies with mutations that eliminate cryptochrome function in addition to all eye structures show an almost complete failure to entrain to light-dark cycle (Helfrich-Forster *et al.*, 2001; Mealey-Ferrara *et al.*, 2003).

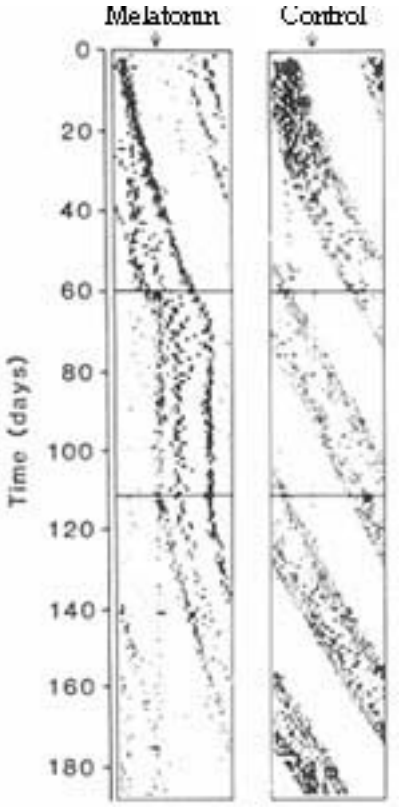

Figure 2. Circadian rhythms entrainment by a rhythmic zeitgeber. Entrainment of a rat with daily injections of melatonin. Locomotor activity records of rats are shown. Dark areas indicate activity. Both rats were in constant darkness for 60 days, after that the rat in the left received daily melatonin injections for 52 days (the time of the injection is marked by arrow), the rat on the right received a control solution. The melatonin rat's clock entrains to the injection, while the control clock continues to free-run. After the injections the activity was monitored for the next 30 days. Adapted from (Redman *et al.*, 1983).

4. Why are circadian clocks needed?

"Circadian resonance hypothesis" by Pittendright and Bruce states that organisms with clocks having periodicities matching those of cycling environment perform "better" compared to those whose periodicities that do not match the period of the environmental cycle (Pittendrigh CS, 1959).

It has been hypothesized that when the life began, the Earth did not have an ozone layer. It has been postulated that DNA synthesis occurred at night to limit radiation damage by the sun. Ancient clocks could have evolved to separate incompatible biochemical reactions like reductions/oxidation, photosynthesis and nitrogen fixation into separate phases of the daily rhythm. For example, the circadian clock of plants allows production of photo-system I and photo-system II components before sunrise, therefore allowing photosynthesis to start as soon as sun energy is available (Harmer *et al.*, 2000). *Synechococcus elongatus* can separate nitrogen fixation and photosynthesis, which allows for very effective nitrogen fixation, since the nitrogenase enzyme is oxygen sensitive (Berman-Frank *et al.*, 2001). Likewise circadian oscillators in mammalian liver cells help to separate glycogen synthesis and degradation (Ishikawa and Shimazu, 1976). Expression of many rate-limiting enzymes in metabolic cascades is under circadian clock control (Panda *et al.*, 2002). The coordination of the first steps in biological pathways help to ensure circadian control of the biochemical cascades. Neuronal signaling and neurotransmitter synthesis are regulated by circadian clock. Some biochemical processes produce harmful byproducts such as free radicals during redox reactions. Clock controlled cytochrome p450 enzymes are involved in the hepatic detoxification of xenobiotic substances that generate reactive oxygen species (Lavery *et al.*, 1999).

5. About molecular clocks

Circadian rhythms are generated by intracellular molecular clocks. Experiments with inhibitors of RNA and protein synthesis showed that these processes were required for the circadian mechanism (Feldman, 1967; Karakashian and Hastings, 1962). All studied clocks are built up as oscillators. The oscillation process is designed by negative and positive elements. Transcription of clock genes yields clock proteins, is driven by positive elements whose role is to activate the clock genes that keeps oscillators running. In contrast, the negative elements slow down the process. The interaction between positive and negative loops keeps the clocks running.

Drosophila and *Neurospora* clockworks have long been the leading model systems to circadian clocks (Bargiello *et al.*, 1984; Dunlap, 1990; Dunlap, 1999). More recently, the mammalian clock has greatly benefited from findings in flies due to structural homologies between many proteins in the two

clockworks. Despite the structural homologies in fly and mammalian clocks the functions of similar proteins are not necessarily conserved.

The clockworks of *Drosophila* and mammals are described in more detail below.

5.1. Molecular basis of *Drosophila* circadian clock

The clock mechanism underlying circadian rhythms were first shown have a genetic basis by the isolation of clock mutants in *Drosophila melanogaster* (Konopka and Benzer, 1971). The first gene involved in circadian timekeeping to be cloned was the *Drosophila* clock gene *period* (Bargiello *et al.*, 1984; Reddy *et al.*, 1984). It functions as a PAS domain-containing transcriptional repressor and forms heterodimer with the product of the second clock gene cloned in fruit flies, *timeless* (tim). *tim* was identified by interaction with PER and by positional cloning of a novel arrhythmic mutation (Gekakis *et al.*, 1995; Myers *et al.*, 1995). Both TIM and PER proteins localize to the nucleus each night where PER down regulates the mRNA production of both genes. Cycling and nuclear localization of PER is blocked by the arrhythmic *timeless* null mutant flies (Vosshall *et al.*, 1994). Thus, *timeless* function has been proposed as a regulator of PER subcellular localization.

The mRNA and protein levels of both *per* and *tim* oscillate with a circadian period. Peak levels of mRNA of both genes occur around CT14 (Hardin *et al.*, 1990; Sehgal *et al.*, 1995). Protein levels of PER and TIM are at their highest in the middle of the night at CT18 with a 4-hr delay with respect to mRNA accumulation (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Siwicki *et al.*, 1988; Zeng *et al.*, 1996; Zerr *et al.*, 1990).

Transcriptional feedback loops of the *Drosophila* molecular clock are shown in Figure 3. The cycling expression of *per* and *tim* is regulated by two PAS domain-containing helix-loop-helix type transcription factors *Cycle* (CYC) and *Clock* (CLK) identified in an EMS mutagenesis screen (Allada *et al.*, 1998; Rutila *et al.*, 1998).

In *Clk* and *cyc* mutants, the mRNA levels of *per* and *tim* are constitutively produced at low levels (Allada *et al.*, 1998). The mRNA and protein levels of CLK cycle with a circadian rhythm with the peak levels produced around dawn (Bae *et al.*, 2000; Bae *et al.*, 1998; Darlington *et al*., 1998).

CYC mRNA and protein however are constitutively produced (Bae *et al*., 2000; Rutila *et al.*, 1998). In cell culture experiments CLK and CYC are able to activate *per* and *tim* promoters given the presence of their binding sites (Darlington *et al.*, 1998; Hogenesch *et al.*, 1998). This activation of gene activity by CLK and CYC is suppressed by the activities of PER and TIM (Darlington *et al.*, 1998). In the middle of the night PER-TIM dimers translocate to the nucleus. PER and TIM can bind to the CLK-CYC complex which inhibits their DNA binding activity without disrupting the dimer of CLK

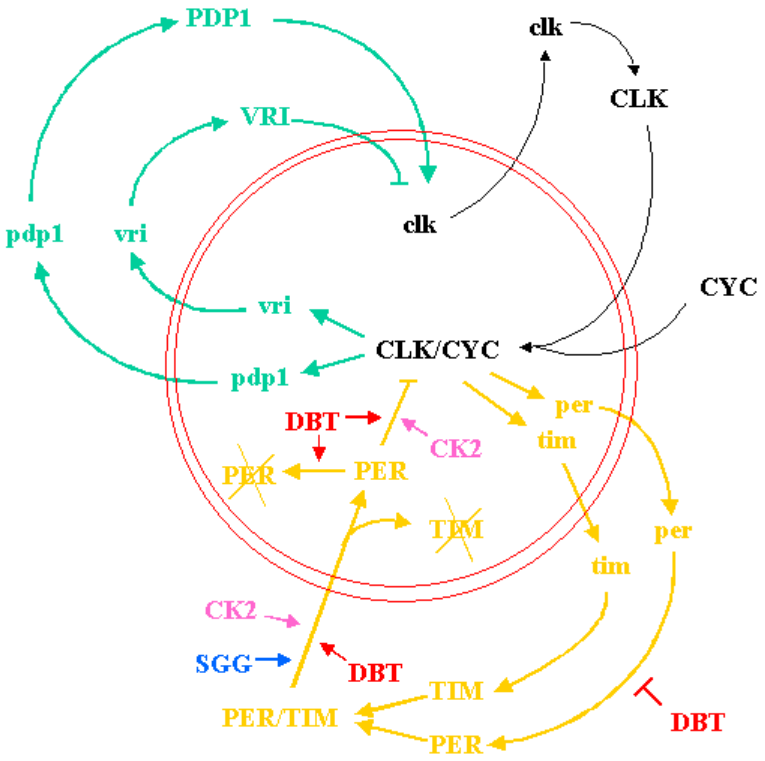

Figure 3. Model of *Drosophila* clockwork**.** The model shows the regulatory relationships between the genes and proteins in the negative and positive transcriptional feedback loops. Positive feedback loop is color coded in green, negative feedback loop is in yellow. Lines ending with bars indicate negative regulation, arrows designate positive regulation. Protein names are in upper case, genes and mRNAs are written in lower case. Two red concentric circles represent the nuclear membrane. Briefly, CLK/CYC heterodimer activates *per* and *tim* genes as well as *vri* and *pdp1* genes during late day, early evening. With a 4-hr delay relative to their mRNAs, PER and TIM proteins are produced and after heterodimerization translocate to the nucleus in the middle of the night. In the nucleus PER represses CLK/CYC dependent gene activation when released from complex with TIM causing a decline in *per* and *tim* mRNA levels. This closes the negative feedback loop that controls transcription of CLK/CYC target genes. At the same time rhythmic CLK expression is regulated by a positive feedback loop that involves repression of *clk* expression by VRI and subsequent activation by PDP1. Protein accumulation phases of VRI and PDP1 are temporally segregated. VRI accumulates in early evening whereas PDP1 accumulates in late evening. In the early morning highly accumulated CLK is initially inhibited from activating its target genes by PER monomers from the previous night. By midday PER monomers have been degraded and new cycle of transcription can start. CYC protein is constitutively expressed. PER monomer stability is negatively regulated in the cytoplasm and nucleus by DBT. Association with TIM stabilizes PER. In addition, DBT and CK2 regulate nuclear translocation of PER/TIM dimers and enhance PER transcriptional repressor activity in the nucleus. SGG positively regulates nuclear translocation of PER/TIM dimers.
Abbreviations: per- *period*, tim–*timeless*, clk–*clock*, dbt–*doubletime*, sgg-*shaggy*, vri– *vrille*, pdp1–*PAR domain protein1*, cyc–*cycle*, ck2–*casein kinase 2*.

and CYC (Lee *et al.*, 1998). The repressive activity is to be carried out primarily by PER monomers, as TIM appears to be dispensable for transcriptional inhibition *in vivo* (Rothenfluh *et al.*, 2000). Thus a transcriptional feedback loop is formed that regulates cycling expression of PER and TIM as negative regulators of their own expression through inhibiting CLK and CYC activity.

A second transcriptional feedback loop regulates the oscillating transcription of the *Clk* gene that cycles in antiphase compared to *per* and *tim*. PER and TIM are required for high-level expression of *clk* mRNA (Bae *et al.*, 1998). This requirement is lost in *Clk* mutant flies. Such genetic interaction suggests the presence of a *clk* repressor that itself is expressed under CLK control (Glossop *et al.*, 1999). The PAR domain containing bzip protein Vrille (VRI) fulfills the requirements for the *Clk* repressor. Overexpression of VRI leads to long behavioral rhythms or arrhythmicity. Its mRNA is expressed under CLK control and oscillates in phase with *per* and *tim* (Blau and Young, 1999). In flies overexpressing *vri, clk* mRNA levels are suppressed and *in vitro* VRI binds to sites in the *Clk* promoter (Cyran *et al.*, 2003; Glossop *et al.*, 2003). Another PAR domain containing bzip transcription factor Pdp1 (PAR domain protein 1) functions to activate *Clk* promoter. Pdp1 mRNA and protein are also expressed with a circadian rhythm under CLK-CYC control but with a later peak phase than *vri* around CT18. Loss of *Pdp1* stops the clock indicating its essential role in oscillator function (Cyran *et al.*, 2003). Pdp1 can also activate *Clk* promotor in reporter assays.

Pdp1 and VRI compete for the same binding sites in the *Clk* promoter (Cyran *et al.*, 2003). The changing balance between initial VRI-dependent suppression and subsequent Pdp1-dependent activation later in the cycle determines the cycling expression of *Clk*. The newly produced CLK at the end of the night and early morning is inhibited by the feedback of high levels of PER induced by the previous wave of CLK activity. The two feedback loops regulating *per* and *Clk* expression are linked and simultaneously restarted by CLK activity after PER is degraded during the day.

In addition to regulation at the transcriptional level many clock components in *Drosophila* are also regulated posttranscriptionally and posttranslationally. Constitutive transcription of *per* and *tim* genes still allows behavioral rhythms to be manifested (Cyran *et al.*, 2003; Frisch *et al.*, 1994; Kaneko *et al.*, 2000; Vosshall and Young, 1995; Yang and Sehgal, 2001). Comparison of *per* transcription and mRNA accumulation rates shows a delay specific to the transcript buildup phase in the morning suggesting regulated mRNA turnover (So and Rosbash, 1997; Stanewsky *et al.*, 1997). *per* RNA splicing is also a regulated process. There is a temperature-sensitive spliced intron in the 3' UTR of *per* mRNA. In cold temperatures this intron is preferentially removed which correlates with earlier rise in *per* mRNA and protein levels (Majercak *et al.*, 1999). Such temperature dependent regulation may regulate seasonal activity.

Most proteins involved in the transcriptional feedback loops are phosphorylated in a time of day dependent manner. Three kinases, CK1ε/*dbt*,

CK2 and GSK3/*shaggy*, have been identified as clock components in *Drosophila*.

Mutations in the gene *doubletime*, a *Drosophila* ortholog of CK1ε, have been found to shorten or lengthen the period of the behavioral rhythms or abolish them all together (Price *et al.*, 1998; Rothenfluh *et al.*, 2000; Suri *et al.*, 2000). DBT is constitutively expressed at the mRNA and protein level and is an essential gene for fly development regulating cell survival and proliferation (Kloss *et al.*, 1998; Kloss *et al.*, 2001; Zilian *et al.*, 1999). Loss of DBT activity is associated with high levels of hypophosphorylated PER indicating its role in determining the rate of PER degradation (Price *et al.*, 1998). DBT has been shown to interact with PER *in vitro* and *in vivo* and create a stable complex with PER throughout the circadian cycle (Kloss *et al.*, 1998; Kloss *et al.*, 2001; Price *et al.*, 1998). PER phosphorylated by DBT is recognized by the Slimb protein (Ko *et al.*, 2002)**.** Slimb is a component of the SCF (Skp1/ Cullin/ F-box protein) ubiquitin ligase complex, which marks proteins for proteosomal degradation in a phosphorylation-dependent manner (Deshaies, 1999). Fly mutants that have reduced levels of Slimb accumulate hyperphosphorylated forms of TIM and PER proteins suggesting that the stability of both proteins is Slimb-dependent (Grima *et al.*, 2002). Overexpression of Slimb leads mostly to loss of locomotor activity rhythms with a few animals displaying long period rhythmicity (Grima *et al.*, 2002; Ko *et al.*, 2002). Reduction of Slimb levels also lengthens behavioral rhythms (Grima *et al.*, 2002). Long period rhythms in both overexpression and underexpression conditions have been explained by a hypothesized Slimb function both in cytoplasm and in the nucleus (Grima *et al.*, 2002). Enhanced PER degradation in the cytoplasm would delay nuclear translocation of PER and thus delay the overall cycle. Reduction of Slimb function may delay the degradation of nuclear PER, which also would delay the start of the next cycle. The short and long period length alleles of DBT enhance or attenuate, respectively, PER degradation in the nucleus further demonstrating the importance of timely PER degradation as a critical determinant in establishing 24-hr rhythmicity. In addition to protein degradation, DBT appears to affect also the timing of nuclear translocation of PER. A short period mutant dbt^{S} shows delayed PER nuclear translocation that is apparently independent of PER protein stability (Bao *et al.*, 2001). The mechanism by which DBT affects the nuclear entry of PER is currently unknown. In cultured cells reduction of DBT activity by RNAi has been demonstrated to reduce the transcriptional repressor activity of PER (Nawathean and Rosbash, 2004). DBT appears to affect different regulatory aspects of PER function in multiple steps throughout the circadian cycle.

Another protein kinase required in the clock mechanism, CK2, was identified by mutations in the catalytic subunit (*timekeeper*) and in the regulatory β-subunit (*andante*) (Akten *et al.*, 2003; Lin *et al.*, 2002). Both mutations of CK2 lead to long locomotor activity rhythms. At the molecular level the protein levels of PER and TIM are elevated in the mutants. In cultured

Drosophila S2 cells CK2 has been shown to cooperate with DBT positively regulating the transcriptional repressor activity of PER (Nawathean and Rosbash, 2004). Reduction of CK2 activity in the mutant animals may elevate PER and TIM levels due to inefficient repression of CLK dependent transcription. Additionally, the nuclear translocation time of PER and TIM is delayed in CK1 mutants. The expression and activity levels of CK2 itself are constant over the circadian cycle.

An overexpression screen of transposon-tagged *Drosophila* genes revealed a role for *shaggy* (*sgg*), a developmentally required protein kinase and a fly homolog of GSK-3, in the circadian clock. Like *dbt* and CK2, the mRNA and protein levels of *sgg* do not cycle. Raising or lowering the levels of SGG leads to shortening or lengthening, respectively, of the period length of locomotor activity rhythms (Martinek *et al.*, 2001). Overexpression of SGG is correlated with a faster rate of nuclear translocation of the PER-TIM complex and hyperphosphorylation of TIM *in vivo* (Martinek *et al.*, 2001). Thus, at least one of SGG functions in the clock is to control the rate of PER nuclear entry. *In vitro* SGG phosphorylates TIM. Direct phosphorylation may serve as a signal for nuclear translocation. Additionally, hyperphosphorylated forms of TIM that are found in SGG overexpressing flies, are specifically light sensitive (Martinek *et al.*, 2001) suggesting a possible role for SGG in light resetting as well.

Protein dephosphorylation also plays a critical role in *Drosophila* clock. *twins* (*tws*) and *widerborst* (*wdb*), two regulatory subunits of protein phosphatase 2A (PP2A), are transcriptionally under circadian clock control. Reduction of expression levels of either gene in cultured cells leads to destabilization of transfected PER (Sathyanarayanan *et al.*, 2004). Over-expression of *wdb* lengthens the period of locomotor activity rhythms *of* mutant animals whereas overexpression of *tws* leads to shortened rhythms that degrade into arrhythmia. PP2A activity appears to be required also for appropriately timed nuclear translocation of PER. In a strong *tws* hypomorphic mutant, PER nuclear translocation is delayed compared to wild type in addition to low protein levels. Altering expression levels of *mutagenic star* (*mts*), the catalytic subunit of PP2A, has effects similar to manipulations of *tws* levels. Overexpression of *mts* shortens while decrease of activity by overexpression of a dominant negative *mts* lengthens the period of behavioral rhythms. PER protein in *mts* overexpressors can be detected exclusively in the nucleus. Similar to *tws* hypomorphic allele, overexpression of dominant negative *mts* leads to very low levels of PER.

5.2. Molecular basis of mammalian circadian clock

Overall, the mammalian circadian oscillator appears very similar to that of the *Drosophila*. Orthologs of most fly clock proteins have been found in the mammalian pacemaker. The current list of mammalian clock genes with their respective function is shown in Table 1. Like in *Neurospora* and fly clocks, the mammalian clock also displays interlocked positive and negative transcriptional feedback loops (Ueda *et al.*, 2005). A model showing the transcriptional feedback loops in the mammalian molecular clock is shown in Figure 4. Two PAS domain containing basic helix-loop-helix DNA binding proteins Clock and BMAL1 function to rhythmically drive clock gene transcription (Bunger *et al.*, 2000; Gekakis *et al.*, 1998; King *et al.*, 1997). The *clock* gene was identified in a forward genetic screen looking for mutations with altered wheel running activity rhythms. The *clock* gene mutation causes long period rhythmicity in heterozygous and loss of rhythms in homozygotes mice (Vitaterna *et al.*, 1994). The mutant *clock* gene has a premature stop codon that eliminates the C-terminal transcriptional activation domain resulting in a dominant negative form of the protein (King *et al.*, 1997; King *et al.*, 1997). The *clock* gene product dimerizes with BMAL1 that binds specifically to E-box element to activate gene expression (Gekakis *et al.*, 1998; Hogenesch *et al.*, 1998). Unlike its ortholog in flies, Clock protein in mammals is produced constitutively (Maywood *et al.*, 2003). In contrast, BMAL1 mRNA and protein cycle in their abundance. In the SCN neurons, the peak levels of *Bmal1* mRNA are expressed in the middle of the night (Maywood *et al.*, 2003). The maximum in protein levels of BMAL1 is delayed to early morning hours in the pacemaker cells. In peripheral organs the mRNA and protein levels are delayed by approximately six hours relative to the SCN.

The Clock-BMAL1 heterodimers activate rhythmic transcription of three *Period* genes (*Per1*, *Per2* and *Per3*), homologs of insect PER, and two *Cryptochrome* genes (*Cry1* and *Cry2*), members of the blue-light photoreceptor/photolyase family (Jin *et al.*, 1999; Kume *et al.*, 1999; Maywood *et al.*, 2003). Transcript levels of all *per and cry* genes peak in mid to late circadian day, antiphase to the *Bmal1* mRNA while *cry* transcripts are delayed by about 4-hr. The PER1 and PER2 proteins form heteromultimeric complexes with CRY1 and CRY2 that when translocated to the nucleus negatively feed back to the Clock-BMAL1 complex (Kume *et al.*, 1999). The inhibitory activity is carried out primarily by the CRY proteins while association between the PER and CRY proteins is required for their nuclear translocation (Griffin *et al.*, 1999; Kume *et al.*, 1999; Lee *et al.*, 2001). Single knockouts of either *Cry1* or *Cry2* shorten or lengthen, respectively, activity rhythms while their combined loss leads to loss of both behavioral and molecular circadian rhythms (van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). Loss of *Per1* function leads to subtle shortening of period length and some animals become arrhythmic in constant darkness (Bae *et al.*, 2001; Cermakian *et al.*, 2001; I). A more dramatic

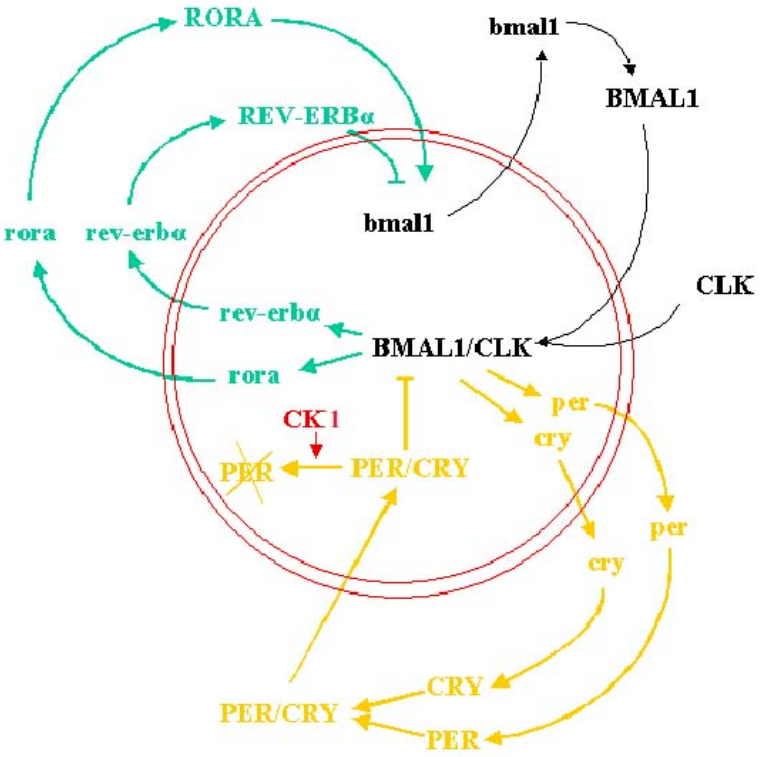

Figure 4. Model of mammalian clockwork. Positive and negative transcriptional feedback loops are color coded as in Figure 3. Two concentric red circles represent nuclear membrane. Lines ending with bars indicate negative regulation, arrows designate positive regulation. Protein names are in upper case, genes and mRNAs are written in lower case. Briefly, in the negative feedback loop, CLK/BMAL1 heterodimers activate the transcription of *cry* and *per*. PER and CRY proteins are produced in the cytoplasm and after heteromultimerization translocate to the nucleus during the night. In the nucleus PER and CRY protein complexes interact with CLK/BMAL1 heterodimers on clock-controlled promoters and inhibit gene activation causing a decline in *per* and *cry* mRNA levels. At the same time cycling *bmal1* expression is regulated by a positive feedback loop that involves rhythmically expressed REV-ERB/ROR family of transcription factors. REV-ERBα (likely to function redundantly with other REV-ERB family proteins) promotes repression of *bmal1* transcription while RORa activates (probably redundantly with other ROR family proteins) *bmal1* transcription. By midday PER proteins have been targeted for degradation by phosphorylation-dependent ubiquitination. Repressive protein complexes at circadian promoters are disassembled and new cycle of transcription can start. Unlike in flies, CLK mRNA and protein in mammals are constitutively expressed. Most clock proteins in mammals are hyperphosphorylated during the night in the nucleus. CK1 phosphorylates PER and also CRY proteins when in multimeric complexes.
Abbreviations: per–*period* (represents in this model both *per1* and *per2* genes), cry–*cryptochrome* (represents here both *cry1* and *cry2* genes), clk–*clock*, bmal1–*brain and muscle ARNT1 like*, rora-*retinoic acid-related orphan receptor a*, CK1–casein kinase 1ε and δ isoforms.

phenotype is apparent in *Per2* mutant mice. Animals lacking functional *Per2* have locomotor rhythms shortened by about 2-hr that eventually degrade to arrhythmicity in constant conditions (Bae *et al.*, 2001; Zheng *et al.*, 1999). *Per1* and *Per2* double mutant mice have no locomotor activity rhythms that are accompanied by a lack of rhythmic clock directed gene expression (I). *Per3* is not required for core clock mechanism as its function can be eliminated in either *Per1* or *Per2* knockout background without noticeable additional effects (Bae *et al.*, 2001). Interestingly *Per1* and *Per2* have differential effects on the expression of clock output genes suggesting there may be diversity in the protein complexes that bind circadian promoters during the negative feedback in the nucleus (I). The positive action of Clock-BMAL1 and negative feedback from PER-CRY are connected by nuclear receptor family of proteins Rev-Erbα and RORa that are required for cycling *Bmal1* transcription (Preitner *et al.*, 2002; Sato *et al.*, 2004). *Rev-Erb*α gene is activated by CLK-BMAL1 heterodimers that activate also *Per* and *Cry* genes (Preitner *et al.*, 2002). *Rev-Erb*α mRNA and protein are expressed in the same phase in the middle of the circadian day. Rise of *Rev-Erb*α causes *Bmal1* transcription to decrease to trough levels at the end of the day (Preitner *et al.*, 2002). REV-ERBα binds to retinoic acid-related orphan receptor response elements in the *Bmal1* promoter and inhibits transcription. In *Rev-erb*α deficient animals *Bmal1* mRNA cycling is dampened and not repressed during the day (Preitner *et al.*, 2002). *Rora*, a transcriptional activator of the REV-ERB/ROR family participates in the activation of the *Bmal1* gene in the SCN (Sato *et al.*, 2004). *In vitro* and in luciferase reporter assay REV-ERBα and RORa compete for the same binding sites in the *Bmal1* promoter (Sato *et al.*, 2004). *Rora* peak levels in the SCN are delayed compared to *RevErb*α suggesting successive repression and activation by REV-ERBα and RORa respectively (Sato *et al.*, 2004; Ueda *et al.*, 2002; Ueda *et al.*, 2004). Both the *RevErb*α and *Rora* deficient mice have a subtle 20–30 min period shortening in activity rhythms (Preitner *et al.*, 2002; Sato *et al.*, 2004). These surprisingly similar phenotypes have been speculated to be a result of reduced amplitude of *Bmal1* rhythms (Sato *et al.*, 2004). The weak behavioral phenotype suggests that other factors contribute to *Bmal1* expression. In fact other members of the REV-ERB/ROR family that all can bind to the same DNA binding sites are also under clock control (Preitner *et al.*, 2002; Ueda *et al.*, 2002). *RevErb*α cycles in the same phase in the periphery and in the SCN. *Rorb* knockout mice display behavioral rhythms with lengthened period (Andre *et al.*, 1998). *Rorb* shows low amplitude rhythms in the SCN with a delayed phase similar to *Rora* but the third activator in this protein family, *Rorc* is absent in the SCN (Ueda *et al.*, 2002). In the peripheral clock, in the liver *Rorc* cycles while *Rora* is constitutively produced and *Rorb* is undetectable (Preitner *et al.*, 2002; Sato *et al.*, 2004; Ueda *et al.*, 2002). Different REV-ERB/ROR family members may act redundantly which could explain the weak behavioral phenotypes of the single knockouts.

Both Clock-BMAL1 and REV-ERB/ROR family proteins together control a subclass of clock-controlled genes exemplified by *Cry1*. *Cry1* promoter contains both E-boxes and REV-ERB/ROR binding sites (Etchegaray *et al.*, 2003). Maximal Clock-BMAL1 binding to E-boxes in the *Cry1* promoter occurs surprisingly in the middle of the day while peak transcription takes place in early night. Rhythmic REV-ERBα binding to *Cry1* promoter, which extends the activation of transcription, explains the apparent discrepancy in activator binding and actual peak activation of *Cry1* promoter (Etchegaray *et al.*, 2003). In RevErbα knockout mice *Cry1* mRNA peaks predictably prematurely and in *Clock* mutants mice shows reduced *Cry1* levels (Kume *et al.*, 1999; Preitner *et al.*, 2002). Expression of *Per* genes is unaltered in *RevErbα* deficient mice but also reduced in the *Clock* mutant (Jin *et al.*, 1999; Preitner *et al.*, 2002). Such combinatorial control produces the delayed phase of *Cry1* expression compared to the *Per* genes that are also activated by Clock-BMAL1 heterodimers.

Clock-dependent transcriptional activation is accompanied by chromatin modification in mammals. Histone H3 acetylation is cycling on Clock responsive promoters in synchrony with RNA polymerase activity on these promoters (Etchegaray *et al.*, 2003). Clock protein associates specifically with a histone acetyltransferase p300 in a circadian fashion with peak levels of interaction in the middle of the day. Rhythmic chromatin acetylation is accompanied also by cycling histone H3 phosphorylation in *Per1* and *Per2* promoters (Etchegaray *et al.*, 2003).

Many of the transcription factors involved in circadian feedback loops are themselves phosphorylated with a circadian rhythm. Protein phosphorylation serves several functions including regulation of nuclear translocation, protein stability and activity. Two casein kinase one isoforms, CK1ε and CK1δ have been placed in the clock mechanism by genetic and biochemical evidences. A point mutation in CK1ε was identified in the Syrian hamster mutant *tau* (Lowrey *et al.*, 2000). Homozygote *tau* animals have a 20-hr locomotor activity period and have altered response to entraining light treatment (Ralph and Menaker, 1988). The mutated kinase in these animals has reduced activity *in vitro* but it remains unclear how this contributes mechanistically to the observed phenotype. The phosphorylation pattern of PER and Clock proteins is similar in the mutant and normal hamsters. In the mutant hamster, the molecular cycle is compressed to 20-hr period without the 4-hr asymmetry in protein accumulation that could account for the period change (Lee *et al.*, 2001). CK1ε and CK1δ have also been shown to directly associate with clock protein complexes *in vitro* and *in vivo* via direct associations with PER proteins (Camacho *et al.*, 2001; Keesler *et al.*, 2000; Lee *et al.*, 2001; Takano *et al.*, 2004; Takano *et al.*, 2004; Vielhaber *et al.*, 2000).

It is unclear how the behavioral phenotypes described above are generated by defective clock protein phosphorylation. However, several regulatory processes have been identified, primarily using cultured cells that help to define

the roles of phosphorylation in circadian clock. Nuclear entry or exit of PER proteins, apparently depending on the cell line used, can be forced by overexpression of CK1ε (Takano *et al.*, 2004; Takano *et al.*, 2000; Vielhaber *et al.*, 2000). Mammalian clock proteins are phosphorylated predominantly during the night in the nucleus (Lee *et al.*, 2001; Takano *et al.*, 2004; Takano *et al.*, 2000; Vielhaber *et al.*, 2000). PER proteins are highly phosphorylated at late night when bound to heteromultimeric complexes with Clock-BMAL1 dimers at circadian promoters and repressing transcription (Lee *et al.*, 2001). Clock and BMAL1 are also phosphorylated primarily when in the nucleus (Lee *et al.*, 2001). CK1 dependent phosphorylation of PER proteins leads to destabilization and degradation (Akashi *et al.*, 2002; Keesler *et al.*, 2000). Phosphorylated PER proteins are ubiqutinated and targeted to the proteosome (Akashi *et al.*, 2002; Yagita *et al.*, 2002). Protein-protein interactions regulate the phosphorylation of clock proteins. For example CK1ε phosphorylates CRY and PER proteins only once they form heterotrimeric complexes (Eide *et al.*, 2002). Thus one of the benefits of large clock protein complexes at night on circadian promoters may be formation of scaffolds that bring together substrates and kinases. Reporter assays suggest that BMAL1 dependent transcription may also be modulated by phosphorylation. In cultured cells repressing CK1ε or CK1δ activity reduces BMAL1 dependent transactivation of *Per1* promoter (Eide *et al.*, 2002). BMAL1 can be phosphorylated *in vitro* by mitogen-activated kinase (MAPK) (Sanada *et al.*, 2002). In cell culture MAPK activity moderately inhibits avian BMAL1 activity (Sanada *et al.*, 2002). Specific roles for protein phosphorylation have not yet been demonstrated in the mammalian clock, however, given the conservation between different clock model systems this mechanism is likely preserved.

Table 1. List of current mammalian clock genes. Protein motifs, promoter DNA regulatory elements, functions, mutation phenotypes, RNA and protein expression peak in the SCN and peripherial tissues has been shown.
Abbreviations: E-box: CACGTG, E'-box: CACGTT, RRE: Reverb/ROR binding element, D-box: DBP/E4BP4 binding element, ND: not done.

Gene	Characteristic Protein Motifs	Promoter Regulatory Elements	Function	Mutation Phenotype	Peak CT Expression (RNA/Protein)	
					SCN	Periphery
Clock	bHLH-PAS	RRE	transcription factor	4.0-hr longer period in LD arrhythmic in DD	not cycling	21-03/not cycling
Bmal1 (Mop3/Arnt1)	bHLH-PAS	RRE	transcription factor	arrhythmic in DD	15-18/0-8	21-03/15-03
Per1	PAS domain	E-box, D-box	PER/CRY interaction, CLOCK/BMAL1 inhibitor	1.1-hr shorter period 20% arrhythmic in DD	4-6/10-14	10/15-18
Per2	PAS domain	E'-box, D-box	PER/CRY interaction, CLOCK:BMAL1 inhibitor, NPAS2:BMAL1 enhancer	1.5-hr shorter period arrhythmic in DD	6-12/10-14	15/18
Per3	PAS domain	D-box	PER/CRY interaction	0-0.5-hr shorter period	4-9/10	12-15/ND
Cry1	Flavoprotein	E-box /E'-box; RRE	Interaction with PERs, CLOCK:BMAL1 inhibitor	1.0-hr shorter period	8-12/12-18	14-17/21-24
Cry2	Flavoprotein	ND	Interaction with PERs, CLOCK:BMAL1 inhibitor	1.0-hr longer period	8-16/12-18	8-10/15-21
Rev-erbα (Nr1d1)	Orphan nuclear receptor	E-box, D-box	BMAL1 inhibitor, links negative and positive feedback loops	0.4-hr shorter period	2-6/ND	2-6/6-8

Gene	Characteristic Protein Motifs	Promoter Regulatory Elements	Function	Mutation Phenotype	Peak CT Expression (RNA/Protein) SCN	Periphery
CK1ε (Csnk1ε)	Casein kinase	ND	Phosphorylation of PERs, CRYs, BMAL1	4.0-hr shorter period (tau mutant hamster)	not cycling	not cycling
Timeless	PER interaction	ND	dimerize with PERs	embryonic lethal	not cycling/12	ND
Npas2 (Mop4)	bHLH-PAS	RRE	Transcription factor, CLOCK paralog	0.2-hr shorter period	22-2/ND	0-4/ND
Rora	Orphan nuclear receptor	D-box	BMAL1 activator links negative and positive feedback loops	0.4-hr shorter period, some arrhythmic in DD	4-8/not cycling	
Dec1 (Bhlhb2)	bHLH	E-box /E'-box	BMAL1 inhibitor	ND	0-4	
Dec2 (Bhlhb3)	bHLH	E-box /E'-box	BMAL1 inhibitor	ND	2-6	

6. Entraining input to circadian oscillators

It is postulated that input signals shift the state of the clockwork by increasing or decreasing the amount of a single clock component (Crosthwaite *et al.*, 1995; Myers *et al.*, 1996). In most organisms light is the strongest clock-resetting agent. There are two components of light input to the clock; photoreception and the signaling cascade that results in resetting the oscillator. The mechanism of light dependent phase resetting varies between different clock models. In mammals the circadian pacemaker tissue in the SCN is not directly accessible to light. SCN light responses are mediated by retinal ganglion cells expressing photoreceptor melanopsin and the conventional visual photoreceptor cells that innervate the SCN via the retinohypothalamic tract. Previously, rods and cones were considered the only photoreceptors of the eye. However, melanopsin is a putative opsin-family photopigment, signaling through the G proteins.

How is the light information transmitted to the SCN? Light pulses activate calcium dependent signaling cascades that ultimately lead to the phosphorylation of transcriptional activators like CREB, which activates genes with Ca/cAMP response elements. Among these genes are clock genes *Per1* and *Per2* that mediate phase resetting (Travnickova-Bendova *et al.*, 2002). Also nontranscriptional mechanisms are likely to contribute to resetting. Recently, genetic deletion of Ras like G protein DEXRAS1 reduces photic entrainment by eliminating a pertussis-sensitive circadian response to NMDA. Mechanistically, DEXRAS1 couples NMDA and light input to $G_{i/0}$ and ERK activation (Cheng *et al.*, 2004).

Three anatomical pathways have been postulated that mediate light-dependent resetting of the *Drosophila* clock. These include poorly understood retinal and extra-retinal pathways that mediate the phase resetting of *Drosophila* pacemaker by light. The entrainment pathway of *Drosophila* is a cell autonomous cryptochrome (CRY) photopigment dependent degradation of TIM. As described in the phase response curve, short light pulses in the early night delay the phase of behavioral rhythms whereas advances are produced during late night. Thee behavioral responses can be explained with the subcellular localization of TIM at the time of degradation. Considering TIM as a transporter of PER to the nucleus, degradation of TIM in the accumulation phase would delay the nuclear translocation of PER and thus the transcriptional repression phase of the cycle. A transition from delays to advances occurs at the time of TIM and PER nuclear translocation in the middle of the night. This can be explained by the release of PER from the complex with TIM upon light exposure which has been demonstrated to enhance the repressor activity of PER and thus advancing the end of the molecular cycle (Kloss *et al.*, 2001; Myers *et al.*, 1996; Rothenfluh *et al.*, 2000).

CRY binds a pterin (a folate derivative) chromophore which acts as the blue light sensor and a flavin molecule that is required for redox process dependent

conformational change upon light exposure. A conformationally active form of CRY binds TIM and targets it for ubiquitination (Lin *et al.*, 2001; Naidoo *et al.*, 1999). An unknown protein kinase is also involved in light induced TIM degradation as it is phosphorylated at tyrosine residue(s) prior to degradation (Naidoo *et al.*, 1999). Light activation causes CRY itself to be degraded as well (Lin *et al.*, 2001). CRY mRNA and protein levels are under circadian control cycling in the phase of CLK with maximum levels just after dawn (Emery *et al.*, 1998).

In the absence of functional CRY circadian rhythms can still be entrained with lower efficiency indicating alternative pathways for photoentrainment. Extra-ocular photoreception from anatomically diverse sources appears to contribute to the photoentrainment of *Drosophila* clock. Genetically ablating the function of conventional photoreceptors with a phospholipase C mutant $norpA^{P41}$ causes diminished but persistent responsiveness to the entraining light indicating extra-ocular photoreception. Flies with a combination of *cry* and *norpA* loss of function mutations retain reduced entrainability suggesting photopigments additional to CRY are involved in the entrainment of rhythms (Stanewsky *et al.*, 1998). Phase shifting of circadian locomotor activity by light exposure is lost with elimination of both *cry* function and the genetic removal of external photoreceptor cells and a structure known as the Hofbauer-Buchner eyelet located underneath the compound eye that makes neuronal contacts with the pacemaker cells in the brain (Helfrich-Forster *et al.*, 2001).

In the *Neurospora crassa* clock light exposure leads to WCC (White Collar complex, a complex of White Collar-1 and White Collar-2 proteins) dependent activation of *frq (*frequency) gene transcription (Crosthwaite *et al.*, 1995). Transcriptional activation by WCC is mediated by two light-response elements (LRE) in the *frq* promoter (Froehlich *et al.*, 2002; Froehlich *et al.*, 2003). DNA bound WCC undergoes a multimerization step upon light exposure that promotes its activity. The FAD (flavin adenine dinucleotide) bound WC-1 protein in WCC functions as a blue light receptor (Froehlich *et al.*, 2002; He *et al.*, 2002).

7. Circadian clock-controlled genes expression and their feedback on the core clock mechanism

In the past several years, transcriptional profiling using DNA microarray has been extensively applied to study circadian clock dependent gene expression. Genome-wide expression analysis described in more than a dozen studies from *Arabidopsis*, *Drosophila*, *Neurospora* and mammals has shown that hundreds of genes covering a wide variety of functions from basic metabolism to synaptogenesis are under the control of the circadian oscillators (see the

microarray details in Table 2). In mouse liver for example glycolysis, glyconeogenesis, fatty acid metabolism and cholesterol metabolism are all under circadian clock control (Panda *et al.*, 2002).

Charactization of the circadian output genes in the SCN revealed that its principle role in neuropeptide release is recapitulated by the transcriptional output there. One of the largest group of cycling transcripts in the SCN contains genes involved in peptide synthesis, processing and release (Panda *et al.*, 2002). These transcripts contain several neuropeptides — pro-opiomelanocortin, cholecystokinin, platelet-derived growth factor (PDGF), somatostatin and many others.

Interestingly circadian control of gene expression is highly tissue specific. In any tissue approximately 10% of the transcripts are under circadian control. Overlap between two sets of circadian mRNAs between two tissues is only about 10%. About half of the remaining transcripts are present in both tissues but they are rhythmically expressed only in one but not the other (Storch *et al.*, 2002). Given that the circadian control of gene activity is tissue specific and each tissue has a mostly separate set of genes under clock control then most of the genome may be under circadian regulation when the whole organism is considered.

Tissue specific regulation in flies and mammals implies that the clock has enlisted the use of the tissue–specific transcriptional machinery to regulate its gene expression (Ceriani *et al.*, 2002; Duffield, 2003; Panda *et al.*, 2002; Storch *et al.*, 2002; Ueda *et al.*, 2002). Clock complex initiates rhythmic transcription of tissue specific transcription factors, which in turn activate their specific target genes to control tissue and phase specific expression.

Core-clock complex itself regulates relatively few transcriptional output genes directly (Panda *et al.*, 2002). Many mRNAs encoding of rate-limiting enzymes are transcribed with a circadian manner. Furthermore within circadianly controlled pathways, genes are transcribed in similar phases allowing the components of the pathway to be expressed at the same time (Panda *et al.*, 2002). Cyclically expressed genes participate in many metabolic pathways (Ceriani *et al.*, 2002; Panda *et al.*, 2002; I, II). In liver and other tissues for example HMG–CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase determines the rate of cholesterol synthesis; δ-aminolevulinate synthase is the rate-limiting enzyme in haem biosynthesis pathway and glycerol kinase is the rate limiting factor in the regulation of the use and uptake of glycerol.

Transcriptional control appears to be the dominant mechanism for controlling clock output genes. There are two ways to control output pathways: 1. Genes directly controlled by the transcription factors in the core oscillator mechanism (Jin *et al.*, 1999). 2. Genes indirectly controlled by the circadian clock. Antagonistic regulation of proline and acidic amino acid-rich basic leucine zipper (PAR bZIP) transcription factor family proteins and E4BP4. DBP (albumin D-site-binding protein), HLF (hepatic leukemia factor) and TEF

(thyrotroph embryonic factor) are the three members of the PAR bZip protein family in mammals. The E4BP4 and PAR proteins are paired components of a reciprocating mechanism wherein E4BP4 suppresses the transcription of target genes during the time of the day when E4BP4 is abundant, and the PAR proteins activate same promoters different time of the day. E4BP4 and the PAR proteins may switch back and forth between the on-off conditions of the target genes (Mitsui *et al.*, 2001).

However, for construction of a systems level description of the output of the clock more work is needed to study the profiling of transcription to the rest of the genomes, tissues, organs and systems.

Table 2. Details of circadian microarray studies. Indicated are organism, tissue, number of genes (#) and array type that were used. Other methods used for chip data verification are indicated in the last column. Modified from (Duffield, 2003).
Abbrevations: cDNA, custom made cDNA microarray; Affymetrics, Affymetrics oligonucleotide chip; qrtRT-PCR, quantitative real-time RT-PCR; qRT-PCR, quantitative RT-PCR; ADDER-Amplification of Double-stranded cDNA End Restriction fragments.

Reference	**Orga-nism**	**Tissue**	**# of genes**	**Array type**	**Validation type**
(I)	mouse	liver	6000	cDNA	Northern
(Panda *et al.*, 2002)	mouse	liver, SCN	10000	Affymetrics	ISH, qrtRT-PCR
(Storch *et al.*, 2002)	mouse	heart, liver	12488	Affymetrics	
(Ueda *et al.*, 2002)	mouse	liver, SCN	12488	Affymetrics	ISH, qrt RT-PCR
(Akhtar *et al.*, 2002)	mouse	liver, hypothalamus	2122	cDNA	ISH
(Duffield *et al.*, 2002)	cells	rat1 fibroblasts	2124	cDNA	qrt RT-PCR
(Grundschober *et al.*, 2001)	cells	rat1 fibroblasts	9957	Affymetrics	qrt RT-PCR
(Hirota *et al.*, 2002)	cells	rat1 fibroblasts	8000	Affymetrics	qrt RT-PCR
(Kita *et al.*, 2002)	rat	liver, kidney	8448	cDNA	qrt RT-PCR Northern
(Humphries *et al.*, 2002)	rat	pineal gland	1176	cDNA	Northern, ISH, Western
(Ueda *et al.*, 2002)	fly	head	13500	Affymetrics	qrt RT-PCR
(McDonald and Rosbash, 2001)	fly	head	13500	Affymetrics	ADDER
(Claridge-Chang *et al.*, 2001)	fly	head	13500	Affymetrics	Northern, ISH
(Lin *et al.*, 2002)	fly	head	13500	Affymetrics	qRT-PCR
(Ceriani *et al.*, 2002)	fly	head, body	13500	Affymetrics	Northern
(Harmer *et al.*, 2000)	plant	entire organism	8200	Affymetrics	
(Schaffer *et al.*, 2001)	plant	entire organism	7800	cDNA	
(Nowrousian *et al.*, 2003)	fungus	entire organism	1000	cDNA	Northern

8. Overview of haem biosynthesis pathway

Haem as a complex of iron and protoporphyrin IX serves as a prosthetic moiety of numerous hemoproteins that are indispensable for all aerobic life. Hemoproteins are crucial in oxygen binding (hemoglobin and myoglobin), in oxygen metabolism (oxidases, peroxydases, catalases), electron transfer (cytochromes). Haem is the prosthetic group of numerous hemoproteins that synthesize regulatory or signaling molecules including cyclic GMP (guanulate cyclase), steroid hormones (hydroxylases) and nitric oxide (nitric oxide synthase) (Lucas *et al.*, 2000; Ponka, 1999; Stuehr, 1997). Haem controls the expression of many proteins like globin, haem biosynthesis enzymes, cytochromes and haem oxygenases.

Intracellular free haem levels are extremely low because free haem is cytotoxic, therefore haem biosynthesis must be tightly regulated. Haem biosynthesis involves eight enzymes, four of which are cytoplasmic and four are mitochondrial (Fig. 5). The first and rate-limiting step occurs in the mitochondria, where Aminolevulinate synthase (ALAS) catalyses the formation of 5-aminolevulinic acid (ALA) from succinyl coenzyme A (CoA) and glycine. Genes for 5-aminolevulinic acid synthase are responsible for the differences in regulation and rates of haem synthesis in erythroid and nonerythroid cells (Bishop *et al.*, 1990). There are two *aminolevulinate synthase* genes — *Alas1*, which is expressed ubiquitously, and *Alas2*, which is specific to erythroid cells. The ALAS1 enzyme is designated in the literature as ALAS-N (N-nonspecific) and ALAS-H (H-houskeeping). ALAS1 is synthesized in the cytosol as a precursor protein with an N-terminal signal sequence that targets the protein to the mitochondria. Upon entry into the mitochodria, of the precursor fragment is removed to generate the mature protein. The next four-biosynthesis steps take place in the cytosol. It is not known why there is compartmentalization of the enzymes between the mitochondria and cytosol. ALA dehydratase (ALA-D) converts 2 molecules of ALA to a monopyrrole porphobilinogen (PBG). Two subsequent enzymatic steps convert 4 molecules of PBG into the cyclic tetrapyrrole uroporphyrinogen III, which is then decarboxylated to form coproporphyrinogen III. The final 3 steps including the insertion of the ferrous iron into protoporphyrin IX by ferrochelatase occur in the mitochondria. When iron is inserted into the protoporphyrin ring, the iron protoporphyrin or haem is formed in both animals and plants. Magnesium is inserted into the protoporphyrin ring in photosynthetic organisms and as a result Mg-protoporphyrin is formed which is converted in a series of steps to chlorophyll. The pathway to vitamin B12 branches from the pathway to haem at the stage of uroporphyrinogen III (Warren and Scott, 1990). When cobalt is inserted into the protoporphyrin ring metalloporphyrin or vitamin B12 is formed. Vitamin B12 is synthesized primarily in certain bacteria but not in eukaryotes.

The level of cellular haem is tightly controlled, and this is thought to be achieved by a balance between haem synthesis and catabolism, the latter by haem-oxygenases. Two models have been proposed for the regulation of haem biosynthesis. The first model is based on a negative feedback of haem on *Alas*

mRNA stability and on a posttranslational transport control (Hamilton *et al.*, 1991; Kikuchi and Hayashi, 1981). A second model is based on a negative feedback of haem on the transcriptional control of *Alas* expression (Kappas *et al.*, 1968). The observations in this thesis work suggests a model based on a transcriptional control of *Alas1* expression by the circadian clock as a plausible mechanism for regulating levels of ALAS activity, and thereby haem levels, in mice liver (I, Fig. 8; II, Supplementary Fig. 5).

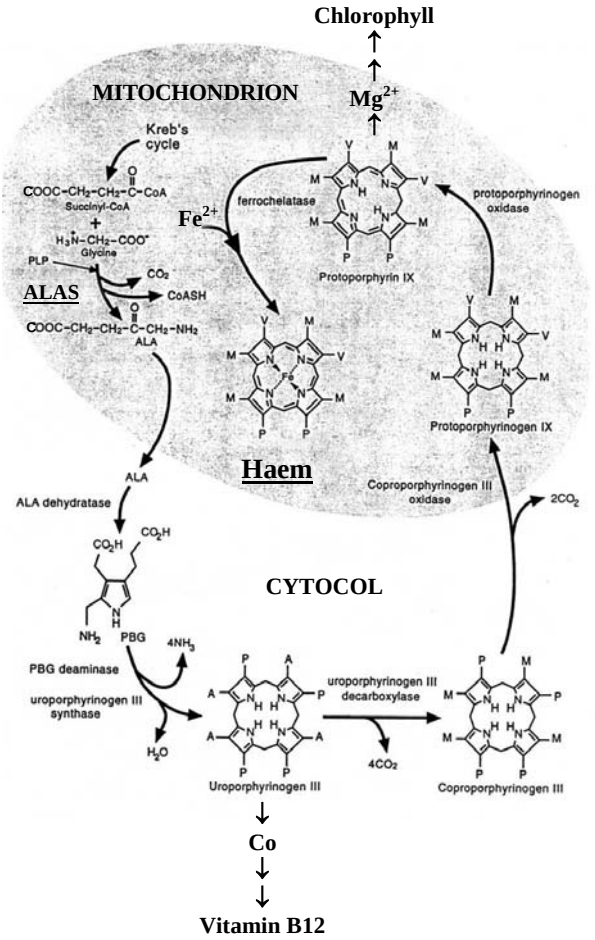

Figure 5. The Pathway of porphyrins biosynthesis. Haem biosynthesis involves eight enzymes, 4 of which are cytoplasmic and 4 are mitochondrial. The rate limiting enzyme and final product of the haem biosynthesis are underlined. Chlorophyll in photosynthetic organisms and vitamin B12 or cobalamin in certain bacteria are produced from the same biosynthetic chain. Metals: Co (cobalt), Fe (iron), Mg (magnesium). Modified from (Ponka, 1999).

8.1. Signal transduction by haem-PAS domain proteins

PAS domain has been identified in over 1100 proteins from bacteria to human (Taylor and Zhulin, 1999). PAS is an acronym from the names of proteins in which the imperfect repeat sequences were initially recognized: the *Drosophila* period clock protein (PER), the vertebrate aryl1 hydrocarbon receptor nuclear translocator (ARNT), and the *Drosphila* single-minded protein (SIM). PAS motifs have highly conserved three-dimensional folds even though the amino acid sequence identity is low. The conserved regions of PAS domains have only ~ 12% of amino acid sequence identity. The biological roles of PAS proteins are very diverse involving kinases, phosphodiesterases and transcription factors. The main role of the PAS domain proteins is sensing of variety of environmental signals such as voltage, xenobiotics, nitrogen availability (Swanson and Bradfield, 1993). Detection of signals such as gases, light and redox potential is known to require cofactors such as haem, FMN and FAD respectively (Christie *et al.*, 1999; Dioum *et al.*, 2002). A broad group of proteins with haem bound PAS domains has been described. For example bacterial PAS domains in AxPDEA1 (*Acetobacter xylinum* phosphodiesterase), DOS (direct oxygen sensor), and FIXL proteins, have been shown to bind haem (Chang *et al.*, 2001; Delgado-Nixon *et al.*, 2000; Gong *et al.*, 1998). Those were the first identified haem containing PAS proteins and their signaling function starts in the heam domain. FIXL is multidomain protein with separates domains employed for oxygen sensing (haem), signaling (catalytic) and membrane integration. Kurakowa *et al.* reported the crystal structures of the haem PAS domain of *E.coli* Dos protein in both inactive Fe^{3+} and active Fe^{2+} states (Kurokawa *et al.*, 2004).

Six PAS domain containing proteins belong to the core clock mechanism some of them contain only PAS domain and some of them are basic helix-loop-helix (bHLH)-PAS proteins that feature PAS domain coupled to a bHLH DNA binding motive (Gu *et al.*, 2000). Eukaryotic PAS domain starts immediately after bHLH domain and extends more than 300 residues. Eukaryotic PAS proteins contain mostly two consecutive PAS domain named as PAS-A and PAS-B. An association of haem to circadian regulation through the haem binding proteins in NPAS2 and PER2 has been described (Dioum *et al.*, 2002; II). The mouse haem-binding protein NPAS2 is highly homologous to Clock. Both NPAS2 and Clock can form heterodimers with BMAL1 protein to form an active transcription complex (Garcia *et al.*, 2000). mPER2 partcipates in accelerating the BMAL1-NPAS2 transcriptional activity. The physiological effects of the haem ligand on NPAS2 are not known, but it has been shown that the haem binding status couples directly to the DNA binding domain in this transcription factor. When NPAS2 is not bound to haem, CO does not affect formation of the productive NPAS2-BMAL1 heterodimers. When NPAS2 is bound to haem, CO concentration range consistent with saturation of the PAS-A domain inhibits formation of NPAS2-BMAL1 heterodimers (Dioum *et al.*, 2002).

Although the haem-binding domains in proteins are relatively well determined, the interaction between the haem-PAS domain and its regulatory targets are mainly unknown.

Recently, PAS domain crystal structure of dPER was solved, which shows a novel PAS domain fold and a ligand binding pocket mediated by inter and intramolecular PAS domain interaction (Yildiz *et al.*, 2005).

9. Medical implications of circadian rhythms

The transcriptional oscillations generated by the clock genes cause many behaviors and physiological processes to cycle with a 24-hr periodicity. Even subtle alterations in clock genes or clock-controlled genes can affect human health.

9.1. Sleep disorders

Several inherited disorders display abnormalities in sleep distribution throughout the 24-hr cycle with few cases suggesting direct involvement of mutations in clock genes being the leading cause. Advanced sleep phase syndrome (ASPS), delayed sleep phase syndrome (DSPS) and irregular-sleep-wake patterns are sleep disorders associated with circadian system dysfunction.

People affected by familial ASPS display approximately four hours advanced activity onset and offset (Jones *et al.*, 1999). Toh *et al*. showed that the familial form of ASPS is associated with a missense mutation that replaces a serine for a glycine in the human *Per2* gene. This point mutation in h*Per2* changes the first serine of five consecutively positioned serines that are aligned as five tandemly positioned CK1 phosphorylation sites. Normally phosphorylation of the first serine could prime CK1 for the downstream serines for processive phosphorylation but when mutated could slow down the phosphorylation of the 5 serines. Consistently with this hypothesis CK1ε phosphorylates the mutated sequence at a slower rate *in vitro* (Toh *et al.*, 2001). It is not clear how the mutation leads to advances in circadian behavior in the patients. The apparent similarities in the phenotypes of both the *tau* hamsters and FASPS patients may indicate similar mechanistic basis for faster clock in both conditions. Therefore, the mutation that affects the phosphoryation state of PER2 disrupts sleep-wake period by altering the circadian timing of sleep. Recently a point mutation changing threonine 44 to alanine (T44A) in the CK1δ protein was shown to cause FASPS in humans (Xu *et al.*, 2005). This mutation reduces CK1δ kinase activity. Short period locomotor activity rhythms are produced in transgenic mice with the engineered T44A mutation in CK1δ. The identification of FASPS mutations in both CK1δ and its substrate PER2 reinforces the hypothesis that reduced phosphorylation of PER2 shortens the

period of transcriptional cycle and leads to advanced onset of rest and wake activity.

The most common circadian sleep disorder is DSPS that has a phenotype opposite to ASPS. Individuals wake around three to six hours later than desired. Ebisawa *et al*. reported a polymorphism in *Per3* gene with a higher frequency in DSPS patients than in control individuals (Ebisawa *et al.*, 2001). The polymorphism is close to the putative Ck1ε phosphorylations site. Another study associated the same polymorphism with self–reported diurnal preference in a different group of patients (Johansson *et al.*, 2003). A recent study identified a length polymorphism in *Per3* gene that is associated both with DSPS and extreme diurnal preference (Archer *et al.*, 2003). Putative Ck1ε phosphorylations sites are also located in that repetitive region. The PER3 polymorphism that is correlated with extreme diurnal preference has two forms, the longer allele associated with morningness while the shorter allele is associated with eveningness. The shorter allele was strongly associated with the delayed sleep phase syndrome patients, 75% of who were homozygous.

People with irregular sleep disorders, including Smith-Magenis syndrome (SMS), display sleep patterns that are often heavily disrupted leading to reduced amount of rest.

Smith-Magenis syndrome and dup (17)(p11.2p11.2) are contiguous gene syndromes associated with a deletion or a duplication of band p11.2 of chromosome 17, respectively. First described in 1986 (Smith *et al.*, 1986), Smith-Magenis syndrome has a birth prevalence estimated at 1 in 25,000 (Greenberg *et al.*, 1991). The clinical phenotype has been well described and includes craniofacial abnormalities, brachydactyly, self-injurious behavior and mental retardation. Less commonly reported are cleft palate, congenital heart defects, seizures, hearing impairment, hypercholesterolemia and urinary tract anomalies (Greenberg *et al.*, 1996). Major behavioral and severe sleep disturbances occur in almost all cases. Specific sleep disturbances include difficulty in falling and staying asleep and frequent awakening during the night. Moreover the “sleep attach” at the end of the day may represent the endogenous sleep onset of the patients, therefore these patients are regarded as having a sleep phase advance. SMS patients display a phase shift of their circadian rhythm of melatonin, abnormally secreted during the day (De Leersnyder *et al.*, 2001). Normally melatonin secretion increases after the onset of darkness peaking at midnight and gradually decreases during the late night. The treatment of SMS patients with β1-adrenergic antagonists improves their sleep, by suppressing the abnormal rhythms of melatonin secretion. The therapeutic approach using beta-blockers in the morning and melatonin in the evening, reset circadian rhythm of melatonin improved behavior and restored sleep (De Leersnyder *et al.*, 2003). The mouse model of Smith-Magenis syndrome that had been created displayed a circadian phenotype (III). Molecular mechanism underlying the circadian phenotype in the mouse models may relate to the sleep disturbances observed in the SMS patients.

9.2. Metabolic disorders

Many metabolic events are temporally controlled with respect to the time of day. Discoveries that clock genes regulate circadian rhythms *in vitro* and in peripheral tissues indicate that the circadian and metabolic processes are linked at multiple levels (Balsalobre *et al.*, 1998; Yoo *et al.*, 2004). In mammals glucose and lipid homeostasis oscillate with a circadian rhythm. The risk of acute myocardial infarction is 40% higher in the morning, thrombosis and silent ischemia also happens more likely in the morning hours (Cohen *et al.*, 1997; Rudic *et al.*, 2005; Ueda *et al.*, 2002). Allergia responses are most likely occurrence with a peak in the late evening hours.

A metabolic crisis happens in tissues, when the circadian cycle of metabolic genes is lost, leading to circadian metabolic disorders. For example diabetics exhibit deviations from the normal circadian metabolic physiology. Patients with type I diabetes exhibit an exaggerated counter regulatory response to nocturnal hypoglycemia, resulting in early morning hyperglycemia (DeLawter, 1991). In another example, shift workers have increased incidence of cardio-vascular disease, which has been hypothesized to relate to an inversion of metabolic rhythms. Night shift workers in Antarctica exhibit higher level of postprandial glucose, insulin and triacylglycerol responses during the night compared to daytime workers. Meals at night cause abnormal metabolic responses, which could contribute to the documented cardiovascular morbidity associated with shift workers (Lund *et al.*, 2001).

McKnight's group linked cellular metabolism to circadian clock, showing that clock can be influenced by the redox states of NAD(H) or NADP(H) (Rutter *et al.*, 2001). NADP(H) stimulates the DNA binding of CLOCK and NPAS2, whereas NAD(P) inhibits this process (Rutter *et al.*, 2001). The balance of NAD(H) and NADP(H) cofactors in the cell depends on the intracellular concentrations of glucose, oxygen and lactate dehydrogenase (LDHA). This balance is itself subject to daily oscillations. LDHA is activated by NPAS2/BMAL1 heterodimers and influences cellular redox potential and thereby changes the activity of its activator (Rutter *et al.*, 2002).

Adapting metabolic assays to assess circadian variations has been used recently in several studies of clock mutants. Changes in feeding times shift the circadian pattern of gene expression in the liver but not in the SCN (Damiola *et al.*, 2000). Interestingly, when the feeding time is shifted, the *Npas2* mutant mice cannot adapt their feeding behavior to the change in feeding schedule, they eat less and loose 25% of their body weight (Dudley *et al.*, 2003). Diurnal variations in glucose and triglycerides are disrupted in *Bmal* $^{-/-}$ and *Clock* mutant mice (Rudic *et al.*, 2004). Clock mutant mice are obese, they develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia and hypoinsulinemia (Turek *et al.*, 2005).

Identification of local circadian clocks and local clock controlled genes will provide new ways to look of metabolic disorders. This emphasizes the

importance of local CCGs as direct or indirect mediators of circadian prevalence and allows local tissue specific therapy, independent from master and core clock mechanism. To restore circadian control of metabolic gene expression would be potential therapy of metabolic disorders.

PRESENT INVESTIGATIONS AND DISCUSSION

1. Aims of the present thesis

The work described in this thesis aimed to answer the following questions:

1. What is the role of *Period* 1 and *Period* 2 genes in the mammalian circadian clock? What is the mammalian *Period* 1 and *Period* 2 role in regulating clock dependent downstream gene expression?
2. What constitute the clock feed back mechanism to the central oscillator? What is the relationship between genes of haem biosynthesis pathway and circadian clock?
3. Which behavioral phenotypes are phenocopied in the mouse models of the Smith-Magenis syndrome? Do the mouse models of Smith-Magenis syndrome have a circadian phenotype similar to the human disorder?

2. Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock (Publication I)

Here, the phenotypic and molecular analysis of targeted mutations of *mPer1* and *mPer2* are described.

To investigate the role of *mPer1* in circadian clock function, a *mPer1* mutant mouse was generated by gene targeting (I, Fig. 1). The 4.3 kb region of *mPer1* gene was replaced with *Hprt* minigene. *mPer1* mutation was concluded as a null allele, because the remaining mPer1 N-terminal region does not contain any recognizable sequence motif. The *mPer1* mutants display a moderately shortened circadian locomotor activity period of about one hour compared to wild type littermates (I, Fig. 2). It was shown previously that *mPer2* mutation that affects the expression of central clock components leads to two hour shortening of behavioral rhythms (Zheng, *et al.*, 1999). Thus, the phenotype of the *mPer1* mutant is mild in contrast with that of the $mPer2^{m/m}$ (Zheng *et al.*, 1999) or the $mPer2^{-/-}$ (Fu *et al.*, 2002) which exhibits an gradual loss of circadian rhythmicity. The unique feature of the *mPer1* mutant is that the clock has reduced precision and stability. Loss of rhythmicity in constant darkness was occasionally observed in *mPer1* mutant. To further address their role in circadian clock control, $mPer1^{m/m}mPer2^{m/m}$ double mutants were created and assayed at the behavioral (I, Fig. 3) and molecular level (I, Fig. 7). The $mPer1^{m/m}mPer2^{m/m}$ double mutant mice displayed no circadian rhythms. This result implicates *mPer1*, along with *mPer2*, in the central clock mechanism. Loss of mPER1 did not disrupt the rhythmic expression of its mutant transcript, the mutant *mPer1* transcript oscillates with a higher peak and an apparent phase delay compared with the wild type controls, which may simply reflect an increased stability of the mutant transcript (I, Fig. 5a). Consistent with this

possibility is the observation that the level of the noncycling *mPer1* transcripts throughout the brain is elevated in the *mPer1* mutant (data not shown). None of the other clock genes, including *mPer2*, *mCry1*, and *mBmal1*, show any obvious change in expression levels in the absence of mPER1. These observations deviate from the expected role for mPER1 in the negative feedback that has been demonstrated for *dPer* in *Drosophila* and inferred from *in vitro* studies with mammalian cell cultures (Darlington *et al.*, 1998; Kume *et al.*, 1999; Sangoram *et al.*, 1998). Indeed, among the three mPERs, only mPER2 appears to play a key role in the transcriptional control of clock genes (Shearman *et al.* 2000; Zheng *et al.* 1999). Furthermore, the transcriptional control by mPER2 appears to be in the opposite direction to its *Drosophila* counterpart: loss of mPER2 function results in significantly reduced peak expression of *mPer1*, *mPer2*, and *mCry1* (Shearman *et al.*, 2000), indicating that mPER2 exerts a positive regulation on clock gene expression. Thus, the involvement of mammalian PERs in the clock appears to be mechanistically different from that of the *Drosophila Per*. These observations raise the question as to how mPER1 regulates the mammalian circadian clock. The studies of the peripheral clock suggest that mPER1 has a role in the clock at a posttranscriptional level. The loss of mPER1 results in an enhanced level of mPER2 protein in the mutant, suggesting that mPER1 normally represses mPER2 levels *in vivo*. As mPER1 and mPER2 have been shown to interact *in vitro* and *in vivo* (Field *et al.*, 2000; Kume *et al.*, 1999), this posttranscriptional regulation of mPER2 by mPER1 may be mediated through a direct protein-protein interaction. Whether posttranscriptional regulation by mPER1 mediates the circadian phenotype of the *mPer1* mutants remains to be determined.

Despite the high degree of sequence similarity (Albrecht *et al.*, 1997), the wheel-running activity experiments of *mPer1* and *mPer2* individual mutants showed different behavioral phenotypes suggesting at least partially non-overlapping functions. cDNA microarray technology was employed to investigate how clock-controlled gene expression was affected by the loss of mPER. A collection of 6000 cDNAs from UniGene cDNA clones from the *Mus musculus* Genome Project at Baylor College of Medicine were used. Comparative measurements of mRNA levels between mutant and wild type mice through a 24-hr period in constant darkness revealed several hundreds candidate genes (data not shown). Detailed analysis based on more rigorous parameters including duplicate difference of less than 10 percent variation led to 53 of the most highly oscillating genes as candidates for further analysis. Furthermore, these genes showed at least 2.5 fold expression difference in mutant versus wild type comparison. These candidate genes were then analyzed by Northern analysis of liver RNA over a circadian cycle in wild types and single and double $mPer1^{m/m}mPer2^{m/m}$ mutants. Only 16 clock-controlled transcripts out of 53 candidate genes were confirmed by Northern blot analysis. The amount of false positive transcripts from these studies shows that microarray studies must be verified by an independent method. Based on the

expression pattern from the Northern blot analysis, the CCGs were divided into four groups (Table 3). I, Fig. 7 shows differential expression of four representative clock-controlled genes in *mPer1*$^{m/m}$, *mPer2*$^{m/m}$ *and* *mPer1*$^{m/m}$*mPer2*$^{m/m}$ double mutant. Importantly, mRNAs showed differential response to mutations in *mPer1* or *mPer2* suggesting non-redundant functions for the mammalian *Period* genes in regulating circadian clock dependent transcription. Although loss of circadian expression of CCGs in the *mPer2* mutants can be attributed partially to the loss of rhythms, this cannot apply to the *mPer1* mutants. The presence of CCGs whose circadian expression is abolished in *mPer1* but not in *mPer2* mutants suggests that while mPER1 is not a major contributor of the RNA oscillation of the clock genes, it regulates some output pathways that are not shared by mPER2. Regardless of whether a particular CCG loses circadian expression in the *mPer1* or the *mPer2* mutant, all CCGs simultaneously lost circadian expression in the double mutants further indicating a complete loss of clock function in these animals. At a molecular level, a complete loss of rhythmic behavior is also reflected at the transcriptional level. Thus, the loss of circadian function is a loss of temporal transcriptional control.

Table 3. Differential expression of clock-controlled genes in *mPer1* and *mPer2* mutants. Clock-controlled genes are classified into four groups (I-IV) according to their transcriptional profile (cycling or not cycling with 24-hr period) in 4 genotypes: wild type, *mPer1* mutant (*mPer1*$^{m/m}$), *mPer2* mutant (*mPer2*$^{m/m}$) and *mPer1/mPer2* double mutant (*mPer1*$^{m/m}$*mPer2*$^{m/m}$).

Groups	**Wild type**	***mPer1*$^{m/m}$**	***mPer2*$^{m/m}$**	***mPer1*$^{m/m}$*mPer2*$^{m/m}$**	**Clock Controlled Genes** (GeneBank accession ID)
I	cycling	not cycling	cycling	not cycling	*mCrbp1* (X60367) *s11-6* (AI594922) *Inter α trypsin inhibitor heavy chain 3* (AA062129)
II	cycling	cycling	not cycling	not cycling	*Ng27* (AAC97965) *Klf9* (AA472299) *Slc39a4* (W18585)
III	cycling	cycling	cycling	not cycling	*Alas1* (W15813) *P311* (W62819) Est, similar to Ca dependent protease (AA118977)
IV	cycling	not cycling	not cycling	not cycling	*Alas2* (M63244) *Serine protease inhibitor* IV (AI430332) *Methallothionin* (A1430332) *Vdup1* (W34721) *Scpep1* (W10703) *Cyt P450 2b13* (A1325330) *Procolipase* (AI 385475)

One of the CCGs identified is cellular retinal binding protein 1 (mCrbp1), that encodes a protein involved in vitamin A homeostasis. It has been shown that mice deficient in mCRBP1 are essentially normal but when raised on a vitamin A-deficient diet, develop abnormalities characteristic of postnatal hypo-vitaminosis (Ghyselinck *et al.*, 1999). In the absence of vitamin A, *mCrbp1* mutant animals display a markedly altered electroretinogram and the normal intimate contact between retinal pigment epithelium and outer segment is disrupted (Ghyselinck *et al.*, 1999). Given the observation that expression of mCRBP1 is under circadian control, it would be interesting to test whether mCRBP1 is involved in circadian photo response. Recently it has been shown that mCRBP1 modulates photo-length response in Siberian hamster (Ross *et al.*, 2004).

Another of the CCGs identified encodes for angiopoietin like protein 14 (Angpt14, NG27). Recently it has been shown that induction of NG27 in the heart inhibits lipoprotein lipase derived fatty acid delivery (Yu *et al.*, 2005). Inhibition of lipoprotein lipase by NG27 has potential therapeutic benefit of reducing arterioscleroses risk by excess of dietary lipids.

The observation that the genes encoding the rate-limiting enzymes for haem biosynthesis, *aminolevulinate synthases* (*Alas1* and *Alas2*) are under circadian control is of particular interest. Circadian expression of *Alas1* and *Alas2* is completely deregulated in the $mPer1^{m/m}mPer2^{m/m}$ double mutant mice (I, Fig. 7), indicating that mPER1 and mPER2 regulate the biosynthesis and availability of haem. The level of cellular haem is tightly controlled, and this was thought to be regulated by a balance between haem synthesis and catabolism, the latter by haem-oxygenase. The observations presented in this thesis suggest a model based on transcriptional control of *Alas1* expression by the circadian clock as a plausible mechanism for regulating levels of ALAS activity, and thereby haem levels. Northern analysis of the expression of *haem-oxygenase2* shows constitutive expression in wild type, $mPer1^{m/m}$ and $mPer2^{m/m}$ mutants (data not shown), suggesting that unlike its biosynthesis, the rate of haem catabolism by haem-oxygenase 2 occurs at a constant rate. The clock control of the availability of haem may have a wider implication for temporal control of the biochemical and physiological processes of an organism. It is well known that haem serves as a prosthetic moiety for many haem proteins that are involved in a vast array of biological functions. Among those proteins that contain haem are proteins involved in oxygen metabolism (myoglobin, hemoglobin, catalase, etc), electron transfer (cytochrome c and p450), and signaling (guanylyl cyclases, nitric oxide synthase) (Lucas *et al.*, 2000; Ponka, 1999; Stuehr, 1997). Thus, it is possible that temporal control of haem biosynthesis could be a basis for a wider range of cascades in physiological processes.

In summary, the data provide compelling behavior and molecular evidence that *mPer1* and *mPer2* have distinct roles in the clock. mPER2 regulates the circadian cycle via a transcriptional control while mPER1 may operate via a posttranscriptional mechanism. The loss of mPER1 and mPER2 results in a

complete loss of circadian rhythms and is reflected at the molecular level by the loss of the temporal control for rhythmic expression of CCGs. The data demonstrate that some CCGs are differentially regulated by mPER1 and mPER2. Together with earlier studies that implicate roles for mPER1 and mPER2 in the input pathway of the clock (Albrecht *et al.*, 2001), these studies indicate that mPER1 and mPER2 play nonredundant roles in circadian clock control. Finally, the studies imply that the regulation of cellular haem is under circadian control. The connection to haem biosynthesis would suggest both circadian control of physiological processes by regulating haem metabolism and a possible regulatory role of haem metabolism on the clock mechanism itself, possibly via PAS proteins that are haem binding. Those aspects are investigated next.

3. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals (Publication II)

Here a novel biological finding of a reciprocal regulation between the mammalian circadian clock mechanism and haem biosynthesis *in vivo* is described.

In the previous section a central role was postulated for haem in the regulation of the mammalian circadian clock (I). Recent observations that NPAS2, a paralog of Clock and a partner of BMAL1 is a haem-binding protein (Dioum *et al.*, 2002) and electrophysiological studies which show that hemin can phase shift neuronal firing in SCN slices further implicate haem as an component of the core clock mechanism (Artinian *et al.*, 2001). RNA *in situ* hybridization showed that *Alas1* mRNA is rhythmically expressed in the SCN (peak at CT 6-12), which is a hallmark for circadian genes (data not showed). An interesting question was raised whether the haem itself is important in regulating the clock mechanism. An independent link of haem to circadian regulation is that some PAS proteins are haem-binding proteins. Recently, three bacterial PAS proteins, AxPDEA1, DOS, and FIXL, have been shown to be haem binding proteins (Chang *et al.*, 2001; Delgado-Nixon *et al.*, 2000; Gong *et al.*, 1998). PAS motifs from bacteria to mammals have highly conserved three-dimensional folds even though the amino acid sequence identity is low (Pellequer *et al.*, 1999; Pellequer *et al.*, 1998). Thus, it is possible that other PAS proteins, including those in mammals, are potentially haem-binding proteins. The regulation of haem levels via mPER dependent regulation of Alas expression may be part of an interface between the core clock mechanism and the cellular/subcellular environment. It can be envisaged that the availability of haem controls the function of haem binding PAS proteins like NPAS2, which in turn regulates their activity with their transcriptional partner protein BMAL1. The transcriptional activity of the BMAL1-NPAS2 or BMAL1-Clock heterodimers would then regulate the expression of clock genes such as the *mPer's*. The levels of mPER proteins in turn would regulate haem biosynthesis

via control of *Alas* expression. Such theoretical model would provide a plausible interactive regulation between the biochemical/physiological conditions and the clock mechanism, that was investigated experimentally.

First it was demonstrated that haem (hemin) transiently resets clock gene rhythms in cultured cells (II, Supplementary Fig. 1). Next it was shown that intra-peritoneal injection of hemin altered *mPer1* and *mPer2* expression in the liver (II, Fig. 1). In wild type animals, expression of other clock genes such as *Bmal1, Cry1, Npas2* and *Clock* are apparently not affected by hemin (II, Fig. 1). The effects of hemin on *mPer1* and *mPer2* expression were most prominent during subjective night. At the behavioral level, wheel-running activity was suppressed by hemin given during subjective night period (II, Supplementary Fig. 2b). In contrast, locomotor activity was normal when hemin was injected during the subjective day (II, Supplementary Fig. 2a). Together these observations suggest that the effects of hemin are dependent on temporally regulated factors.

Key regulators of *mPer1* and *mPer2* expression are the bHLH transcription factor complexes of BMAL1/NPAS2 and BMAL1/Clock. Recent studies demonstrate that haem is a regulator of BMAL1/NPAS2 transcriptional activity (Dioum *et al.*, 2002), thus NPAS2 was focused on as a target of hemin. Using mutant *Npas2* animals it was shown that the effects of hemin on *mPer1* and *mPer2* expression were attenuated compared to wild type animals during subjective night implicating its role in modulating haem response (II, Supplementary Fig. 3). The mutant NPAS2 has an in-frame deletion of the bHLH region (Garcia JA, 2000). Thus the attenuated hemin response may reflect residual NPAS2 function and/or redundant regulators. Redundant regulators include mPER2 since the loss of mPER2 function also affects *mPer1* induction by hemin with similar expression pattern of *Npas2* mutant (II, Supplementary Fig. 3). The mutation of *Npas2* dampened *mPer2* expression in the liver and is consistent with previous findings in the forebrain. Northern analysis of liver RNA from $mPer2^{m/m}$ and $mPer2^{-/-}$ mice showed an absence of *Npas2* expression or a loss of *Npas2* mRNA transcript stability (II, Fig. 2a). Therefore, these studies showed that mPER2 is a major regulator of NPAS2 function *in vivo*.

Mouse and human genetic studies have established PER2 as a major circadian regulator (Toh *et al.*, 2001; Zheng *et al.*, 1999, I). The loss of mPER2 function results in the down regulation of core clock genes *Bmal1, Cry1* and *mPer1* in the SCN and in peripheral tissues (Zheng *et al.*, 1999; Sherman *et al.*, 2000; I). Therefore, genetic evidence implicates mPER2 as a positive regulator of the mammalian circadian clock mechanisms. Although mPER2 is a PAS domain protein, it does not have the classical bHLH motif found in other circadian PAS domain transcription factors such as BMAL1, Clock and NPAS2 (Albrecht *et al.*, 1997). *In vitro* studies using mammalian genes have failed to establish mPER2 partnership with other bHLH-PAS proteins such as BMAL1 and Clock nor does it enhance BMAL1/Clock transcription complex activity as

predicted by genetic studies (Yamaguchi *et al.*, 2000). In fruit flies, the auto-regulatory mechanism is modeled on a negative feedback function of dPER (Young and Kay, 2001), however, in the mouse, *in vivo* and *in vitro* studies have identified CRY proteins as the major negative regulators (Kume *et al.*, 1999). Thus, the molecular target and function of mPER2 is an enigma.

Current observation that *Npas2* expression or mRNA stability is dependent on mPER2 led us to analyze whether NPAS2 could be its target in the circadian clock mechanism. Using *mPer2* promoter reporter assay, the addition of mPER2 significantly enhanced the BMAL1/NPAS2 complex transcription activity that was repressed by CRY1 addition (II, Fig. 2b). In contrast, mPER2 does not stimulate BMAL1/Clock complex transcriptional activity directly indicating that mPER2 action is NPAS2 specific (II, Fig. 2b). It is consistent with an *in vivo* observation that loss of mPER2 function does not alter *Clock* gene expression in the SCN and in peripheral tissues and Clock is not a direct target of mPER2 (Zheng *et al.*, 1999). The target of mPER2 requires a heterodimeric complex of BMAL1 and NPAS2. Monomer of BMAL1 or NPAS2 was not stimulated by mPER2 (II, Fig. 2b). Therefore mPER2 is an enhancer of the BMAL1/NPAS2 transcriptional complex activity. The activation and repressing effect of haem is likely gene specific. Both BMAL1/NPAS2 and BMAL1/Clock transcription complexes can activate or repress gene promoter activity in a gene specific fashion. The BMAL1/NPAS2 complexes activate *mPer1* promoter, but act as a repressor of the *c-Myc* promoter (Fu *et al.*, 2002). Similarly BMAL1/Clock heterodimer is an activator of *mPer1* but acts as a repressor of the TTF-1 promoter (Kim *et al.*, 2002). Thus the inverse effect of haem on *mPer1* and *mPer2* promoter activity may reflect gene specific response of the same transcription complex machineries.

The functional link between mPER2 and NPAS2 ties key regulators of mammalian circadian clock to the haem control of the BMAL1/NPAS2 transcription complex activity (Dioum *et al.*, 2002). The observation that *Npas2* mutation dramatically dampened *Alas1* expression (II, Fig. 3a) implicates NPAS2 as a key regulator of ALAS1 activity and by extension the biosynthesis of haem *in vivo*. Consistent with the genetic observations, *in vitro* studies showed that BMAL1/NPAS2 complexes directly activate the reporter construct of *Alas1* promoter and this activity is enhanced by mPER2 (II, Fig. 3b). Therefore, BMAL1/NPAS2 complex dependent control of *Alas1* transcription is an example of a reciprocal regulation between circadian clock regulation of haem biosynthesis and haem control of circadian clock complexes regulating transcription. In this model (Figure 6), expression of *Alas1* is dependent on the BMAL1/NPAS2 transcription activity that is amplified in the presence of mPER2 protein. Via biosynthesis *Alas1* expression levels control the amount of haem. In addition haem biosynthesis is coordinated with the expression of many circadianly controlled genes that encode haem binding protein such as nitric oxide synthase, catalase and others that are regulators of biochemical and physiological cascades (Agapito *et al.*, 1999; Stevenson *et al.*, 2001; Tunctan *et*

al., 2002; Ueda *et al.*, 2003). Increased level of haem eventually induces its own degradation via haem-oxygenase enzymes that are induced by free haem (Ndisang *et al.*, 2003). Degradation of haem by haem-oxygenases results in the formation of carbon monoxide, biliverdin and free iron (Artinian *et al.*, 2001). In the presence of carbon monoxide, DNA binding of haem bound BMAL1/NPAS2 transcription complex is inhibited and thus gene transcription is downregulated (Dioum *et al.*, 2002). The decreased in transcription complex activity is further amplified by the decline in mPER2 levels. The released transcription complex components are then presumably targeted for degradation. The decline in haem abundance eventually reaches trough levels that in turn allow basal BMAL1/NPAS2 transcription complex to bind DNA that restarts transcription again.

The evolution of porphyrin molecules as regulators of mammalian circadian clock mechanism is likely very ancient. In different phylogenies, the common substrate for porphyrin biosynthesis is aminolevulinate acid (ALA). In plants, ALA is biosynthesized in the presence of magnesium ion into the porphyrin product chlorophyll, the main floral circadian photo-sensor. In bacteria, ALA is biosynthesized in the presence of cobalt to form another porphyrin product cobalt-hemin (cobalamin) (Roth *et al.*, 1996). It has been observed over the past decade in clinical trials and in animal studies that cobalamin phase shifts human and rodent circadian clocks but the mechanism is unclear (Hashimoto *et al.*, 1996; Ikeda *et al.*, 1998; Nakamura *et al.*, 1997; Uchiyama *et al.*, 1995). Present studies demonstrate that cyanocobalamin, an analog of vitamin B12 inhibits haem binding to NPAS2 and mPER2 (II, Fig. 4 a & c). Injection of cyanocobalamin has an inverse effect of haem on *mPer1* and *mPer2* expression indicating that it targets clock regulators in a differential manner or suggests involvement of other molecules such as gas mediators *in vivo* (II, Fig. 4d).

Previously it has been shown that *mPer2* deficient mice have abnormal sensitivity to genotoxic agents and have an enhanced cell proliferation phenotype (Fu *et al.*, 2002). Cell division cycle regulators such as *c-Myc, Cyclin D, Cyclin* A and others are under circadian control *in vivo* (Fu *et al.*, 2002). Hemin also differentially affects expression of *c-Myc* (II, Supplementary Fig. 6) and other cell division cycle regulators (Zhu *et al.*, 2002) implicating the involvement of the same regulators. Furthermore, in *Npas2* mutant animals *c-Myc* expression is elevated and deregulated as was observed in *mPer2* deficient animals (II, Supplementary Fig. 6; Fu *et al.*, 2002). Analogues of vitamin B12 are known to have differential effects on proliferation rate of leukemia cells (McLean *et al.*, 1997). The timing of vitamin B12 given to mice undergoing ionizing radiation therapy for Ehrlich Sarcoma has a significant impact on the efficacy and toxicity response of the animals (Levitman *et al.*, 2002). The authors postulated that vitamin B12 acts as a synchronizer of cell division. Newly released clinical trials demonstrate that giving vitamin B12 to patients undergoing pemetrexed chemotherapy produced significant reduction in toxicity effect of the drug (Scagliotti *et al.*, 2003). The pharmaceutical target of

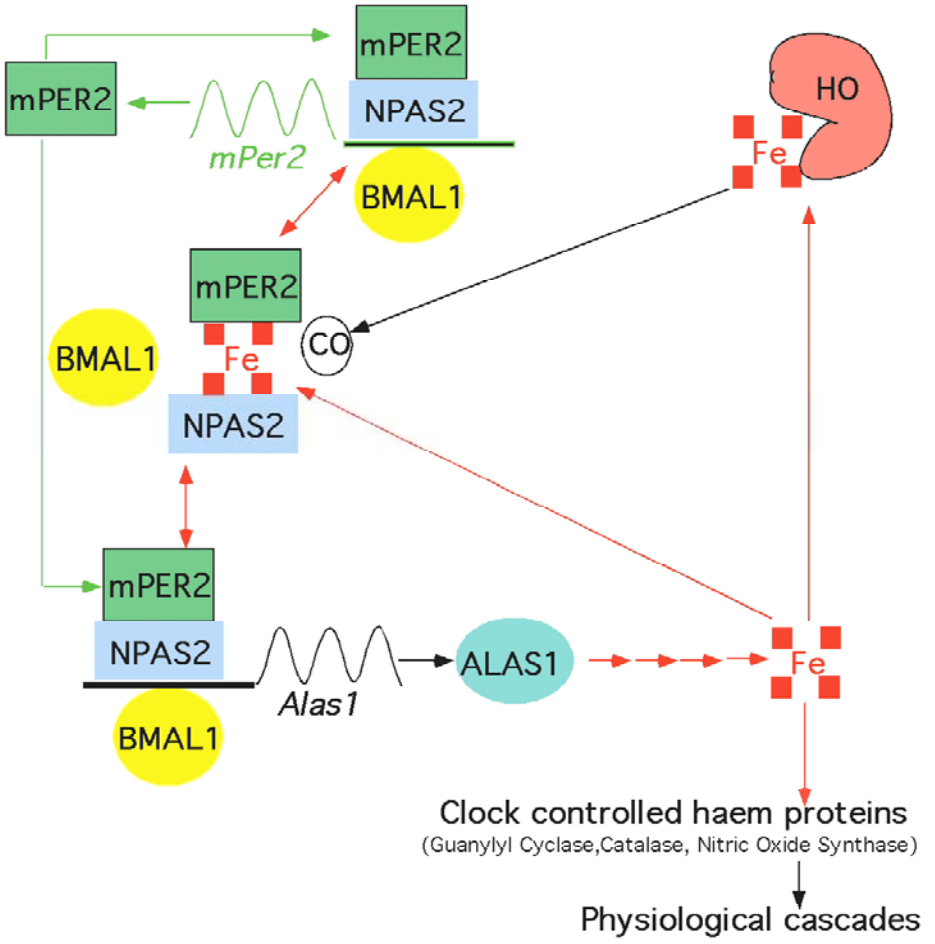

Figure 6. A model of reciprocal regulation between the circadian clock and haem biosynthesis. Expression of *Alas1* is dependent on the BMAL1/NPAS2 transcription activity that is amplified by mPER2 protein. *Alas1* expression levels control the amount of haem. Haem induces its own degradation via haem-oxygenase enzymes that results in the formation of carbon monoxide, biliverdin and free iron. In the presence of carbon monoxide, DNA binding of haem bound BMAL1/NPAS2 transcription complex is inhibited. The decline in haem abundance eventually reaches trough levels that in turn allow basal BMAL1/NPAS2 transcription complex to bind DNA that restarts transcription.

Abbreviations: Mammalian Period protein 2 (mPER2), Neuronal PAS domain protein 2 (NPAS2), Brain Muscle ARNT Like protein 1 (BMAL1), Haem-Oxygenase (HO), Aminolevulinate Synthase 1 (ALAS1), Carbon Monoxide (CO), and Haem (4 red squares with Fe).

pemetrexed is the DNA biosynthesis enzyme thymidylate synthase (Norman, 2001), whose expression is under circadian clock control *in vivo* (Bjarnason *et al.*, 2001; Lincoln *et al.*, 2000). Together these studies would suggest that improvement in the quality of life of cancer patients undergoing chemo and radiation therapy could benefit from therapeutic function of vitamin B12 or other porphyrin derivatives that target circadian regulators which in turn modulate cell division synchronization and regulation.

Taken together a novel biological finding of a reciprocal regulation between the mammalian circadian clock mechanism and haem biosynthesis *in vivo* has been described. The studies based on genetic and molecular methods provide evidence to make several conclusions: haem is a regulator of clock genes *mPer1* and *mPer2 in vivo*; the direct molecular target of mPER2 is BMAL1/NPAS2 and not BMAL1/Clock transcription complex. Furthermore it has been shown that NPAS2 is a major regulator of ALAS1, the rate-limiting enzyme of haem biosynthesis. Together, these studies establish the molecular connection between haem regulation of the BMAL1/NPAS2 transcriptional complex activity and the circadian regulation of haem biosynthesis. In addition these studies show that porphyrin molecules such as haem and cobalamin (vitamin B12) are modulators of circadian clock gene expression and could have therapeutic implication for circadian disorders. The current discoveries are important to fields from multiple disciplines including circadian clock, cancer and porphyrin molecules.

4. Behavioral characterization of mouse models for Smith-Magenis syndrome and dup(17)(p11.2p11.2) (Publication III)

Contiguous gene syndromes (CGS) refer to a group of disorders associated with chromosomal rearrangements in which the phenotype is thought to result from altered copy number of physically linked dosage sensitive genes. Smith-Magenis syndrome (SMS) and dup (17)(p11.2p11.2) are CGSs. Previously Walz *et al*. reported the creation of rearranged chromosomes by chromosomal engineering carrying a deletion (*del(11)17*) or a duplication (*dup(11)17*) of the syntenic region on mouse chromosome 11 that spans the genomic interval commonly rearranged in Smith-Magenis syndrome patients (Walz *et al*., 2003). Human 17p11.2 is highly conserved and syntenic to the mouse chromosome 11, on 32-34 centimorgan region, making it feasible to establish a mouse model of SMS. A deletion of 3.7 Mb genomic fragment in one copy of chromosome 17 band 11.2 has been identified in almost all of the 100 cases of SMS that have been described in the literature (Shelley and Robertson, 2005). SMS patients display numerous neurobehavioral abnormalities including mental retardation, learning disabilities, attention deficits, decreased sensitivity to pain or temperature, sleep disturbances and self-injurious behaviors (Smith *et al.*, 1998; Smith

et al., 1998). The clinical phenotype resulting from duplication [dup (17) (p11.2p11.2)] is milder than the deficiency of this genomic region (Potocki *et al.*, 2000). Duplication individuals have autistic features, hyperactive mild mental retardation and attention deficit. People with Smith-Magenis syndrome display sleep patterns that are often heavily disrupted leading to reduced amount of rest. SMS displays abnormalities in sleep distribution throughout the 24-hr cycle suggesting direct involvement of mutations in clock genes at a leading cause. Although not much is known about how sleep and circadian clock are interconnected, the sleep attack at the end of the day experienced by the patients may represent the endogenous sleep onset, therefore regarded as a sleep phase advance.

Behavioral analysis of two mouse models was performed to determine whether del and dup mutant mice exhibit aspects of the SMS and neurobehavioral phenotypes. Wheel running activity was monitored to uncover defects in the circadian clock of the mutant mice.

The open-field test was performed to assess exploratory activity and anxiety-related responses in a novel arena. Various measures assessed during the open-field test are presented in III, Fig. 1. Male mice, but not female, from both the del and dup mutant models displayed abnormal activity in the open-field compared to their respective wild type mice (III, Fig. 1 a, b). Male del mutants are hypoactive, but male dup mutants are hyperactive (III, Fig. 1 a, b). These results indicate that the locomotor activity is regulated by the gene(s) in the mutated region in a dose dependent manner. The nature of the gender specificity is unknown. There was no overall difference in movement speed or the center distance ratio between the two genotypes in either males or females (III, Fig. 1 c, d).

The del mutants had normal behavior in the light-dark test for anxiety, in prepulse inhibition test for sensorimotor gating. Also the conditioned fear test for learning and memory and hotplate test for analgesia-related responses showed no difference between del mutants and wild type controls. The male dup mice have less anxiety according to the light-dark test (III, Fig. 2). Both genders of dup mice had decreased startle responses, but normal prepulse inhibition (III, Fig. 3). Male dup mutant mice had impaired conditioned fear selective to the context test (III, Fig. 4). Circadian behavioral analyses revealed period length differences for (*del(11)17/*+) mice compared to the wild type littermates. These results indicate that a dosage sensitive gene present in this region is responsible for behavioral abnormalities in the (*del(11)17/*+) mutant. Del mutant mice had significantly shorter locomotor activity period than their wild type littermates (III, Fig. 6e), suggesting that haploinsufficiency of one or more genes in the mutated region is involved in regulation of circadian period. In addition the period distribution among del mice shows reduced accuracy of the circadian period instability.

Interestingly, *Dexras1* (*Dexamethasone–induced Ras1*) gene is located in the affected 3.7 Mb interval. Recently it was shown, that *Dexras1* is expressed

rhythmically in the SCN (Panda *et al.*, 2002; Ueda *et al.*, 2002), which is an hallmark for genes involved in the circadian physiology. Furthermore recent studies showed that DEXRAS1 potentiates photic and suppresses non-photic responses of the circadian clock (Cheng et al; 2004). The circadian locomotor activity behavior of *Dexras*$^{-/-}$ mice is very unstable under constant illumination (Cheng *et al.*, 2004). Therefore, *Dexras1* could be a candidate gene, whose deletion may be involved in the SMS circadian sleep disruption phenotype. Interestingly *Dexras1* maps close to *Rai1* (*Retinoid acid inducible*) gene and a small deletion in *Rai1* gene has been described in some SMS patients (Slager *et al.*, 2003). *Rai1* mutation could be responsible for other SMS associated phenotypes.

In conclusion, heterozygous animals carrying the engineered deletion (*del(11)17/*+) or the duplication (*dup(11)17/*+) are hypoactive or hyperactive, respectively. Both mutations cause learning disabilities. (*del(11)17/*+) mice present impaired analgesia related response. In addition, circadian period length differences were found for (*del(11)17/*+) mice compared to wild type littermates. These results indicate that dosage sensitive gene(s) present in this region are responsible for behavioral abnormalities in the mouse that show similar phenotypes as the patients with the defective syntenic interval.

CONCLUSIONS

The results of this thesis can be summarized as follows:

I *mPer1* and *mPer2* play nonredundant roles in the mouse circadian clock mechanism. mPER2 regulates the circadian cycle via a transcriptional control while mPER1 may operate via transcriptional as well as posttranscriptional control. The loss of mPER1 and mPER2 results in a complete loss of circadian rhythms at the behavioral level and this is reflected at the molecular level by the loss of the rhythmic expression of both clock genes and clock-controlled genes. Studies of clock-controlled genes reveal a complex pattern of regulation by mPER1 and mPER2, suggesting independent control by the two proteins of some output pathways. A number of novel and previously known genes were identified that are transcriptionaly regulated in a circadian manner. Among them, Aminolevulinate synthase, the rate-limiting enzyme in haem biosynthesis pathway, was found to be under clock control. It suggests that many metabolic pathways governing physiology and behavior are under circadian control. The clock-controlled genes were found to respond differentially to various *Period* gene mutations in the circadian pacemaker. These observations will enhance future studies to dissect the function of various clock components in regulating output genes.

II Haem biosynthesis is one of the biochemical processes under the control of the circadian clock in mammals, via its rate-limiting enzyme Aminolevulinate synthase. Using genetic and molecular tools, a reciprocal aspect of this relationship has been uncovered; clock genes *mPer1* and *mPer2* are regulated by haem differentially *in vivo* in a pathway involving mPER2 and NPAS2, a protein that is part of the peripheral clock mechanism and also regulates production of *Alas1*. These results implicate another feedback loop between metabolism and circadian timing. In addition, cobalamin (vitamin B12) and haem compete for binding by NPAS2 and mPER2 and have opposite effects on the expression of *mPer1* and *mPer2*. These results identify porphyrin derivatives as potential therapeutics for patients receiving chemotherapy and radiotherapy for cancer via the link between cell-cycle regulation and the circadian clock.

III To find the genetic defect underlying the Smith-Magenis syndrome a mouse model that mimicked the human disorder was analyzed in an exhaustive behavioral analysis. The heterozygous animals carrying the engineered deletion (*del(11)17/*+) or the duplication (*dup(11)17/*+) are hypoactive or hyperactive, respectively, and both have learning disabilities. (*del(11)17/+)* mice present impaired analgesia related response. In addition, a shortening of circadian period in locomotor activity was found in (*del(11)17/+)* mice when compared to wild type littermates. These results indicate that a dosage sensitive gene present in this genetic region is responsible for behavioral abnormalities. Two genes in the affected interval, *Dexras1* and *Rai1* could be potential candidate genes to be involved in the phenotypes of Smith-Magenis syndrome as individual mutations in these genes replicate some of the Smith-Magenis syndrome phenotypes.

REFERENCES

Agapito, M. T., Redondo, I., Plaza, R., Lopez-Burillo, S., Recio, J. M., and Pablos, M. I. (1999). Relationships between melatonin, glutathione peroxidase, glutathione reductase, and catalase. Endogenous rhythms on cerebral cortex in Gallus domesticus. Adv Exp Med Biol *460*, 377–381.

Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002). Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. Mol Cell Biol *22*, 1693–1703.

Akhtar, R. A., Reddy, A. B., Maywood, E. S., Clayton, J. D., King, V. M., Smith, A. G., Gant, T. W., Hastings, M. H., and Kyriacou, C. P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol *12*, 540–550.

Akten, B., Jauch, E., Genova, G. K., Kim, E. Y., Edery, I., Raabe, T., and Jackson, F. R. (2003). A role for CK2 in the Drosophila circadian oscillator. Nat Neurosci *6*, 251–257.

Albrecht, U., Sun, Z. S., Eichele, G., and Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell *91*, 1055–1064.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S., and Lee, C. C. (2001). MPer1 and mper2 are essential for normal resetting of the circadian clock. J Biol Rhythms *16*, 100–104.

Allada, R., White, N. E., So, W. V., Hall, J. C., and Rosbash, M. (1998). A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. Cell *93*, 791–804.

Andre, E., Conquet, F., Steinmayr, M., Stratton, S. C., Porciatti, V., and Becker-Andre, M. (1998). Disruption of retinoid-related orphan receptor beta changes circadian behavior, causes retinal degeneration and leads to vacillans phenotype in mice. Embo J *17*, 3867–3877.

Archer, S. N., Robilliard, D. L., Skene, D. J., Smits, M., Williams, A., Arendt, J., and von Schantz, M. (2003). A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. Sleep *26*, 413–415.

Artinian, L. R., Ding, J. M., and Gillette, M. U. (2001). Carbon monoxide and nitric oxide: interacting messengers in muscarinic signaling to the brain's circadian clock. Exp Neurol *171*, 293–300.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525–536.

Bae, K., Lee, C., Hardin, P. E., and Edery, I. (2000). dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J Neurosci *20*, 1746–1753.

Bae, K., Lee, C., Sidote, D., Chuang, K. Y., and Edery, I. (1998). Circadian regulation of a Drosophila homolog of the mammalian Clock gene: PER and TIM function as positive regulators. Mol Cell Biol *18*, 6142–6151.

Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929–937.

Balsalobre, A., Marcacci, L., and Schibler, U. (2000). Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. Curr Biol *10*, 1291–1294.

Bao, S., Rihel, J., Bjes, E., Fan, J. Y., and Price, J. L. (2001). The Drosophila double-timeS mutation delays the nuclear accumulation of period protein and affects the feedback regulation of period mRNA. J Neurosci *21*, 7117–7126.

Bargiello, T. A., Jackson, F. R., and Young, M. W. (1984). Restoration of circadian behavioural rhythms by gene transfer in Drosophila. Nature *312*, 752–754.

Berman-Frank, I., Lundgren, P., Chen, Y. B., Kupper, H., Kolber, Z., Bergman, B., and Falkowski, P. (2001). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium Trichodesmium. Science *294*, 1534–1537.

Berson, D. M., Dunn, F. A., and Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. Science *295*, 1070–1073.

Bishop, D. F., Henderson, A. S., and Astrin, K. H. (1990). Human -aminolevulinate synthase: assignment of the housekeeping gene to 3p21 and the erythroid-specific gene to the X chromosome. Genomics *7*, 207–214.

Bjarnason, G. A., Jordan, R. C., Wood, P. A., Li, Q., Lincoln, D. W., Sothern, R. B., Hrushesky, W. J., and Ben-David, Y. (2001). Circadian expression of clock genes in human oral mucosa and skin: association with specific cell-cycle phases. Am J Pathol *158*, 1793–1801.

Blau, J., and Young, M. W. (1999). Cycling vrille expression is required for a functional Drosophila clock. Cell *99*, 661–671.

Block, G. D., and McMahon, D. G. (1984). Cellular analysis of the Bulla ocular circadian pacemaker system. III. Localization of the circadian pacemaker. Journal of Comparative Physiology *155*, 387–395.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009–1017.

Camacho, F., Cilio, M., Guo, Y., Virshup, D. M., Patel, K., Khorkova, O., Styren, S., Morse, B., Yao, Z., and Keesler, G. A. (2001). Human casein kinase Idelta phosphorylation of human circadian clock proteins period 1 and 2. FEBS Lett *489*, 159–165.

Ceriani, M. F., Hogenesch, J. B., Yanovsky, M., Panda, S., Straume, M., and Kay, S. A. (2002). Genome-wide expression analysis in Drosophila reveals genes controlling circadian behavior. J Neurosci *22*, 9305–9319.

Cermakian, N., Monaco, L., Pando, M. P., Dierich, A., and Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. Embo J *20*, 3967–3974.

Chang, A. L., Tuckerman, J. R., Gonzalez, G., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., and Gilles-Gonzalez, M. A. (2001). Phosphodiesterase A1, a regulator of cellulose synthesis in Acetobacter xylinum, is a heme-based sensor. Biochemistry *40*, 3420–3426.

Cheng, H. Y., Obrietan, K., Cain, S. W., Lee, B. Y., Agostino, P. V., Joza, N. A., Harrington, M. E., Ralph, M. R., and Penninger, J. M. (2004). Dexras1 potentiates photic and suppresses nonphotic responses of the circadian clock. Neuron *43*, 715–728.

Christie, J. M., Salomon, M., Nozue, K., Wada, M., and Briggs, W. R. (1999). LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide. Proc Natl Acad Sci U S A *96*, 8779–8783.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M. W. (2001). Circadian regulation of gene expression systems in the Drosophila head. Neuron *32*, 657–671.

Cohen, M. C., Rohtla, K. M., Lavery, C. E., Muller, J. E., and Mittleman, M. A. (1997). Meta-analysis of the morning excess of acute myocardial infarction and sudden cardiac death. Am J Cardiol *79*, 1512–1516.

Crosthwaite, S. K., Loros, J. J., and Dunlap, J. C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. Cell *81*, 1003–1012.

Curtin, K. D., Huang, Z. J., and Rosbash, M. (1995). Temporally regulated nuclear entry of the Drosophila period protein contributes to the circadian clock. Neuron *14*, 365–372.

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R., Hardin, P. E., Young, M. W., Storti, R. V., and Blau, J. (2003). vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell *112*, 329–341.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev *14*, 2950–2961.

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D., Weitz, C. J., Takahashi, J. S., and Kay, S. A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science *280*, 1599–1603.

De Leersnyder, H., Bresson, J. L., de Blois, M. C., Souberbielle, J. C., Mogenet, A., Delhotal-Landes, B., Salefranque, F., and Munnich, A. (2003). Beta 1-adrenergic antagonists and melatonin reset the clock and restore sleep in a circadian disorder, Smith-Magenis syndrome. J Med Genet *40*, 74–78.

De Leersnyder, H., de Blois, M. C., Vekemans, M., Sidi, D., Villain, E., Kindermans, C., and Munnich, A. (2001). beta(1)-adrenergic antagonists improve sleep and behavioural disturbances in a circadian disorder, Smith-Magenis syndrome. J Med Genet *38*, 586–590.

DeLawter, D. E. (1991). The management of early morning hyperglycemia: is it due to Somogyi effect or dawn phenomenon? Md Med J *40*, 391.

Delgado-Nixon, V. M., Gonzalez, G., and Gilles-Gonzalez, M. A. (2000). Dos, a heme-binding PAS protein from Escherichia coli, is a direct oxygen sensor. Biochemistry *39*, 2685–2691.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol *15*, 435–467.

Dioum, E. M., Rutter, J., Tuckerman, J. R., Gonzalez, G., Gilles-Gonzalez, M. A., and McKnight, S. L. (2002). NPAS2: a gas-responsive transcription factor. Science *298*, 2385–2387.

Dudley, C. A., Erbel-Sieler, C., Estill, S. J., Reick, M., Franken, P., Pitts, S., and McKnight, S. L. (2003). Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. Science *301*, 379–383.

Duffield, G. E. (2003). DNA microarray analyses of circadian timing: the genomic basis of biological time. J Neuroendocrinol *15*, 991–1002.

Duffield, G. E., Best, J. D., Meurers, B. H., Bittner, A., Loros, J. J., and Dunlap, J. C. (2002). Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. Curr Biol *12*, 551–557.

Dunlap, J. C. (1990). Closely watched clocks: molecular analysis of circadian rhythms in Neurospora and Drosophila. Trends Genet *6*, 159–165.

Dunlap, J. C. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Ebisawa, T., Uchiyama, M., Kajimura, N., Mishima, K., Kamei, Y., Katoh, M., Watanabe, T., Sekimoto, M., Shibui, K., Kim, K., *et al.* (2001). Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. EMBO Rep *2*, 342–346.

Eide, E. J., Vielhaber, E. L., Hinz, W. A., and Virshup, D. M. (2002). The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon. J Biol Chem *277*, 17248–17254.

Emery, P., So, W. V., Kaneko, M., Hall, J. C., and Rosbash, M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell *95*, 669–679.

Etchegaray, J. P., Lee, C., Wade, P. A., and Reppert, S. M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177–182.

Feldman, J. F. (1967). Lengthening the period of a biological clock in Euglena by cycloheximide, an inhibitor of protein synthesis. Proc Natl Acad Sci U S A *57*, 1080–1087.

Field, M. D., Maywood, E. S., O'Brien, J. A., Weaver, D. R., Reppert, S. M., and Hastings, M. H. (2000). Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. Neuron *25*, 437–447.

Frisch, B., Hardin, P. E., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1994). A promoterless period gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the Drosophila nervous system. Neuron *12*, 555–570.

Froehlich, A. C., Liu, Y., Loros, J. J., and Dunlap, J. C. (2002). White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science *297*, 815–819.

Froehlich, A. C., Loros, J. J., and Dunlap, J. C. (2003). Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. Proc Natl Acad Sci U S A *100*, 5914–5919.

Fu, L., Pelicano, H., Liu, J., Huang, P., and Lee, C. (2002). The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell *111*, 41–50.

Garcia JA, Z. D., Estill SJ, Michnoff C, Rutter J, Reick M, Scott K, Diaz-Arrastia R, McKnight SL. (2000). Impaired cued and contextual memory in NPAS2-deficient mice. Science *288*, 2226–2230.

Gekakis, N., Saez, L., Delahaye-Brown, A. M., Myers, M. P., Sehgal, A., Young, M. W., and Weitz, C. J. (1995). Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. Science *270*, 811–815.

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Ghyselinck, N. B., Bavik, C., Sapin, V., Mark, M., Bonnier, D., Hindelang, C., Dierich, A., Nilsson, C. B., Hakansson, H., Sauvant, P., *et al.* (1999). Cellular retinol-binding protein I is essential for vitamin A homeostasis. Embo J *18*, 4903–4914.

Glossop, N. R., Houl, J. H., Zheng, H., Ng, F. S., Dudek, S. M., and Hardin, P. E. (2003). VRILLE feeds back to control circadian transcription of Clock in the Drosophila circadian oscillator. Neuron *37*, 249–261.

Glossop, N. R., Lyons, L. C., and Hardin, P. E. (1999). Interlocked feedback loops within the Drosophila circadian oscillator. Science *286*, 766–768.

Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998). Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. Proc Natl Acad Sci U S A *95*, 15177–15182.

Greenberg, F., Guzzetta, V., Montes de Oca-Luna, R., Magenis, R. E., Smith, A. C., Richter, S. F., Kondo, I., Dobyns, W. B., Patel, P. I., and Lupski, J. R. (1991). Molecular analysis of the Smith-Magenis syndrome: a possible contiguous-gene syndrome associated with del(17)(p11.2). Am J Hum Genet *49*, 1207–1218.

Greenberg, F., Lewis, R. A., Potocki, L., Glaze, D., Parke, J., Killian, J., Murphy, M. A., Williamson, D., Brown, F., Dutton, R., *et al.* (1996). Multi-disciplinary clinical study of Smith-Magenis syndrome (deletion 17p11.2). Am J Med Genet *62*, 247–254.

Griffin, E. A., Jr., Staknis, D., and Weitz, C. J. (1999). Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science *286*, 768–771.

Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limbourg-Bouchon, B., and Rouyer, F. (2002). The F-box protein slimb controls the levels of clock proteins period and timeless. Nature *420*, 178–182.

Grundschober, C., Delaunay, F., Puhlhofer, A., Triqueneaux, G., Laudet, V., Bartfai, T., and Nef, P. (2001). Circadian regulation of diverse gene products revealed by mRNA expression profiling of synchronized fibroblasts. J Biol Chem *276*, 46751–46758.

Gu, Y. Z., Hogenesch, J. B., and Bradfield, C. A. (2000). The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol *40*, 519–561.

Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F., Alcedo, J. A., and Wetterhahn, K. E. (1991). Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. Arch Biochem Biophys *289*, 387–392.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1990). Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., and Kay, S. A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290*, 2110–2113.

Hashimoto, S., Kohsaka, M., Morita, N., Fukuda, N., Honma, S., and Honma, K. (1996). Vitamin B12 enhances the phase-response of circadian melatonin rhythm to a single bright light exposure in humans. Neurosci Lett *220*, 129–132.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., and Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science *295*, 1065–1070.

He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K. H., and Liu, Y. (2002). White collar-1, a DNA binding transcription factor and a light sensor. Science *297*, 840–843.

Helfrich-Forster, C. (2004). The circadian clock in the brain: a structural and functional comparison between mammals and insects. J Comp Physiol A Neuroethol Sens Neural Behav Physiol *190*, 601–613.

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J. C., and Stanewsky, R. (2001). The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron *30*, 249–261.

Hirota, T., Okano, T., Kokame, K., Shirotani-Ikejima, H., Miyata, T., and Fukada, Y. (2002). Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. J Biol Chem *277*, 44244–44251.

Hogenesch, J. B., Gu, Y. Z., Jain, S., and Bradfield, C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci U S A *95*, 5474–5479.

Humphries, A., Klein, D., Baler, R., and Carter, D. A. (2002). cDNA array analysis of pineal gene expression reveals circadian rhythmicity of the dominant negative helix-loop-helix protein-encoding gene, Id-1. J Neuroendocrinol *14*, 101–108.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the Drosophila protein timeless suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677–685.

Ikeda, M., Asai, M., Moriya, T., Sagara, M., Inoue, S., and Shibata, S. (1998). Methylcobalamin amplifies melatonin-induced circadian phase shifts by facilitation of melatonin synthesis in the rat pineal gland. Brain Res *795*, 98–104.

Ishikawa, K., and Shimazu, T. (1976). Daily rhythms of glycogen synthetase and phosphorylase activities in rat liver: influence of food and light. Life Sci *19*, 1873–1878.

Jin, X., Shearman, L. P., Weaver, D. R., Zylka, M. J., de Vries, G. J., and Reppert, S. M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell *96*, 57–68.

Johansson, C., Willeit, M., Smedh, C., Ekholm, J., Paunio, T., Kieseppa, T., Lichtermann, D., Praschak-Rieder, N., Neumeister, A., Nilsson, L. G., *et al.* (2003). Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference. Neuropsychopharmacology *28*, 734–739.

Jones, C. R., Campbell, S. S., Zone, S. E., Cooper, F., DeSano, A., Murphy, P. J., Jones, B., Czajkowski, L., and Ptacek, L. J. (1999). Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. Nat Med *5*, 1062–1065.

Kaneko, M., Park, J. H., Cheng, Y., Hardin, P. E., and Hall, J. C. (2000). Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. J Neurobiol *43*, 207–233.

Kappas, A., Levere, R. D., and Granick, S. (1968). The regulation of porphyrin and heme synthesis. Semin Hematol *5*, 323–334.

Karakashian, M. W., and Hastings, J. W. (1962). The inhibition of a biological clock by actinomycin D. Proc Natl Acad Sci U S A *48*, 2130–2137.

Keesler, G. A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C., and Yao, Z. (2000). Phosphorylation and destabilization of human period I clock protein by human casein kinase I epsilon. Neuroreport *11*, 951–955.

Kikuchi, G., and Hayashi, N. (1981). Regulation by heme of synthesis and intracellular translocation of delta-aminolevulinate synthase in the liver. Mol Cell Biochem *37*, 27–41.

Kim, M. S., Hur, M. K., Son, Y. J., Park, J. I., Chun, S. Y., D'Elia, A. V., Damante, G., Cho, S., Kim, K., and Lee, B. J. (2002). Regulation of pituitary adenylate cyclase-activating polypeptide gene transcription by TTF-1, a homeodomain-containing transcription factor. J Biol Chem *277*, 36863–36871.

King, D. P., Vitaterna, M. H., Chang, A. M., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1997). The mouse Clock mutation behaves as an antimorph and maps within the W19H deletion, distal of Kit. Genetics *146*, 1049–1060.

King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., *et al.* (1997). Positional cloning of the mouse circadian clock gene. Cell *89*, 641–653.

Kita, Y., Shiozawa, M., Jin, W., Majewski, R. R., Besharse, J. C., Greene, A. S., and Jacob, H. J. (2002). Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. Pharmacogenetics *12*, 55–65.

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., and Young, M. W. (1998). The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. Cell *94*, 97–107.

Kloss, B., Rothenfluh, A., Young, M. W., and Saez, L. (2001). Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the Drosophila clock. Neuron *30*, 699–706.

Ko, H. W., Jiang, J., and Edery, I. (2002). Role for Slimb in the degradation of Drosophila Period protein phosphorylated by Doubletime. Nature *420*, 673–678.

Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S., and Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. Science *266*, 1233–1236.

Konopka, R. J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A *68*, 2112–2116.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., and Reppert, S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193–205.

Kurokawa, H., Lee, D. S., Watanabe, M., Sagami, I., Mikami, B., Raman, C. S., and Shimizu, T. (2004). A redox-controlled molecular switch revealed by the crystal structure of a bacterial heme PAS sensor. J Biol Chem *279*, 20186–20193.

Lavery, D. J., Lopez-Molina, L., Margueron, R., Fleury-Olela, F., Conquet, F., Schibler, U., and Bonfils, C. (1999). Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. Mol Cell Biol *19*, 6488–6499.

Lee, C., Bae, K., and Edery, I. (1998). The Drosophila CLOCK Protein Undergoes Daily Rhythms in Abundance, Phosphorylation, and Interactions with the PER-TIM Complex. Neuron *21*, 857–867.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. Cell *107*, 855–867.

Levitman, M., Kublik, L. N., Shaposhnikova, V. V., Akatov, V. S., and Korystov Iu, N. (2002). [Antitumor effect of combined treatment with ionizing radiation and vitamin B12-C complex]. Radiats Biol Radioecol *42*, 511–514.

Lin, F. J., Song, W., Meyer-Bernstein, E., Naidoo, N., and Sehgal, A. (2001). Photic signaling by cryptochrome in the Drosophila circadian system. Mol Cell Biol *21*, 7287–7294.

Lin, J. M., Kilman, V. L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M., and Allada, R. (2002). A role for casein kinase 2alpha in the Drosophila circadian clock. Nature *420*, 816–820.

Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T. M., Amarakone, A., Awad, T. A., Stormo, G. D., Van Gelder, R. N., and Taghert, P. H. (2002). Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in Drosophila melanogaster. Proc Natl Acad Sci U S A *99*, 9562–9567.

Lincoln, D. W., 2nd, Hrushesky, W. J., and Wood, P. A. (2000). Circadian organization of thymidylate synthase activity in normal tissues: a possible basis for 5-fluorouracil chronotherapeutic advantage. Int J Cancer *88*, 479–485.

Liu, C., Weaver, D. R., Strogatz, S. H., and Reppert, S. M. (1997). Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. Cell *91*, 855–860.

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., Menaker, M., and Takahashi, J. S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science *288*, 483–492.

Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000). Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev *52*, 375–414.

Lund, J., Arendt, J., Hampton, S. M., English, J., and Morgan, L. M. (2001). Postprandial hormone and metabolic responses amongst shift workers in Antarctica. J Endocrinol *171*, 557–564.

Majercak, J., Sidote, D., Hardin, P. E., and Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron *24*, 219–230.

Martinek, S., Inonog, S., Manoukian, A. S., and Young, M. W. (2001). A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. Cell *105*, 769–779.

Maywood, E. S., O'Brien, J. A., and Hastings, M. H. (2003). Expression of mCLOCK and other circadian clock-relevant proteins in the mouse suprachiasmatic nuclei. J Neuroendocrinol *15*, 329–334.

McDonald, M. J., and Rosbash, M. (2001). Microarray analysis and organization of circadian gene expression in Drosophila. Cell *107*, 567–578.

McLean, G. R., Quadros, E. V., Rothenberg, S. P., Morgan, A. C., Schrader, J. W., and Ziltener, H. J. (1997). Antibodies to transcobalamin II block in vitro proliferation of leukemic cells. Blood *89*, 235–242.

Mealey-Ferrara, M. L., Montalvo, A. G., and Hall, J. C. (2003). Effects of combining a cryptochrome mutation with other visual-system variants on entrainment of locomotor and adult-emergence rhythms in Drosophila. J Neurogenet *17*, 171–221.

Mihalcescu, I., Hsing, W., and Leibler, S. (2004). Resilient circadian oscillator revealed in individual cyanobacteria. Nature *430*, 81–85.

Mitsui, S., Yamaguchi, S., Matsuo, T., Ishida, Y., and Okamura, H. (2001). Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. Genes Dev *15*, 995–1006.

Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M. W. (1996). Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. Science *271*, 1736–1740.

Myers, M. P., Wager-Smith, K., Wesley, C. S., Young, M. W., and Sehgal, A. (1995). Positional cloning and sequence analysis of the Drosophila clock gene, timeless. Science *270*, 805–808.

Nagoshi, E., Saini, C., Bauer, C., Laroche, T., Naef, F., and Schibler, U. (2004). Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell *119*, 693–705.

Naidoo, N., Song, W., Hunter-Ensor, M., and Sehgal, A. (1999). A role for the proteasome in the light response of the timeless clock protein. Science *285*, 1737-1741.

Nakamura, T., Uchida, K., Moriguchi, Y., Okamoto, N., and Morita, Y. (1997). Transient fluctuation of serum melatonin rhythm is suppressed centrally by vitamin B12. Chronobiol Int *14*, 549–560.

Nawathean, P., and Rosbash, M. (2004). The doubletime and CKII kinases collaborate to potentiate Drosophila PER transcriptional repressor activity. Mol Cell *13*, 213–223.

Ndisang, J. F., Wu, L., Zhao, W., and Wang, R. (2003). Induction of heme oxygenase-1 and stimulation of cGMP production by hemin in aortic tissues from hypertensive rats. Blood *101*, 3893–3900.

Norman, P. (2001). Pemetrexed disodium (Eli Lilly). Curr Opin Investig Drugs *2*, 1611–1622.

Nowrousian, M., Duffield, G. E., Loros, J. J., and Dunlap, J. C. (2003). The frequency gene is required for temperature-dependent regulation of many clock-controlled genes in Neurospora crassa. Genetics *164*, 923–933.

Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S., and Johnson, C. H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci U S A *95*, 8660–8664.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*, 307–320.

Panda, S., Hogenesch, J. B., and Kay, S. A. (2002). Circadian rhythms from flies to human. Nature *417*, 329–335.

Panda, S., Provencio, I., Tu, D. C., Pires, S. S., Rollag, M. D., Castrucci, A. M., Pletcher, M. T., Sato, T. K., Wiltshire, T., Andahazy, M., *et al.* (2003). Melanopsin is required for non-image-forming photic responses in blind mice. Science *301*, 525–527.

Pellequer, J. L., Brudler, R., and Getzoff, E. D. (1999). Biological sensors: More than one way to sense oxygen. Curr Biol *9*, R416–418.

Pellequer, J. L., Wager-Smith, K. A., Kay, S. A., and Getzoff, E. D. (1998). Photoactive yellow protein: a structural prototype for the three-dimensional fold of the PAS domain superfamily. Proc Natl Acad Sci U S A *95*, 5884–5890.

Pittendrigh, C. (1993). Temporal organization: reflections of a Darwinian clockwatcher. Annu Rev Physiol *55*, 16–54.

Pittendrigh CS, B. V. (1959). Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism.).

Plautz, J. D., Kaneko, M., Hall, J. C., and Kay, S. A. (1997). Independent photoreceptive circadian clocks throughout Drosophila. Science *278*, 1632–1635.

Ponka, P. (1999). Cell biology of heme. Am J Med Sci *318*, 241–256.

Ponka, P. (1999). Cellular iron metabolism. Kidney Int Suppl *69*, S2–11.

Potocki, L., Chen, K. S., Park, S. S., Osterholm, D. E., Withers, M. A., Kimonis, V., Summers, A. M., Meschino, W. S., Anyane-Yeboa, K., Kashork, C. D., *et al.* (2000). Molecular mechanism for duplication 17p11.2 — the homologous recombination reciprocal of the Smith-Magenis microdeletion. Nat Genet *24*, 84–87.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell *110*, 251–260.

Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M. W. (1998). double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell *94*, 83–95.

Ralph, M. R., Foster, R. G., Davis, F. C., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975–978.

Ralph, M. R., and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225–1227.

Reddy, P., Zehring, W. A., Wheeler, D. A., Pirrotta, V., Hadfield, C., Hall, J. C., and Rosbash, M. (1984). Molecular analysis of the period locus in Drosophila melanogaster and identification of a transcript involved in biological rhythms. Cell *38*, 701–710.

Redman, J., Armstrong, S., and Ng, K. T. (1983). Free-running activity rhythms in the rat: entrainment by melatonin. Science *219*, 1089–1091.

Ross, A. W., Webster, C. A., Mercer, J. G., Moar, K. M., Ebling, F. J., Schuhler, S., Barrett, P., and Morgan, P. J. (2004). Photoperiodic regulation of hypothalamic retinoid signaling: association of retinoid X receptor gamma with body weight. Endocrinology *145*, 13–20.

Roth, J. R., Lawrence, J. G., and Bobik, T. A. (1996). Cobalamin (coenzyme B12): synthesis and biological significance. Annu Rev Microbiol *50*, 137–181.

Rothenfluh, A., Abodeely, M., and Young, M. W. (2000). Short-period mutations of per affect a double-time-dependent step in the Drosophila circadian clock. Curr Biol *10*, 1399–1402.

Rothenfluh, A., Young, M. W., and Saez, L. (2000). A TIMELESS-independent function for PERIOD proteins in the Drosophila clock. Neuron *26*, 505–514.

Rudic, R. D., Curtis, A. M., Cheng, Y., and Fitzgerald, G. (2005). Peripheral clocks and the regulation of cardiovascular and metabolic function. Methods Enzymol *393*, 524–539.

Rudic, R. D., McNamara, P., Curtis, A. M., Boston, R. C., Panda, S., Hogenesch, J. B., and Fitzgerald, G. A. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol *2*, e377.

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., and Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. Cell *93*, 805–814.

Rutter, J., Reick, M., and McKnight, S. L. (2002). Metabolism and the control of circadian rhythms. Annu Rev Biochem *71*, 307–331.

Rutter, J., Reick, M., Wu, L. C., and McKnight, S. L. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science *293*, 510–514.

Sanada, K., Okano, T., and Fukada, Y. (2002). Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. J Biol Chem *277*, 267–271.

Sangoram, A. M., Saez, L., Antoch, M. P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E. M., Vitaterna, M. H., Shimomura, K., King, D. P., *et al.* (1998). Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. Neuron *21*, 1101–1113.

Sathyanarayanan, S., Zheng, X., Xiao, R., and Sehgal, A. (2004). Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. Cell *116*, 603–615.

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., Naik, K. A., FitzGerald, G. A., Kay, S. A., and Hogenesch, J. B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron *43*, 527–537.

Scagliotti, G. V., Shin, D. M., Kindler, H. L., Vasconcelles, M. J., Keppler, U., Manegold, C., Burris, H., Gatzemeier, U., Blatter, J., Symanowski, J. T., and Rusthoven, J. J.

(2003). Phase II study of pemetrexed with and without folic acid and vitamin B12 as front-line therapy in malignant pleural mesothelioma. J Clin Oncol *21*, 1556–1561.

Schaffer, R., Landgraf, J., Accerbi, M., Simon, V., Larson, M., and Wisman, E. (2001). Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell *13*, 113–123.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M. P., and Young, M. W. (1995). Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. Science *270*, 808–810.

Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., and Reppert, S. M. (2000). Interacting molecular loops in the mammalian circadian clock. Science *288*, 1013–1019.

Shelley, B. P., and Robertson, M. M. (2005). The neuropsychiatry and multisystem features of the Smith-Magenis syndrome: a review. J Neuropsychiatry Clin Neurosci *17*, 91–97.

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron *1*, 141–150.

Slager, R. E., Newton, T. L., Vlangos, C. N., Finucane, B., and Elsea, S. H. (2003). Mutations in RAI1 associated with Smith-Magenis syndrome. Nat Genet *33*, 466–468.

Smith, A. C., Dykens, E., and Greenberg, F. (1998). Behavioral phenotype of Smith-Magenis syndrome (del 17p11.2). Am J Med Genet *81*, 179–185.

Smith, A. C., Dykens, E., and Greenberg, F. (1998). Sleep disturbance in Smith-Magenis syndrome (del 17 p11.2). Am J Med Genet *81*, 186–191.

Smith, A. C., McGavran, L., Robinson, J., Waldstein, G., Macfarlane, J., Zonona, J., Reiss, J., Lahr, M., Allen, L., and Magenis, E. (1986). Interstitial deletion of (17)(p11.2p11.2) in nine patients. Am J Med Genet *24*, 393–414.

So, W. V., and Rosbash, M. (1997). Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. Embo J *16*, 7146–7155.

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the Drosophila clock gene period and a lacZ reporter: mapping elements of the PER protein involved in circadian cycling. J Neurosci *17*, 676–696.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., Rosbash, M., and Hall, J. C. (1998). The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell *95*, 681–692.

Stevenson, T. H., Gutierrez, A. F., Alderton, W. K., Lian, L., and Scrutton, N. S. (2001). Kinetics of CO binding to the haem domain of murine inducible nitric oxide synthase: differential effects of haem domain ligands. Biochem J *358*, 201–208.

Storch, K. F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F. C., Wong, W. H., and Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78–83.

Stuehr, D. J. (1997). Structure-function aspects in the nitric oxide synthases. Annu Rev Pharmacol Toxicol *37*, 339–359.

Suri, V., Hall, J. C., and Rosbash, M. (2000). Two novel doubletime mutants alter circadian properties and eliminate the delay between RNA and protein in Drosophila. J Neurosci *20*, 7547–7555.

Swanson, H. I., and Bradfield, C. A. (1993). The AH-receptor: genetics, structure and function. Pharmacogenetics *3*, 213–230.

Sweeney, B. M., and Hastings, J. W. (1960). Effects of temperature upon diurnal rhythms. Cold Spring Harb Symp Quant Biol *25*, 87–104.

Takano, A., Isojima, Y., and Nagai, K. (2004). Identification of mPer1 phosphorylation sites responsible for the nuclear entry. J Biol Chem *279*, 32578–32585.

Takano, A., Shimizu, K., Kani, S., Buijs, R. M., Okada, M., and Nagai, K. (2000). Cloning and characterization of rat casein kinase 1epsilon. FEBS Lett *477*, 106–112.

Takano, A., Uchiyama, M., Kajimura, N., Mishima, K., Inoue, Y., Kamei, Y., Kitajima, T., Shibui, K., Katoh, M., Watanabe, T., *et al.* (2004). A Missense Variation in Human Casein Kinase I Epsilon Gene that Induces Functional Alteration and Shows an Inverse Association with Circadian Rhythm Sleep Disorders. Neuropsychopharmacology *29*, 1901–1909.

Taylor, B. L., and Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev *63*, 479–506.

Thain, S. C., Hall, A., and Millar, A. J. (2000). Functional independence of circadian clocks that regulate plant gene expression. Curr Biol *10*, 951–956.

Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., Ptacek, L. J., and Fu, Y. H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040–1043.

Travnickova-Bendova, Z., Cermakian, N., Reppert, S. M., and Sassone-Corsi, P. (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc Natl Acad Sci U S A *99*, 7728–7733.

Tunctan, B., Weigl, Y., Dotan, A., Peleg, L., Zengil, H., Ashkenazi, I., and Abacioglu, N. (2002). Circadian variation of nitric oxide synthase activity in mouse tissue. Chronobiol Int *19*, 393–404.

Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R., *et al.* (2005). Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. Science.

Uchiyama, M., Mayer, G., Okawa, M., and Meier-Ewert, K. (1995). Effects of vitamin B12 on human circadian body temperature rhythm. Neurosci Lett *192*, 1–4.

Ueda, H. R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., *et al.* (2002). A transcription factor response element for gene expression during circadian night. Nature *418*, 534–539.

Ueda, H. R., Chen, W., Minami, Y., Honma, S., Honma, K., Iino, M., and Hashimoto, S. (2004). Molecular-timetable methods for detection of body time and rhythm disorders from single-time-point genome-wide expression profiles. Proc Natl Acad Sci U S A *101*, 11227–11232.

Ueda, H. R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet *37*, 187–192.

Ueda, H. R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., and Hashimoto, S. (2002). Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. J Biol Chem *277*, 14048–14052.

Ueda, M., Kinoshita, H., Maeda, S. I., Zou, W., and Tanaka, A. (2003). Structure-function study of the amino-terminal stretch of the catalase subunit molecule in oligomerization, heme binding, and activity expression. Appl Microbiol Biotechnol *61*, 488–494.

van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., *et al.* (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature *398*, 627–630.

Vielhaber, E., Eide, E., Rivers, A., Gao, Z. H., and Virshup, D. M. (2000). Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. Mol Cell Biol *20*, 4888–4899.

Vitaterna, M. H., King, D. P., Chang, A. M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science *264*, 719–725.

Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., *et al.* (1999). Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proc Natl Acad Sci U S A *96*, 12114–12119.

Vosshall, L. B., Price, J. L., Sehgal, A., Saez, L., and Young, M. W. (1994). Block in nuclear localization of period protein by a second clock mutation, timeless. Science *263*, 1606–1609.

Vosshall, L. B., and Young, M. W. (1995). Circadian rhythms in Drosophila can be driven by period expression in a restricted group of central brain cells. Neuron *15*, 345–360.

Walz, K., Caratini-Rivera, S., Bi, W., Fonseca, P., Mansouri, D. L., Lynch, J., Vogel, H., Noebels, J. L., Bradley, A., and Lupski, J. R. (2003). Modeling del(17)(p11.2p11.2) and dup(17)(p11.2p11.2) contiguous gene syndromes by chromosome engineering in mice: phenotypic consequences of gene dosage imbalance. Mol Cell Biol *23*, 3646–3655.

Warren, M. J., and Scott, A. I. (1990). Tetrapyrrole assembly and modification into the ligands of biologically functional cofactors. Trends Biochem Sci *15*, 486–491.

Wever, R. A. (1979). The circadian system of man: results of experiments under temporal isolation (New York, Springer-Verlag).

Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K., and Johnson, C. H. (2004). The adaptive value of circadian clocks; an experimental assessment in cyanobacteria. Curr Biol *14*, 1481–1486.

Xu, Y., Padiath, Q. S., Shapiro, R. E., Jones, C. R., Wu, S. C., Saigoh, N., Saigoh, K., Ptacek, L. J., and Fu, Y. H. (2005). Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature *434*, 640–644.

Yagita, K., Tamanini, F., Yasuda, M., Hoeijmakers, J. H., van der Horst, G. T., and Okamura, H. (2002). Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. Embo J *21*, 1301–1314.

Yamaguchi, S., Isejima, H., Matsuo, T., Okura, R., Yagita, K., Kobayashi, M., and Okamura, H. (2003). Synchronization of cellular clocks in the suprachiasmatic nucleus. Science *302*, 1408–1412.

Yamaguchi, S., Mitsui, S., Yan, L., Yagita, K., Miyake, S., and Okamura, H. (2000). Role of DBP in the circadian oscillatory mechanism. Mol Cell Biol *20*, 4773–4781.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., and Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science *288*, 682–685.

Yang, Z., and Sehgal, A. (2001). Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron *29*, 453–467.

Yildiz, O., Doi, M., Yujnovsky, I., Cardone, L., Berndt, A., Hennig, S., Schulze, S., Urbanke, C., Sassone-Corsi, P., and Wolf, E. (2005). Crystal structure and interactions of the PAS repeat region of the Drosophila clock protein PERIOD. Mol Cell *17*, 69–82.

Yoo, S. H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M., Hong, H. K., Oh, W. J., Yoo, O. J., *et al.* (2004). PERIOD2:LUCIFERASE real-time

reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A *101*, 5339–5346.

Young, M. W., and Kay, S. A. (2001). Time zones: a comparative genetics of circadian clocks. Nat Rev Genet *2*, 702–715.

Yu, X., Burgess, S. C., Ge, H., Wong, K. K., Nassem, R. H., Garry, D. J., Sherry, A. D., Malloy, C. R., Berger, J. P., and Li, C. (2005). Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. Proc Natl Acad Sci U S A *102*, 1767–1772.

Zeng, H., Qian, Z., Myers, M. P., and Rosbash, M. (1996). A light-entrainment mechanism for the Drosophila circadian clock. Nature *380*, 129–135.

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. J Neurosci *10*, 2749–2762.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., Lee, C. C., and Bradley, A. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature *400*, 169–173.

Zhu, Y., Sun, Y., Jin, K., and Greenberg, D. A. (2002). Hemin induces neuroglobin expression in neural cells. Blood *100*, 2494–2498.

Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., Bryant, P. J., and Noll, M. (1999). double-time is identical to discs overgrown, which is required for cell survival, proliferation and growth arrest in Drosophila imaginal discs. Development *126*, 5409–5420.

SUMMARY IN ESTONIAN

Ööpäeva rütme tekitavate geenide roll imetajate bioloogilises kellas, ainevahetuses ja käitumises.

Maakera pöörlemisest ümber oma telje tingitud öö ja päeva vaheldumine põhjustab tsirkadiaanseid rütme (*circa*-ligikaudu, *diem*-päev) peaaegu kõikide organismide käitumises ja füsiloogias. Need 24-tunnise perioodiga rütmid on endogeensed, keskkonna tingimustest (valgus, temperatuur) sõltumatud. Ööpäeva rütme tekitab endogeenne ehk bioloogiline kell, mis piltlikult öeldes tagab, et organismis tehakse "õigeid asju õigel ajal". Imetajatel asub tsirkadiaanne kell hüpotaalamuses *nucleus chiasmaticus superior*'s, koosnedes ligikaudu 10000 neuronist. Selleks, et füsioloogilised (toitumine, ainevahetus) ja käitumuslikud (ärkvelolek ja magamine) protsessid toimuksid kooskõlastatult ja sobival ajal, on igas koes ja rakus oma kellad, mis ajastavad lokaalseid biokeemilisi protsesse ja on sünkroniseeritud hüpotaalamuse kella poolt.

Molekulaarsete ja geneetiliste meetoditega on näidatud, et ööpäeva rütmide perioodi pikkuse tagab kella põhikomponentide negatiivne transkriptsiooniline tagasiside. Imetajatel BMAL1/Clock valkude kompleks aktiveerib *Per* ja *Cry* geenide transkriptsiooni tuumas. PER/CRY valkude kompleks omakorda interakteerub BMAL1/Clock valkude kompleksiga, et blokeerida viimaste aktiivsus. BMAL1/Clock valkude kompleks aktiveerib *Rev-Erbα* geeni, mis omakorda represseerib *Bmal1* ekspressiooni läbi Rev-Erbα/Ror seostumise järjestuse *Bmal1* geeni promootoris. Negatiivne transkriptsiooniline tagasiside seisneb selles, et PER ja CRY valgud blokeerivad oma enda geenide transkriptsiooni. Uus transkriptsiooni aktivatsioon saab alata kui suurem osa PER, CRY ja REV-ERBα valke on degradeeritud. Ainult mRNA ossileerumisest ei piisa kella "tiksumiseks", selleks on vaja ka posttranslatsioonilisi modifikatsioone, mis reguleerivad valkude stabiilsust ja aktiivsust.

Käesoleva töö eesmärgiks oli uurida kellageenide ja nendest sõltuvate geenide transkriptsiooni hiires ja kuidas kellageenid mõjutavad metabolismi (heemi biosünteesi) ja käitumist (Smith-Magenis'e sündroomi näitel).

Hiire PER1 ja PER2 valgud on järjestuselt homoloogsed ja siiani arvati, et üks kompenseerib teist. Selleks, et selgitada PER1 ja PER2 rolli imetajate kellas uuriti *Per1* ja/või *Per2* geenimutatsiooniga hiiri. Võrreldes normaalsete hiirtega on *Per1* ja *Per2* geenimutatsiooniga hiirtel tsirkadiaanse lokomotoorse aktiivsuse perioodi pikkus konstantsetes keskkonna tingimustes vastavalt üks ja kaks tundi lühem. Hiirtel, kellel on nii *Per1* kui ka *Per2* geenimutatsioon, puudub tsirkadiaanne rütm lokomotoorses aktiivsuses. *Per1* ja *Per2* üksikmutantsed hiired käituvad erinevalt ja tõenäoliselt on ka PER1 ja PER2 valkude molekulaarne roll kellas erinev. Selle hüpoteesi testimiseks uuriti nende hiirte tsirkadiaanseid rütme molekulaarsel tasemel ja leiti, et *Per1* ja *Per2* kaksikmutatsiooniga hiirte kõikidel kellageenidel ja kella kontrolli all olevatel geenidel

puudus ööpäevaringselt rütmiline ekspressioon. Analüüsides kella kontrolli all olevaid geene *Per1* ja *Per2* üksikmutantidest, leiti, et kesksed kella komponendid PER1 ja PER2 aktiveerivad nii ühiseid kui ka erinevad geene, näidates, et lisaks sarnastele rollidele on *Per1* ja *Per2* geenidel hiire kellas ka erinevaid, üksteisest sõltumatuid funktsioone. DNA mikrokiibi meetodiga leiti, et paljude geenide ekspressioon on rütmiline. Näiteks heemi biosünteesi limiteeriva ensüümi aminolevulinaadi süntaasi ekspressioon ossileerub 24-tunnise perioodiga normaalses hiires, kuid mutantides on ekspressiooni rütm ebanormaalne. Siit tekkis huvi uurida kuidas heemi biosüntees on reguleeritud tsirkadiaanse kella poolt. Esiteks katsetati heemi mõju kellageenide transkriptsioonile normaalsete hiirte maksas. Heemi (heemkloriid) süstid indutseerisid *Per1*, kuid represseerisid *Per2* ekspressiooni. Teiste uuritud kellageenide (*Bmal1*, *Npas2*, *Cry1*, *Clock*) transkriptsiooni heem ei mõjutanud. Heemi mõju kellale väljendus nii molekulaarsel tasemel kui ka hiirte käitumises, inhibeerides lokomotoorse aktiivsuse rütmi spetsiifiliselt öösel. Seega, molekulaarsete ja käitumuslike tulemuste põhjal võib järeldada, et heemi mõju kellale avaldub läbi rütmiliselt reguleeritud faktorite. Edasi uuriti Clock valgu homoloogi NPAS2, sest varem oli näidatud, et NPAS2 valgu PAS domäänid interakteeruvad heemiga. *Per1*, *Per2* ja *Npas2* geenimutantide uurimisel selgus, et heem võib mõjutada kella läbi NPAS2 ja PER2 valgu. Üllatusena selgus, et *Npas2* ei ekspresseeru *Per2*$^{-/-}$ hiire maksas, näidates, et PER2 valk on *Npas2* geeni positiivne regulaator. Koekultuuri katsed kinnitasid, et PER2 valk on NPAS2 valgu positiivne regulaator kui viimane on kompleksis BMAL1 valguga. Siiani avaldatud tulemused olid näidanud, et PER2 valgul on imetaja kellas repressori roll, kuid kõik need põhinesid BMAL1/Clock transkriptsiooni kompleksi ja PER2 valgu omavahelisel transkriptsioonilise tagasiside uurimisel. *In vivo* (vähemalt maksas) on Npas2 vajalik *Alas1* rütmiliseks ekspressiooniks. *Npas2* hiiremutandis on *Alas1* mRNA represseeritud ja BMAL1/NPAS2/PER2 valkude kompleks aktiveerib *Alas1* promootorit koekultuuris. Järgnevalt uuriti, kas PER2 võib mõjutada NPAS2 aktiivsust läbi heemi. PER2 seostumise katsetest heemi agaroosiga selgus, et PER2 valgu PAS domään seob heemi. Teine uuritud porfüriini molekul, vitamiin B12, mille struktuur sarnaneb heemile, võistles seostumises PER2 valguga *in vitro*. *In vivo*, vitamiin B12, erinevalt heemist, aktiveerib *Per2* ja inhibeerib *Per1* geeni ekspressiooni. Nende tulemuste põhjal võib järeldada, et ainevahetus ja tsirkadiaanne kell on omavahel vastastikku reguleeritud ning spekuleerida porfüriini molekulide kasutusest tsirkadiaansete haiguste raviks.

Käesoleva töö viimases osas uuriti kas Smith-Magenis'e sündroomi sümptomid esinevad ka hiirtel, kellel on inimese haigusega assotsieerunud DNA piirkonnale vastav genoomi osa muteeritud. Smith-Magenis'e sündroom ja dup (17)(p11.2p11.2) on külgnevate geenide haigus, mida on seostatud 17. kromosoomi lühikese õla p11.2 piirkonna duplikatsiooni või deletsiooniga. Töös uuriti hiire 11. kromosoomi sünteense piirkonna deletsiooniga ja duplikatsiooniga loomade käitumist. Analüüsiti heterosügootseid hiiri, sest sündroomi põhjustavas piirkonnas on ligikaudu 30 geeni ja terve piirkonna eemaldamine on letaalne.

Lisaks uuriti kas tsirkadiaanne kell on rikutud, sest peaaegu kõigil Smith-Magenis'e sündroomiga patsientidel on kirjeldatud unehäireid. Osadel patsientidel on kirjeldatud ka unefaasi nihet varasemasse ajavahemikku ööpäevas. Varem oli näidatud, et *Per2* geenimutatsioon põhjustab unefaasi nihet varasemasse ajavahemikku ööpäevas. Lokomotoorse aktiivsuse testi tulemused näitasid, et deletsiooniga hiirtel oli lühem tsirkadiaanne periood kui normaalsetel hiirtel. Sellest tulenevalt võiks edasi uurida *Dexras1* geeni, mis oli üks deleteeritud geenidest, kui võimalikku kandidaatgeeni. Hiljuti avastati, et *Dexras1* ekspressioon on rütmiline ja *Dexras1*$^{-/-}$ hiirtel on lühem lokomotoorse aktiivsuse rütmi pikkus konstantsetes tingimustes võrreldes normaalsete hiirtega. Teised uuritud käitumistestid näitasid, et nii nagu patsientidel on ka vastavalt muteeritud hiirtel õppimisraskused. Duplikatsiooniga hiired olid hüpoaktiivsed, samas kui deletsiooniga hiired käitusid hüperaktiivselt. Seega Smith-Magenis'e sündroomi tunnustega hiiremudelid mimikeerivad seda haigust inimesel ning uuritud regioonis paikneb haigust põhjustav geenidoosist sõltuv piirkond.

Käesoleva töö tulemuste põhjal võib järeldada: 1. *Period1* ja *Period2* geenidel on imetajate kellas erinev roll, nad reguleerivad erinevalt kellast sõltuvaid füsioloogilisi ja käitumuslikke protsesse; 2. Heemi biosüntees ja bioloogiline kell on omavahel vastastikku reguleeritud; 3. Smith-Magenis'e sündroomi unehäired võivad olla tingitud ebanormaalselt ekspresseeritud tsirkadiaanset kella reguleerivatest geenidest.

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PUBLICATIONS

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Scientific Interests

During my undergraduate and master studies I investigated human inherited disorders such as cystic fibrosis, familial breast cancer and Alzheimer disease.

During my graduate studies I got fascinated about circadian rhythms. My research subject transferred towards mouse genetics. My current thesis is based on four years of studies on molecular mechanisms of mammalian circadian clock using molecular and behavioral methods.

PUBLICATIONS

Kaasik K and Lee CC. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature*. 2004 Jul 22; 430 (6998): 467–71.

Walz K, Spencer C, **Kaasik K**, Lee CC, Lupski JR, Paylor R. Behavioral characterization of mouse models for Smith-Magenis syndrome and dup (17)(p11.2p11.2). *Hum Mol Genet.* 2004 Feb 15; 13(4): 367–78.

Greenfield JP, Leung LW, Cai D, **Kaasik K**, Gross RS, Rodriguez-Boulan E, Greengard P, Xu H. Estrogen lowers Alzheimer beta-amyloid generation by stimulating trans-Golgi network vesicle biogenesis. *J Biol Chem*. 2002 Apr 5; 277(14): 12128–36.

Zheng B, Albrecht U**, Kaasik K**, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*. 2001 Jun 1; 105(5): 683–94.

Teder M, Klaassen T, Oitmaa E, **Kaasik K**, Metspalu A. Distribution of CFTR gene mutations in cystic fibrosis patients from Estonia. *J Med Genet*. 2000 Aug; 37(8): E16.

Tonisson N, Kurg A, **Kaasik K,** Lohmussaar E, Metspalu A. Unravelling genetic data by arrayed primer extension. *Clin Chem Lab Med*. 2000 Feb; 38(2):165–70.

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Teadustegevus

Üliõpilasena ja magistrandina tegelesin inimese pärilike haiguste — tsüstiline fibroos, perekondlik rinnakasvaja ja Alzheimeri haiguse, uurimisega. Doktorantuuri õpingutes lisandus minu huviorbiiti hiiregeneetika ja mu uurimistöö teemaks oli imetajate tsirkadiaanne kell. Käesolevas väitekirjas on uuritud molekulaarsete ja geneetiliste meetotidega bioloogilise kella mehhanisme ja selle poolt reguleeritud füsioloogilisi ja käitumuslikke protsesse.