Functional Analysis of Mammalian Cryptochromes

A Matter of Time

The studies presented in this thesis were performed in the Department of Genetics, Chronobiology and Health Researcher Group at the Erasmus University Medical Centre in Rotterdam, The Netherlands. The Department is a member of the Medisch Genetisch Centrum Zuid-West Nederland (MGC).
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Functionele analyse van zoogdier cryptochromen
Een kwestie van tijd

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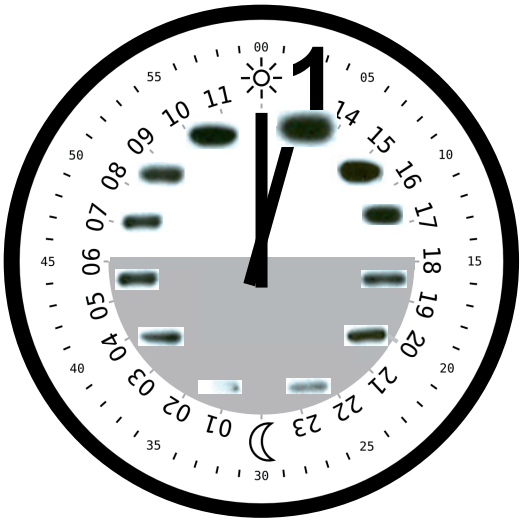
Introduction



I The circadian clock

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...a rose is not necessarily and unqualifiedly a rose...
it is a very different biochemical system at noon and at midnight.
Colin Pittendrigh, 1965

We live in a clockwork universe – dictated by the particular configuration of our solar system, such as the movements of planets and gravity. The annual rotation of the Earth around the Sun is the primary cause of seasonal fluctuations in temperatures, whereas the Earth's rotation around its axis causes daily alterations in temperature and light conditions. Other phenomena, such as tides, are influenced by the monthly revolution of the Moon around the Earth. As one complete rotation of the Earth takes 24 hours, all living organisms have adapted by evolving their own internal clockwork tuned to a 24-hour day/night cycle to adapt their behaviour, physiology and metabolism.

Circadian rhythms, as their name indicates ("*circadian*" comes from the Latin *circa*, meaning "around" and *dies*, meaning "a day"), are endogenous rhythms with a period of about 24 hours that regulate all aspects of the physiology of most organisms (e.g. blood pressure, body temperature, hormonal levels), behaviour (e.g. alertness, sleep cycle) and metabolism (Gachon et al., 2004; Lowrey et al., 2004; Reppert and Weaver, 2001). These internal clocks are reset to light/dark cycles and other daily external cues known as *zeitgebers* (German meaning time-givers), and therefore circadian rhythms are synchronized with the external environment. In mammals, the light is the major *zeitgeber* for circadian rhythms (Dijk et al., 1995). In rodents, circadian rhythms of wheel-running activity are presented by doubleplotted actograms (Figure 1). When environmental cues are eliminated by moving organism to constant darkness, the circadian clock is no longer reset each day and its endogenous periodicity, called free-running period, is revealed and can be determined.

As light is the major *zeitgeber* of the circadian clock it can reset the clock in accordance with the phase response curve (PRC). The PRC illustrates the relationship between the timing and the effect of a treatment designed to affect the circadian clock. Depending on the timing, light can advance or delay the circadian rhythm. Exposure to bright light early or late in the subjective night induces phase delay or phase advance of the circadian clock, respectively (Daan and Pittendrigh, 1976).

Historical background

Daily rhythms in plants and animals have been observed since early times. In 1729, the French astronomer Jean Jacques d'Ortous de Marian performed the first known experiment on biological rhythms (De Marian, 1773). He noticed that the mimosa plant leaves are open during the day and are folded at night. By moving the plant to the

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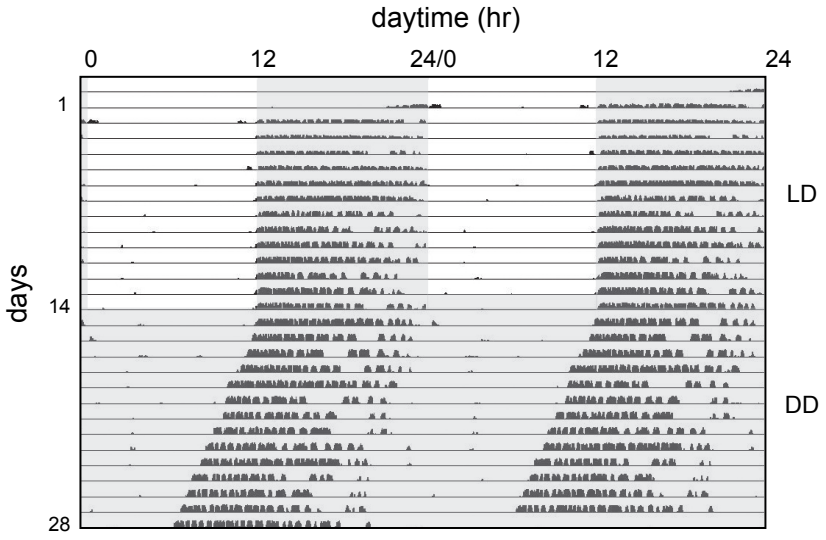

Figure 1. Representative double-plotted actogram of wild-type mice
C57BL/6J mice under LD (12:12) – Light (12 h) / Dark (12 h) and DD – Dark/Dark condition. Shading indicates darkness and the black vertical lines represent animal activity.

dark, he observed that the daily rhythmic motions of the mimosa leaves persisted even in the absence of sunlight. Also in the 18th century, Swedish naturalist Carolus Linnaeus designed a living clock – a garden with flowers which opened at various times around the clock (Linnaeus, 1758). A hundred years later, Charles Darwin described in his book "The power of movement in plants" (Darwin and Darwin, 1880) the folded-leaf state of plants at night as "sleep" and hypothesized that it was a way for plants to reduce exposure, and thus conserve energy. The first proof of the existence of a biological clock came from studies performed by Gustav Kramer and Karl von Frisch. They showed that birds and bees can maintain a given direction throughout the day using a time-compensated sun compass in navigation (Kramer G., 1952 a, b; Von Frisch K., 1950,1974). To use the sun as a compass, an organism must compensate for the movement of the sun. Therefore it was concluded that birds and bees have an internal clock which helps them to recognize the direction on the basis of the sun. Klaus Hoffmann showed that the clock persists in dim light, as birds can use the sun as a compass at a time of the day at which they had never seen the sun, thus the clock is endogenous (Hoffman, 1960). Inspired by the hypothesis that circadian systems are conserved throughout evolution, Colin Pittendrigh demonstrated that circadian clocks are temperature compensated (Pittendrigh, 1954). He demonstrated that temperature changes did not affect the clock

system controlling the time at which Drosophila adults emerge from puparia. Since then (1954), temperature compensation has been shown to be a universal feature of circadian oscillations. Jürgen Aschoff formulated the "Circadian Rule", which states "that in light-active animals: the spontaneous frequency (Aschoff, 1958), the ratio of activity time to rest time (Aschoff, 1960), and the total activity (Aschoff, 1963) all increase with increasing intensity of continuous illumination." Finally it was shown that circadian rhythms are present in all major groups of organisms (Pittendrigh and Minis, 1964; Evans, 1966; Menaker, 1969).

Circadian oscillator mechanisms

The circadian clock is based on an oscillating system – one that changes back and forth between two extreme states in a regular rhythm, in much the same way as a pendulum drives the clock (changing position between two extreme physical positions). The system has an input (primarily in the form of light signals), an output (biochemically controlled cycles), and one or more core oscillatory mechanisms. On the basis of studies in *Drosophila* and mice, the core circadian oscillator is thought to consist of one or more transcription/translation feedback loops.

The first key finding in our understanding of the genetic basis of circadian rhythms came with the identification of the first clock mutant flies (*Drosophila*) with different behavioural phenotypes, i.e. abnormally long or short rhythms and arrhythmicity (Konopka and Benzer, 1971). The gene responsible for keeping normal circadian pattern of flies was named *Period* (*Per*). The identification of the *Per* gene (Yu et al., 1987) was followed by the discovery of additional clock genes in *Drosophila* and in other organisms, including mammals.

The second *Drosophila* clock gene identified was *Timeless* (*Tim*) (Myers et al., 1995). The product of this gene, the TIM protein, dimerizes with the PER protein, stabilizes phosphorylated PER and promotes PER localization to the nucleus (Gekakis et al., 1995; Edery, 1999). Work from the Young lab showed that TIM is degraded in response to light (Myers et al., 1996). Soon after it was demonstrated that the circadian transcription of the *Per* and *Tim* genes is induced by the *Drosophila* CLOCK protein, a bHLH-PAS [basic-helix–loop-helix (bHLH) PAS (PER-ARNT-SIM)] transcription factor (Darlington et al., 1998). Another bHLH-PAS transcription factor that binds to CLOCK is CYCLE (Rutila et al., 1998). In *Drosophila* PER, TIM, CLOCK and CYCLE are the core elements of the transcription/translation feedback loops of the circadian clock (discussed in more detail later in relation to the mammalian clock) (Reppert, 1998; Dunlap, 1999) (Figure 2A). In addition, factors that mediate post-transcriptional regulatory events are also required for the circadian oscillator. In *Drosophila,* the DOUBLE-TIME kinase (DBT, a mammalian casein kinase Iε (CKIε) ortholog identified by genetic screening) plays such a role (Price et al., 1998). DBT

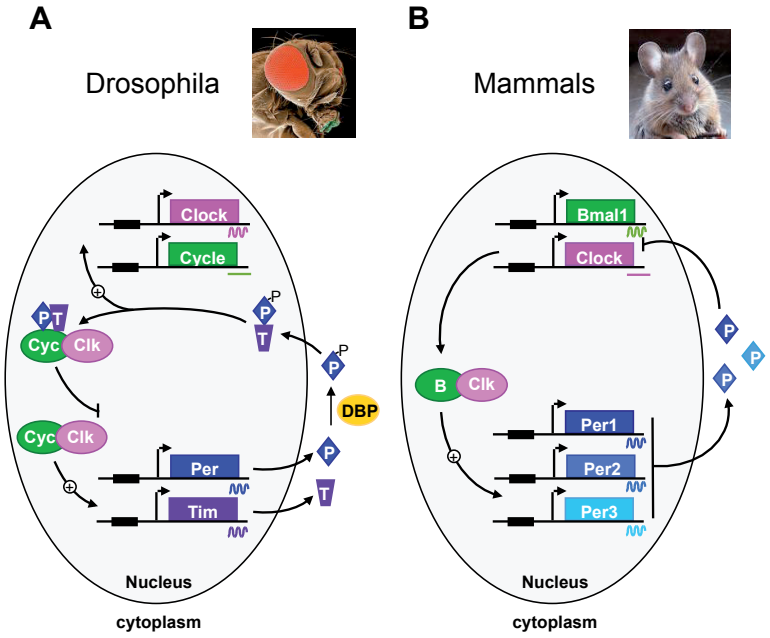

Figure 2. Schematic representation of the molecular model rhythm generation based on the transcription/ translation feedback loops in 1998 A) in Drosophila and B) in mammals

A In Drosophila CLOCK (pink oval) and CYCLE (green oval) heterodimer enhance the transcription of *Period - Per* (indigo rectangle) and *Timless - Tim* (purple rectangle). DOUBLE-TIME kinase – DBT (yellow oval) phosphorylates and consequently destabilizes the PER protein (indigo diamond), which in turn prevents PER from accumulating until it forms a heterodimer with TIM (purple trapezoid). After PER and TIM dimerize and translocate to the nucleus they repress CLOCK/CYCLE mediated transcription.

B In mammals the CLOCK (green oval) and BMAL1 (pink oval) heterodimer enhances the transcription of *Period - Per1* (indigo rectangle), *Per2* (blue rectangle) and *Per3* (cobalt rectangle). After PER proteins (indigo, blue and cobalt diamonds) reach their critical level in the cytoplasm they translocate to the nucleus to repress CLOCK/BMAL1 mediated transcription.

phosphorylates and consequently destabilizes the PER protein, which in turn prevents PER from accumulating until it forms a heterodimer with TIM (Edery, 1999). This post-transcriptional regulation leads to a substantial (approximately 6 hour) delay between *Per* mRNA and PER protein accumulation.

The first circadian gene identified in mammals (as a result of genetic screening for mutant mice with disturbed circadian rhythmicity) was *Clock*. This gene, isolated by positional cloning and transgenic bacterial artificial chromosome (BAC) mutant rescue, was shown to encode the bHLH-PAS transcription factor CLOCK (King et al., 1997; Antoch et al., 1997). CLOCK heterodimerizes with another bHLH-PAS transcription factor named BMAL1 which was discovered using a yeast two-hybrid screen for proteins

that interact with CLOCK (Gekakis et al., 1998; Hogenesch et al., 1998). CLOCK and BMAL1 are homologs of the *Drosophila* CLOCK and CYCLE proteins, respectively. Subsequently, PCR-based molecular screens uncovered three mouse *Per* orthologs (*mPer1, mPer2* and *mPer3*) (Sangoram et al., 1998; King and Takahashi, 2000).

Like in *Drosophila*, CLOCK, BMAL1 and PER act in transcription/translation feedback loops in which CLOCK and BMAL1 are positive elements, and PER is the negative element of the circuit (Whitmore et al., 1998; Wilsbacher and Takahashi, 1998) (Figure 2B). In the conventional model, transcription of the *mPer* genes is driven by the CLOCK/BMAL1 heterodimer that binds to CANNTG E-box enhancer elements in the promoter of the *mPer* gene. Following translation of the *mPer* mRNAs, PER proteins inhibit CLOCK/BMAL1 and accordingly switch down transcription of their own genes (Whitmore et al., 1998; Wilsbacher and Takahashi, 1998).

Soon after, other core clock components were discovered including CRYPTOCHROMES (see introduction part II). In addition to the network of factors involved in transcriptional control, factors that mediate post-transcriptional regulatory events (like *Drosophila* DBT) are also required for the circadian oscillator function in mammals (see introduction part II).

Master clock – suprachiasmatic nucleus

In search for the brain region involved in driving circadian rhythm several brain regions were ablated. Rats with lesions in the suprachiasmatic nuclei (SCN) caused the animal's locomotor activity to become arrhythmic, showing that the SCN is the structure driving circadian rhythms in behaviour (Moore and Eichler, 1972). The suprachiasmatic nuclei (SCN) are located in the brain at the base of the hypothalamus, directly above the optical chiasma. They consist of about 10,000 neurons per nucleus. The same effect of lesions in the SCN was observed in other mammals like golden hamsters (Refinetti et al., 1994) and mice (Schwartz and Zimmerman, 1991). The transplantation experiment of fetal SCN into SCN-lesioned hamsters shows that it can restore behavioural rhythmicity in adult animals which were previously made arrhythmic by SCN lesions (Lehman et al., 1987). The final proof clearly defining the SCN as the master clock in mammals came soon after the discovery of the (natural) *tau* mutant golden hamster, exhibiting circadian behaviour with a periodicity of 20 h (for homozygous animals) as compared to 24 h for wild type animals (Ralph and Menaker, 1988). By transplanting fetal SCN tissue from hamsters with a short period to SCN-lesioned wild type recipients circadian rhythms were restored with a short period, demonstrating that the SCN is the location of the master clock.

Circadian behaviour is driven by SCN-derived electrical signals, which show high firing rates during daytime and low activity during the night (Green and Gillette 1982; Meijer et al., 1997). SCN receives light re-setting signals transmitted from the

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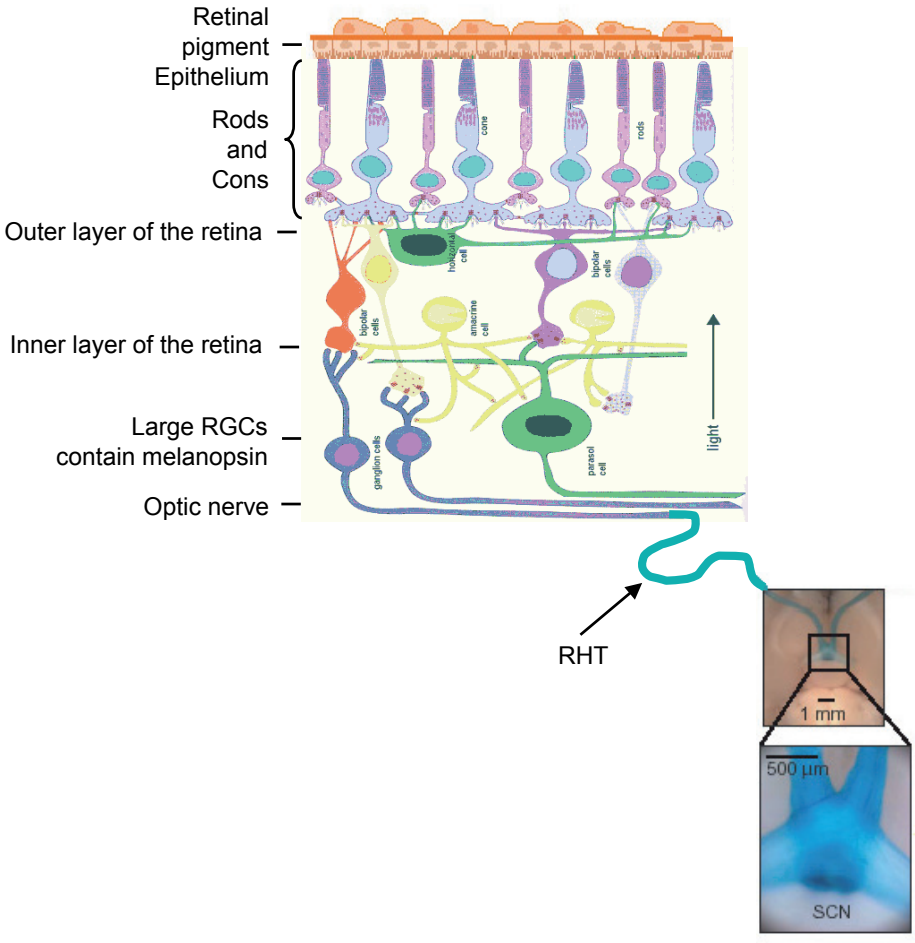

Figure 3. Diagram showing the cellular organization of the primate retina
Visual photoreceptors (rods and cones) located next to the pigment epithelium are stimulated by light that passes various layers of the retina. Retinal ganglion cells (RGCs) are also photosensitive and provide photic input to the circadian system located in the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT).
Retinal connections - Simplified diagram adapted from Dowling (1997) Encyclopedia of Human Biology, vol. 7 pp 571-87.

retina (Rusak, et al., 1989) and in turn, it sends humoral and neuronal signals that will synchronise the peripheral oscillators, located in virtually every cell or tissue (Balsalobre et al., 1998; McNamara et al., 2001; Pando et al., 2002; Yoo et al., 2004).

Photoentrainment

In order to keep pace with the daily light/dark cycle, the SCN neurons receive photic information as electrical signals transmitted via direct synaptic connections with the retina, the retinohypothalamic tract (Ralph and Menaker, 1989; Rusak et al., 1989) (Figure 3). Molecular and genetic studies have been performed in order to characterize the light-input pathway to the circadian oscillator in mammals and several candidate photoreceptors have been proposed including visual photoreceptors, cryptochromes and melanopsin.

Light for vision is absorbed by rhodopsin in rods and color opsins in cones, which are located in the outer layer of the retina (Figure 3). To determine whether retinal rods and cones are required for photoentrainment, the effects of light on the regulation of circadian wheel-running behaviour were examined in mice lacking these photoreceptors (Freedman et al., 1999). It was shown that mice without cones (*cl* mouse model) or without both rods and cones (*rdta*/*cl*) showed normal phase-shifting responses to light, whereas removal of the eyes abolishes this behaviour. Therefore it was concluded that neither rods nor cones are required for photoentrainment, and the eye must contain additional photoreceptors that regulate the circadian clock.

Initially, mammalian cryptochromes (CRY) were proposed to be photoreceptors as they are expressed in both the outer and inner nuclear layer as well as in the ganglion cells of the retina (Miyamoto and Sancar, 1998) and they show homology with blue-light photoreceptors. As CRY proteins are among the core circadian components of CLOCK/BMAL1 transcription (Kume et al., 1999; Griffin et al., 1999), the role of CRY in photoentrainment remains unclear. (Mammalian cryptochromes, for details see chapter 1.III)

At present, an attractive candidate for a photoreceptor is an opsin-like protein called melanopsin, which is expressed by a subset of mouse and human retinal ganglion cells (RGCs) (Provencio et al., 1998, 2000; Gooley et al., 2001; Berson et al., 2002; Hattar et al., 2002). RGCs are in the inner nuclear layer, located in the front part of the retina and project the light signals via the retinohypothalamic tract (RHT) to the SCN (Figure 3). Melanopsin null mice (*Opn4*$^{-/-}$) have been generated and it turned out that melanopsin is required for normal circadian responses to the light, as those mice displayed severely attenuated phase resetting in response to brief pulses of light (Panda et al., 2002). In those animals some residual photoentrainment was still present, suggesting that, in addition to melanopsin, other photoreceptors, perhaps rods and cones, might contribute to circadian photoentrainment in mammals. Indeed, mice lacking both,

rods and cones and deficient in melanopsin, exhibit complete loss of photoentrainment of circadian rhythmicity indicating the importance of both nonvisual and visual photoreceptors for nonvisual photic responses in mammals (Panda et al., 2003).

Peripheral clock

Molecular oscillators are not only restricted to the master clock, but they are also found in other regions of the brain and in most if not all peripheral tissues (Emery et al., 1997; Krishnan et al., 1999; Giebultowicz et al., 2000; Damiola et al., 2000, Yamazaki et al., 2000; Stokkan et al., 2001) where they cycle with a few hours (3-9 h) delay with respect to the oscillation existing in the SCN (Zylka et al., 1998). In contrast to the peripheral clocks of *Drosophila,* which can be entrained directly by light via non-ocular mechanisms (Plautz et al., 1997), mammalian peripheral oscillators do not directly respond to light but are synchronized via neuronal and humoral signals from the SCN (Balsalobre et al., 1998; Yamazaki et al., 2000; McNamara et al., 2001; Pando et al., 2002). Even immortalized fibroblasts treated with serum or chemicals activating a variety of known signal transduction pathways, exhibit rhythmic expression of circadian genes persisting for several days (Balsalobre et al., 2000; Yagita and Okamura, 2000). Additionally, peripheral oscillators also respond to other stimuli (e.g. food availability) independently from the SCN (Cermakian and Sassone-Corsi, 2002). Furthermore, it was shown that the molecular composition of the circadian clock in the SCN and periphery is very similar (Yagita et al., 2001). To investigate the organization of a mammalian circadian system, a transgenic rat line was generated in which luciferase is rhythmically expressed under the control of the mouse *Per1* promoter. Cultured SCN explant of these rats showed robust rhythmicity, persistent up to 32 day whereas in liver explant circadian rhythms damped after several days (Yamazaki et al., 2000). Importantly, as the medium change restarts oscillation in the liver it's shown that the damping was not due to the liver death (Yamazaki et al., 2000). Therefore it was proposed that the SCN is a master clock that synchronises the timing of the peripheral oscillators ("slave" oscillators) according to light input that SCN receives from the environment and in response to this peripheral oscillators regulate local rhythms in physiology and behaviour (Figure 4) (Reppert and Weaver, 2002; Schibler et al., 2003).

A new mouse model was generated in which luciferase was under control of the *mPer2* promoter to monitor circadian dynamics and results using that model challenged the "hierarchical model" of the mammalian circadian system in which the SCN acts as a master clock that is necessary to drive circadian oscillation in peripheral tissues, (Yoo et al., 2003). Surprisingly, it turned out that peripheral tissues in cultures exhibit self-sustained and persistent circadian rhythms, as robust as those present in the SCN. Moreover, the circadian rhythm of bioluminescence in peripheral tissues of SCN-lesioned animals is equivalent to that present in non-lesioned animals. Additionally,

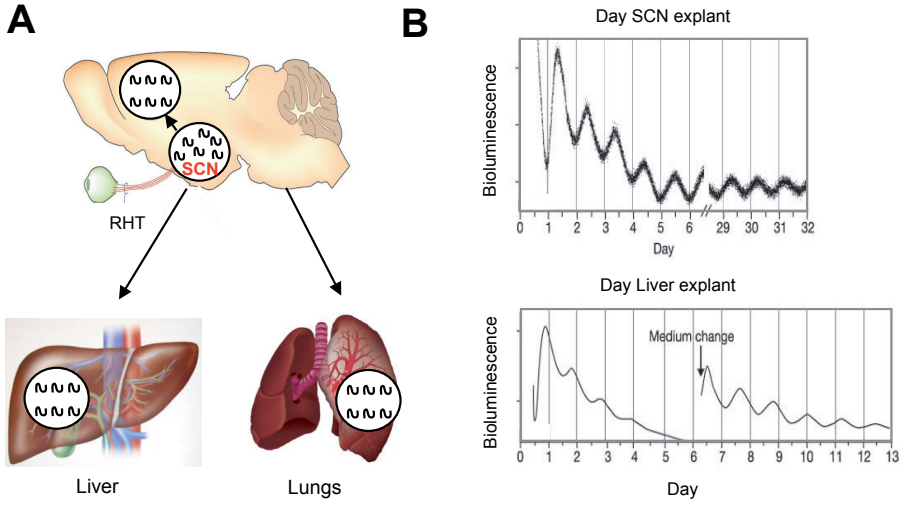

Figure 4. The mammalian circadian system and the communication between the oscillators

A The master clock in the SCN is composed of the numerous clock cells. The clock in the SCN is entrained to the 24 h day/night cycle as it receives the light information via the retinohypothalamic tract (RHT). In turn the SCN coordinates the circadian timing of oscillators in other parts of the brain (e.g. cortex) and in peripheral oscillators (e.g. liver and lungs).

B SCN and liver explants from transgenic rats expressing an *mPer1*-driven luciferase reporter gene exhibit rhythms in culture.

Figure adapted from Reppert and Weaver, 2002, *Nature* **418**:935-41.

new reporters for other clock genes (*mBmal1* and *mPer1*) also supports the idea that circadian oscillations in peripheral tissues are self-sustained (Nishide et al., 2006).

II The photolyase/cryptochrome flavoprotein family

The light of our sun is essential for life on earth. A wide range of physiological, adaptive and biochemical processes are controlled by light energy (Chen et al., 2004). For example, visible light is required for oxygen production by plants via photosynthesis. In mammals and other organisms, even the highly energetic ultraviolet (UV) component of sunlight is required for the synthesis of important substances such as vitamin D. In marked contrast to its beneficial effects, UV light causes DNA lesions, cyclobutane pyrimidine dimers (CPDs) or pyrimidine 6-4 pyrimidine photoproducts [(6-4)PPs], that bring about cell death or, upon replication of a genome, cause somatic mutations (Friedberg, 1996). Along with UV light, other environmental agents like ionizing

radiation and chemicals and endogenously produced cellular metabolites (i.e. reactive oxygen species and other radicals) cause damage in the DNA. To cope with this, cells have developed a variety of DNA repair mechanisms in order to remove damage from the DNA (i.e. nucleotide excision repair (NER), base excision repair (BER), cross-link repair, homologous recombination and non-homologous end joining) with broad substrate specificities (Friedberg, 1996; Hoeijmakers, 2001). One of the first evolved repair systems is a relatively simple mechanism called photoreactivation, where the key role is played by a single enzyme called photolyase, which repairs UV-induced DNA damage.

Another member of the photolyase/cryptochrome flavoprotein family, as the name indicates, includes cryptochromes. Both members of the protein family will be discussed in detail below.

Photolyases

Photolyases (PHL or PL) are monomeric, single-chain flavoproteins with a molecular weight ranging from 50 to 65 kDa. Before photoreactivation, photolyase specifically binds to UV-induced lesions in the DNA (CPDs or (6-4)PPs) but is unable to repair the lesions, this requires activation by light (Mehl and Begley, 1999; Carell et al., 2001; Sancar, 2003). Photolyase are capable of capturing light energy through two, functionally different, non-covalently bound chromophoric cofactors. One such chromophore, present in all photolyases, is a flavin adenine dinucleotide (FAD), which acts as a catalytic cofactor essential for the repair reaction. Various FAD redox states have been found in purified photolyases: oxidized FAD, the half reduced neutral semiquinone radical $FADH^{\cdot}$, and the deprotonated fully reduced form $FADH^{-}$. Only the fully reduced form is biologically active in the repair of UV-lesions (Payne et al., 1987). The conversion of the inactive forms of FAD into $FADH^{-}$ is accomplished by photoreduction involving a unique feature of intraprotein electron transfer along a chain of three tryptophans over a distance of more than 13 Å, (Aubert et al., 2000). The second cofactor is either a reduced folate (5,10-methenyl-tetrahydrofolate, MTHF) or 8-hydroxy-5-deazaflavin (8-HDF), acts as a light-harvesting antenna. Recently, another chromophore that captures light was found in *Thermus thermophilus* photolyase the 7,8-didemethyl-8-hydroxy-5-deazariboflavin (8-HDF) (Klar et al., 2006).

Photolyases are specific for either CPD or (6-4)PP lesions. Yet, the reaction mechanisms are largely the same: shuttling an electron to the lesion in order to destabilize it. Essential driving force for this reaction is the energy acquired by the chromophores by absorption of a photon. This energy is transferred to the FAD and converts it into the fully reduced, active form of flavin $FADH^{-}$ (Kim et al., 1994; Schleicher et al., 2007). Photolyases which do not possess MTHF or 8-HDF are still active in dimer splitting therefore direct excitation of reduced FADH is also effective (Klar et al., 2006).

Structural analysis of photolyases

Several crystal structures are available. The first three-dimensional crystal structure of photolyase has been determined for *E. coli* (Park et al., 1995) and *Anacystis nidulans* (Tamada et al., 1997) photolyase. Their structures are very similar, as they both contain an α/β domain with 5 parallel β-strands and several α-helices and a helical domain containing α-helices only e.g. E. coli photolyase is composed of two domains: an N-terminal α/β domain and a C-terminal α–helical domain. Both domains are interconnected by a 72 amino acid loop. The FAD cofactor is buried inside the α-helical domain and adopts an unusual U-shape conformation. The second chromophore, MTHF, is at the interface between the two domains. The distance between these two cofactors is 16.8 Å and over this distance light energy absorbed by MTHF is transferred to FAD. Across the surface of photolyase runs a positively charged groove, which passes through the cavity were the FAD cofactor is buried. These structural features reveal that binding of photolyases to DNA has to take place in this positively charged groove and is substrate specific (Park et al., 1995). A similar structure was obtained for the core region of cryptochrome (see below). A photolyase - substrate complex structure clearly shows the dimer lesion completely flipped out of the DNA (Mees et al., 2004). Recently the crystal structure of the *Drosophila* (6-4)PP photolyase - substrate and product complex, has become available (Maul et al., 2008).

Photolyase activity is not detected in placental mammals, suggesting that the enzyme has been lost during evolution. Instead, mammals remove UV-induced CPDs and (6-4)PPs (as well as other bulky strand-distorting adducts) by another evolutionary conserved repair mechanism, Nucleotide Excision Repair (NER) (Hoeijmakers, 2001). Whereas (6-4)PPs are rapidly repaired by NER, removal of CPDs is usually slower (Bohr et al., 1985). Despite their absence in placental mammals, it was shown that exogenous photolyases are functional in mice. Transgenic animals expressing the *Arabidopsis thaliana* (6-4)PP)-photolyase and/or the *Potorous tridactylus* (rat kangaroo) CPD-photolyase from the ubiquitously expressed β-actin or keratinocyte-specific K14 promoter are viable and are capable of lesion-specifically repairing UV-induced (6-4)PPs or CPDs in the skin (and cells thereof) in a light-dependent manner (Schul et al., 2002; Jans et al., 2006). It was shown that photoreactivation of CPDs in basal keratinocytes of UV-exposed animals dramatically reduced sunburn, mutation induction, and skin cancer formation. In marked contrast, photoreactivation of (6-4)PPs hardly exerted any effect, which is likely due to the fact that NER repairs CPDs much slower than (6-4)PPs (Jans et al., 2005, 2006; Garinis et al., 2005).

Gene organization and evolutionary history

At present, a vast number of photolyase and photolyase-like genes is known. Products of these genes share a well conserved core domain of about 500 amino acids to which the two

chromophores are bound. Phylogenetic analysis of the amino acid sequence of the core domain of all members of the photolyase family indicates a clear relationship (Yasui et al., 1994). Yet they are functionally very different. The photolyase/cryptochrome protein family, next to the photolyases, includes also cryptochromes, differing from photolyases in having a unique C-terminal extension of various lengths. Despite their sequence and structural homology to photolyases, cryptochromes are not capable of repairing UV-induced DNA damage.

Phylogenetic analysis reveals two distantly related groups (Figure 5), each containing organisms from all three primary kingdoms of life.

The first group comprises a single main cluster with Class II CPD photolyases, mainly but not exclusively, from animals and plants. Placental mammals, in contrast with marsupials, do not possess photolyase genes. Amongst other members of this cluster are several viral photolyases including Cc-*phr2* (Van Oers et al., 2008). It was recently shown that the genome of the *Chrysodeixis chalcites* virus contains two open reading frames (ORF's) encoding CPD DNA photolyase genes, called *phr-1* and *phr-2* (Van Oers et al., 2005). Recently, we have purified and analysed Cc-*phr2* photolyase.

The other group of photolyase/cryptochrome proteins is quite heterogeneous. Separate clusters can be distinguished containing CPD photolyase class I, plant cryptochrome, animal cryptochromes including 6-4 photolyases and cryptochrome DASH. Identification of a new CRY-DASH in bacteria, e.g. *Synechocystis* (Hitomi et al., 2000) *Vibrio cholerae* (Worthington et al., 2003), plants, *Arabidopsis thaliana* (Kleine et al., 2003) and animals, e.g. *Xenopus laevis*, and in several other species (Brudler et al., 2003; Daiyasu et al., 2004) was surprising since it was initially thought that cryptochromes are present only in higher eukaryotes and that prokaryotes possess photolyases but not cryptochromes. The entire family was named CRY-DASH (Cryptochrome-*Drosophila*, *Arabidopsis*, *Synechocystis*, Human) to indicate that members of that family have higher sequence homology to *Drosophila* and human cryptochromes than bacterial photolyases. Nevertheless, it was shown that CRY-DASH are actually photolyases as they show high specificity for CPD lesions and in presence of light catalyze its repair (Selby and Sancar, 2006; Pokorny et al., 2008).

Evolutionary considerations indicate close relationships between animal and plant cryptochromes and it might be assumed that these two proteins are encoded by orthologous genes that originated from a common ancestor CPD photolyase gene. Still, phylogenetic analysis shows that mammalian together with fly cryptochromes are more closely related to 6-4 photolyases than to CPD photolyases and plant cryptochromes. This suggests a close evolutionary relationship, although the proteins are functionally very different. However, the functional differentiation between photolyases and cryptochromes is now being questioned as some fungal photolyases (*Trichoderma atroviride* PHL1, *Aspergillus nidulans* photolyase-like CryA and *Cercospora zeae-maydis* PHL1) perform a dual function: they regulate gene expression, a role usually attributed

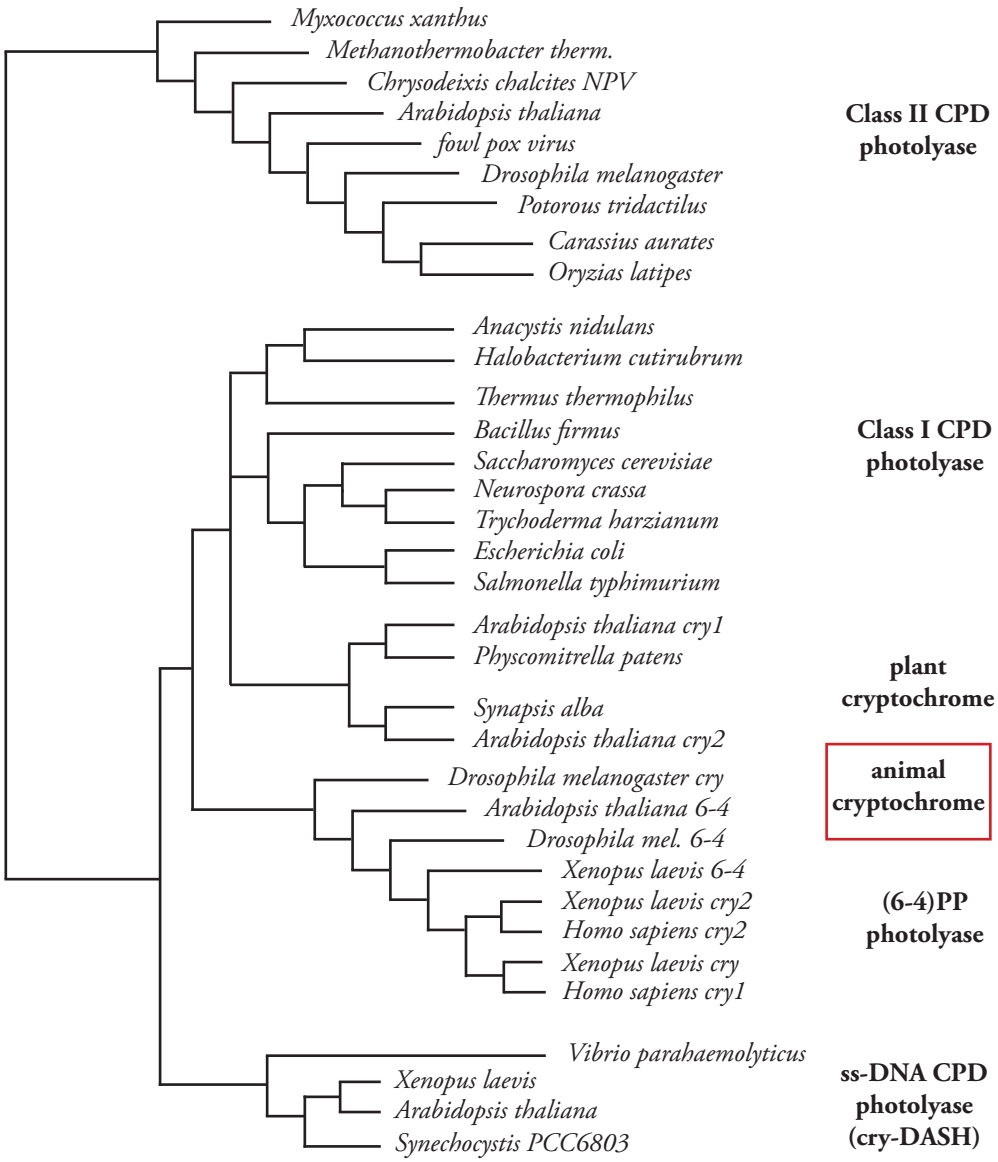

Figure 5. Unrooted phylogenetic tree of the photolyase/cryptochrome family
Amino acid sequences were aligned with ClustalX and a tree was obtained for the core region with the neighbor-joining method (PHYLIP package vs. 3.67 by J. Felsenstein) using the Jones-Taylor-Thornton matrix.
Figure adapted from Eker et al., 2009, *Cell Mol Life Sci* **66:** 968-80.

to cryptochromes, as well as exhibit DNA photorepair activity (Berrocal-Tito et al., 2007; Bayram et al., 2008; Bluhmand Dunkle, 2008). Finally, very recently a missing link in the evolution of the photolyase/cryptochrome protein family was found (Coesel et al., 2009). A new protein from the marine diatom *Phaeodactylum tricornutum* was identified, the *Pt*CPF1 protein, which shows 6-4 photoproduct repair activity and can act as a transcriptional repressor of the circadian clock.

Cryptochromes and phototransduction

Light is not only used as an energy source (e.g. photosynthesis, photoreactivation, etc.), it also allows organisms to perceive and respond to their environment. Evidently, this requires the presence of photoreceptor proteins that translate light information into signals in the organism. Amongst such photoreceptor proteins are cryptochromes, initially discovered as blue-light receptors in *Arabidopsis thaliana* (Cashmore, 2003).

*At*CRY proteins detect light via the core domain of the protein, resulting in activation of the C-terminal domain (Sang et al., 2005). This allows the protein to directly interact with, and subsequently inhibit the COP1 (COnstitutive Photomorphogenic 1) protein. The latter protein serves as a critical component for the activation of the photomorphogenic gene expression program in plants (Wang et al., 2001). Interestingly, overexpression of the C-terminal tails (CT) of *At*CRY1 and *At*CRY2 is sufficient to cause constitutive CRY activity (e.g. repression of COP1) (Yang et al., 2000). Therefore, in plant cryptochromes (e.g. *At*CRYs) the core is mainly a regulator of the CT, while the latter domain carries a major function in phototransduction. Furthermore, light activation of the photoreceptor activity requires homodimerization of the *At*CRY proteins via N-terminal domains in the core domain (Sang et al., 2005). Little is known about the mechanism of light activation of *At*CRY, although (similar to photolyases) the activity of the protein depends on the redox status of the FAD chromophore (Bouly et al., 2007).

The occurrence of cryptochromes is not limited to plants. Emery and coworkers (1998) isolated a fly with a missense mutation at a highly conserved flavin binding residue of *Drosophila* CRY (Emery et al., 1998). Circadian experiments with this mutant strain (*cry*^{b}) revealed that, when kept in constant darkness, flies no longer responded to brief light pulses which induce phase-shifts (Stanewsky et al., 1998). In contrast, flies overexpressing a wild type *cry* gene appeared hypersensitive to light-induced phase-shifting. These data show that *Drosophila* CRY acts as a circadian photoreceptor for resetting of the biological clock. Upon light activation, the *d*CRY protein directly interacts with the clock proteins TIM and PER, causing proteasomal degradation of the PER/TIM protein complexes (Ceriani et al., 1999). In contrast, overexpression of a truncated *d*CRY protein that lacks the CT causes constitutive CRY activity (Busza et al., 2004). Contrary to *At*CRY, the C-terminal domain of *d*CRY plays a regulatory function

by preventing *d*CRY from being active in the dark.

Mammalian cryptochromes are not required for photoentrainment (see for details chapter 1.III).

Cryptochromes and the circadian core clock

In search for mammalian homologues of photolyases, two photolyase-like genes were cloned (Van der Spek et al., 1996; Todo et al., 1997). Since the corresponding gene products do not possess photolyase activity and their core domains lack an N-terminal extension characteristic of eukaryotic photolyases and instead contain a C-terminal extension also present in plant cryptochromes, the genes were designated *mCry1* and *mCry2*.

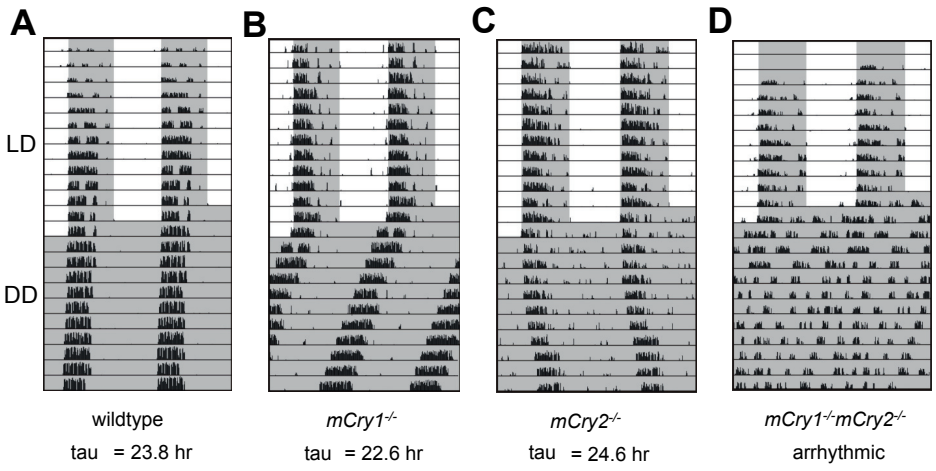

Figure 6. Circadian phenotype of Cry mutant mice

Representative double-plotted actogram of A) wild-type animals (n=14), B) *mCry1* knockout animals (n=9), C) *mCry2* knockout animals (n=5) and D) *mCry1* and *mCry2* double knockout animals (n=8). Mice were under LD (12:12) and DD conditions.

Shading indicates darkness and the black vertical lines represent animal activity.

Studies with genetically modified mice in which the *mCry1* and/or *mCry2* genes were inactivated, surprisingly revealed, that the two mouse cryptochromes are part of the molecular clockwork generating behavioural and molecular rhythms. Notably, the single knock-out mice have opposing circadian phenotypes, as evident from the observation that *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice display short and long behavioural periodicity respectively, as

measured by voluntary wheel-running activity (van der Horst et al., 1999; Vitaterna et al., 1999) (Figure 6A,B,C). Remarkably, behavioural analysis of *mCry1*$^{-/-}$*Cry2*$^{-/-}$ double knockout mice indicated a complete loss of the circadian clock in these animals (Van der Horst et al., 1999) (Figure 6D). Thus, mCRY1 and mCRY2 proteins are integral components of the biological clock as they are essential for maintenance of circadian rhythmicity.

As suggested by the animal experiments, the *mCry1* and *mCry2* genes are expressed in a circadian manner. In the wild type SCN, *mCry1* and *mCry2* mRNA levels peak at the subjective day/night transition, thus following to a large extent expression of the *mPer* genes, whereas in the SCN of *mCry1*$^{-/-}$*mCry2*$^{-/-}$ animals, cycling of *mPer1* and *mPer2* expression is abolished (Okamura et al., 1999). This finding further highlights the indispensable nature of CRY proteins for the generation of circadian rhythmicity. However, the *mPer1* and *mPer2* genes are still induced by light in the SCN of *mCry1*$^{-/-}$*mCry2*$^{-/-}$ animals (Okamura et al., 1999), showing that the *Cry* genes are not required for the photic response in mammals. A key observation in disclosing the function of cryptochrome genes in the core oscillator was the finding that CRY proteins are much stronger inhibitors of CLOCK/BMAL1 driven transcription of E-box containing reporter genes than the mPER proteins (Kume et al., 1999). This observation placed the CRY proteins unequivocally in the core of the circadian oscillator and points to these proteins as being the most important factors to maintain the negative feedback loop of the molecular oscillator, despite other proteins, such as DEC1 and DEC2 having also been proposed to contribute to it (Honma et al., 2002). Interestingly, mCRY1 shows a stronger inhibitory effect than mCRY2 (Kume et al., 1999), which might be an explanation for the opposing phenotypes of the *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ (Oster et al., 2002).

The molecular mechanism regulating mammalian circadian rhythms is composed of ingeniously designed auto-regulatory negative and positive transcription/translation feedback loops, in which CLOCK and BMAL1 are positive elements and PER, CRY are negative elements of the loops (Shearman et al., 2000; Ripperger et al., 2000; Reppert, 2000) (Figure 7).

In the SCN, the intracellular level of the CLOCK protein remains steady throughout the 24-hour cycle, whereas BMAL1 expression levels are high during the subjective night and low during the subjective day. The high level of BMAL1 promotes the formation of CLOCK/BMAL1 complexes. This heterodimer drives the transcription of three mammalian period genes *Per1*, *Per2* and *Per3* and the two cryptochrome genes *Cry1* and *Cry2* by binding to their CANNTG E-Box element. Additionally, the BMAL1/CLOCK heterocomplex activates two orphan nuclear receptors, REV-ERBα (Reverse of Erb alpha) and RORα (RAR-Related Orphan Receptor alpha) which form additional interlocking loops (Preitner et al., 2002; Ueda et al., 2002; Yin et al., 2005). After transcription and translation, the REV-ERBα protein enters the nucleus to suppress the transcription activation of BMAL1 whereas RORα activates BMAL1

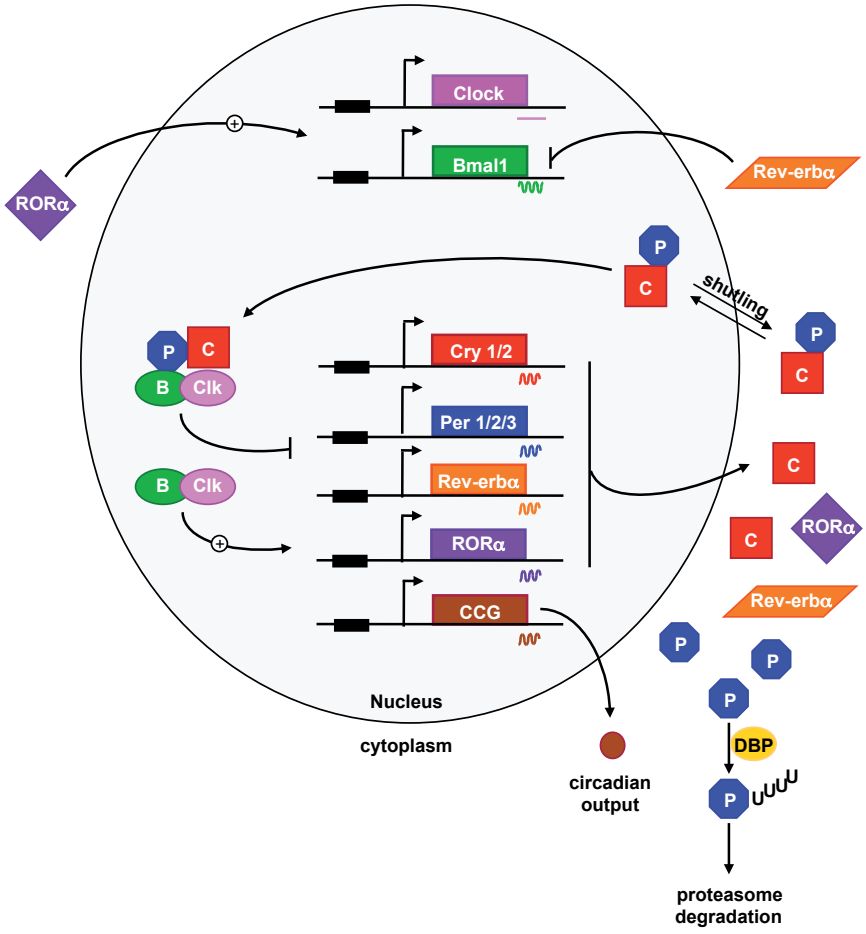

Figure 7. Simplified model of the mammalian circadian clockwork
During the day CLOCK (pink oval) and BMAL1 (green oval) heterodimer enhance the transcription of *Cryptochromes* - *Cry1* and *Cry2* (red rectangle), *Period* – *Per1*, *Per2* and *Per3* (indigo rectangle), *Rev-erbα* (orange rectangle), *Rorα* (magenta rectangle) and others clock control genes (*CCGs*; brown rectangle). During the night once CRY (red square) and PER (indigo hexagon) protein levels reach their maximum, they form a heterodimer and translocate to the nucleus to repress CLOCK/BMAL1 mediated transcription. Opposedly, REV-ERBα (orange parallelogram) levels are high during the day and therefore they inhibit *Clock* (pink rectangle) and *Bmal1* (green rectangle) transcription whereas at night, REV-ERBα levels are low and thus *Clock* and *Bmal1* transcription can take place. Additionally, transcription of *Bmal1* is activated by RORα (magenta diamond).

CRY and PER proteins undergo posttranscriptional and posttranslational modifications. Here is shown Casein Kinase-I-Epsilon - CKIε (yellow oval) which phosphorylates PER proteins and makes them unstable by marking them for degradation through the ubiquitin-proteasome pathway (see text for a detailed explanation).

mediated transcription (Sato et al., 2004, Guillaumond et al., 2005, Akashi et al., 2005). RORα is involved in gene expression during the circadian night, which is in phase with *Bmal1* and in antiphase with *Per2* mRNA oscillations. Late in the subjective day, CRY accumulates in the cytoplasm, promoting the formation of stable PER/CRY complexes, which enter the nucleus at the beginning of a subjective night. Once in the nucleus, CRY1 inhibits the CLOCK/BMAL1 mediated transcription, resulting in the inhibition of *Cry*, *Per* and *Rev-erbα* transcription, and derepression of *Bmal1* transcription. The interacting positive and negative feedback loops of circadian genes ensure low levels of PER and CRY and a high level of BMAL1 at the beginning of a new circadian day. Notably, the circadian core oscillator is coupled to output processes via clock-controlled genes, which also contain E-box elements in their promoter (e.g. the mouse albumin D element-binding protein (Dbp) gene), but differ from true clock genes in that the gene products do not feed back into the core oscillator (Ripperger et al., 2000).

One of the key futures of the self-sustaining circadian oscillation is a time delay of about 6 hours between mRNA and protein peaks for core clock genes (Dunlap, 1999; Young, 2000). Therefore, in addition to factors involved in transcriptional control, factors that mediate posttranscriptional and posttranslational modifications, translocation and regulation of turnover of clock proteins may provide additional regulatory mechanisms by generating time delays necessary for establishing 24-hours periodicities. Identification of mammalian circadian mutation *tau* in hamsters encoding Casein Kinase-I-epsilon (CKIε), a homolog of *Drosophila* DBT, revealed a link between PER and CKIε (Lowrey et al., 2000) placing CKIε within the core clock machinery. CKIε phosphorylates PER proteins and makes them unstable by marking them for degradation through the ubiquitin-proteasome pathway (Akashi et al., 2002). Next to ubiquitin-proteasome dependent degradation, nuclear-cytoplasmic shuttling was shown to add another level at which the delay can be modulated (Yagita et al., 2002). PER proteins shuttle between nucleus and cytoplasm using functional nuclear localisation (NLS) and nuclear export (NES) sequences (Yagita et al., 2002). The subcellular localisation and stability of mPER2 are specifically controlled by mCRY proteins. The mCRY1 protein binds specifically to the C-terminal region of mPER2 (thereby keeping the mPER2 protein in the nucleus) and prevents mPER2 from ubiquitylation and proteasomal degradation (Yagita et al., 2002). In addition to the PER proteins, other core clock proteins, CLOCK, BMAL1 and CRY1 and CRY2 also undergo changes in phosphorylation, abundance, interactions and subcellular localization which plays a vital role in maintenance of the mammalian clock (Lee et al., 2001; Eide et al., 2002; Akashi et al., 2002). Taking together all these data led to the proposition that the time of nuclear entry and the length of time that core clock proteins spend in the nucleus prior to degradation, are essential for maintaining the correct circadian period length in mammals (Tamanini et al., 2005). The important role of phosphorylation in determining the circadian period length was further confirmed by uncovering the molecular basis of

the familial advanced sleep phase syndrome (FASPS), an autosomal dominant condition with early morning awakening and early sleep times, due to a mutation in the hPER2 phosphorylation site (Toh et al., 2001).

The identification of the posttranslational modifications of the clock proteins has revealed new levels of control in maintaining correct timing of the mammalian circadian clock (Lee et al., 2001; Dardente and Cermakian, 2007; Gallego and Virshup, 2007). The postranslational modifications will be discussed in detail in chapter 6.

III Mammalian cryptochromes

Physical and biochemical properties

In general, cryptochromes share a high degree of homology with photolyases (20-25% sequence identity with microbial photolyases and 40-60% sequence identity with 6-4 photolyase (Todo et al., 1999) but lack N-terminal extension containing the nuclear and mitochondrial localization signal, characteristic for eukaryotic photolyases (Sancar, 1994; Todo et al., 1996; Van der Spek et al., 1996; Cashmore, 1999) (Figure 8).

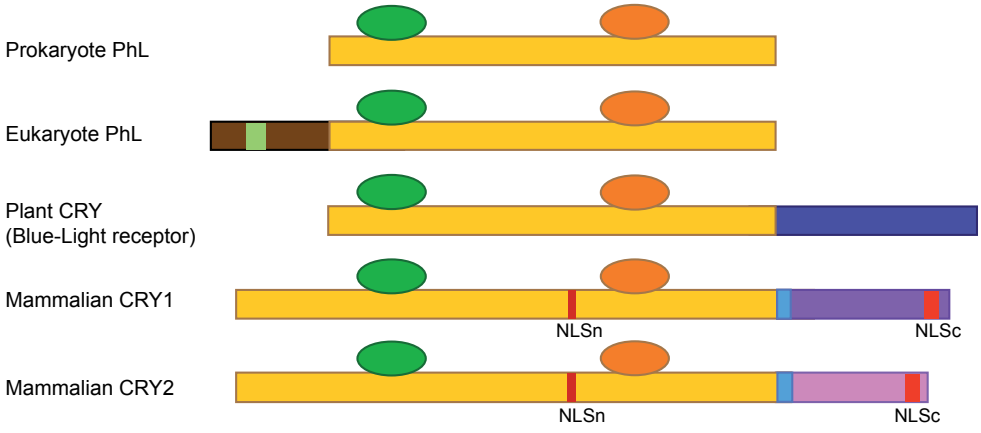

Figure 8. Schematic representation of the photolyase/cryptochrome family of proteins
The homologous core domain shown in yellow, the N-terminal extension of Eukaryotic photolyase (PhL) containing nuclear and mitochondrial localization signals (lime) shown in brown, the C-terminal extension of the plant cryptochrome shown in purple, the C-terminal extension of the mammalian cryptochrome 1 shown in magenta, the C-terminal extension of the mammalian cryptochrome 2 shown in pink, the nuclear localisation signal at the N-terminal extension (NLS_n) shown in dark red, the nuclear localisation signal at the C-terminal extension (NLSc) shown in red, the predicted coiled-coil domain shown in cobalt, the flavin cofactor shown in green and the pterin cofactor shown in orange.

However, comparison of the CRY proteins from various organisms revealed nonhomologous C-terminal extensions (not present in photolyases), ranging from very short in *Drosophila* to very long in plants (240 amino acids). Analysis of the amino acid sequence of the two mammalian CRY proteins revealed 80% homology in the core domain of mCRY1 and mCRY2 (the 500-amino acid region shared by photolyase and cryptochromes) (Kobayashi et al., 1998; Miyamoto and Sancar, 1998). In contrast, the C-terminal extensions of mCRY1 and mCRY2 are unique. The mCRY1 protein is 606 aa in length and encodes a protein of 66 kDa. The mCRY2 protein is 601 aa in length and encodes a protein of 67 kDa. The human CRY proteins are nearly identical to the mouse proteins with the exception of a short region in the C-terminal domain (Hsu et al., 1996; Van der Spek et al., 1996).

Structural/functional analysis of mammalian cryptochromes

Until now, the purification and crystallization of cryptochrome proteins is limited to the *Synechocystis* CRY-DASH protein (Brudler et al., 2003) and the core domain of *Arabidopsis thaliana* cryptochrome 1 (*At*CRY1) (Brautigam et al., 2004). The three-dimensional structures of these proteins show a remarkable similarity to the photolyase structure. Nevertheless, there are some differences between structures of CRY-DASH protein and *At*CRY1 (core domain only). As in photolyase molecules, the surface around the FAD cavity of the CRY-DASH protein has a positive electrostatic potential, suggesting that this region in the CRY-DASH protein is involved in binding to the DNA. Indeed, it has recently been shown that CRY-DASH binds to single stranded DNA with a high degree of specificity for CPD lesions in single stranded DNA (Selby and Sancar, 2006). The core domain of *Arabidopsis thaliana* cryptochrome 1 does not contain this characteristic feature, suggesting that plant cryptochromes do not bind to DNA. The FAD cavity of *At*CRY1 instead is larger and deeper. This is in agreement with findings showing that a single molecule of an ATP analog binds to that particular place (Bouly et al., 2003) [human CRY1 likewise shows the binding of ATP to this cavity (Bouly J.P., 2003)] whereas binding of ATP to the photolyases or CRY-DASH protein is not reported. Later on, another structure of a cryptochrome that belongs to the subfamily of CRY-DASH proteins was solved, namely the structure of *Arabidopsis thaliana* cryptochrome 3 (Klar et al., 2007). It was shown that *At*CRY3 can bind and repair CPD lesions in single-stranded DNA (like conventional DNA photolyases) but as they lack an efficient repair mechanism they are not able to repair the lesion in double-stranded DNA (Pokorny et al., 2008).

Currently, the crystal structure of mammalian cryptochromes is not available whereas the crystal structures of *E. coli* (Park et al., 1995) and *A. nidulans* have been solved (Tamada et al., 1997). As their structures are very similar, it is reasonable to assume that the mammalian cryptochrome structure would be very similar to that of

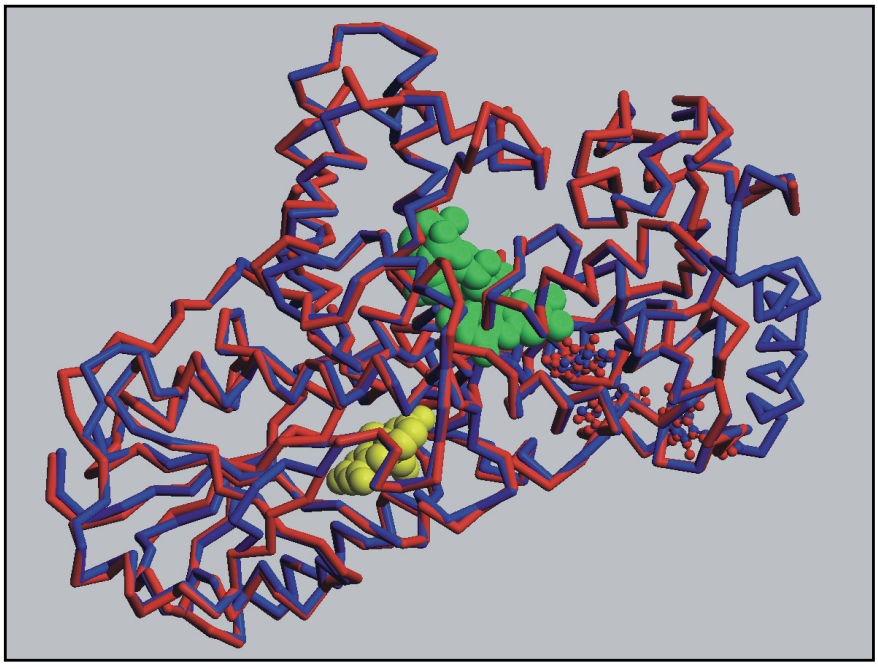

Figure 9. Superposition of the structure of Anacystis nidulans Photolyase and modelled mammalian Cryptochrome 1
The structure of *A. nidulans* photolyase is shown in blue, the modelled structure of human cryptochrome 1 shown in red, the FAD chromophore shown in green, the 8-HDF chromophore shown in yellow.

E. coli. Using the crystal structure of the *A. nidulans* photolyase as a template, a model of hCRY1, excluding the C-terminal extension, was generated using the Swiss-PdbViewer program (A. Eker) (Figure 9). The two structures are superimposible. Such a theoretical model was also determined for hCRY2 (Sancar, 2000).

Remarkably, hCRY2 can bind to DNA *in vitro* and this binding can be further stimulated by the presence of 6-4 photoproducts but light has no effect on such a complex formation as hCRY2, in contrary to conventional photolyases, is deficient in repair activity (Özgür and Sancar, 2003). Similarly, mCRY1 has been suggested to bind to the DNA (Kobayashi et al., 1998) but this aspect of the mammalian cryptochrome function still remains to be solved.

Mammalian cryptochromes are core clock components of the circadian oscillation (Van der Horst et al., 1999) and the primary function of CRY proteins is inhibition of CLOCK/BMAL1 mediated transcription (Kume, K., 1999; Griffin A.E., 1999). As plant and *Drosophila* CRY proteins act as circadian photoreceptors (Cashmore,

2003; Stanewsky et al., 1998) and in addition *Drosophila* CRY are involved in core clock oscillations in peripheral tissues (Emery et al., 1998) it is speculated whether mammalian CRY proteins also display such a function. Unexpectedly, given the anticipated photoreceptor function predicted on the basis of the homology with plant cryptochromes, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice show inhibition of running wheel activity by light and induction of *mPer1* and *mPer2* expression upon exposure to light pulses known to reset the circadian clock, suggesting that mCRY are not required for photoentrainment or light-masking of activity (Okamura et al., 1999). Interestingly, functionally diverse six *Cry* genes are present in zebrafish and each gene has a different role in the molecular clockwork (Kobayashi et al., 2000). On the basis of sequence similarity and the function of the various gene products (as determined *in vitro*), *zfCry* genes can be divided into two groups: one encoding proteins with high sequence similarity to the mammalian *Cry* genes, acting as inhibitors of CLOCK/BMAL1 mediated transcription (repressor type CRY) and one encoding proteins with high sequence similarity to the *Drosophila Cry* gene (encoding circadian photoreceptor) and not involved in transcription inhibition (non-repressor type CRY). Similarly, the Monarch butterfly (*Danaus plexippus*) expresses two functionally different *Cry* genes: a *Drosophila*-like cryptochrome and a repressor type CRY, called *Dp*CRY1 and *Dp*CRY2, respectively (Zhu et al., 2008).

To gain more insight into the biochemical properties of mammalian cryptochromes, a panel of mutant mCRY1 expression constructs (based on domains identified at the sequence level) has been generated which have been instrumental for the identification of functional domains (Chaves et al., 2006). Particular attention was paid to the C-terminal 100-120 amino acids (CT) of mCRY1, which distinguishes the protein most from mCRY2 (as well as from CRY proteins from other organisms; e.g. *Drosophila*), and which is lacking photolyases. A schematic representation of the different mutant proteins, which include mutations in the NLS domains, deletion of the coiled-coil CC, deletion of the C-tail and EGFP (Enhanced Green Fluorescent Protein) fused to the extended C-terminal domain (CT) of cryptochrome 1 (aa 371-606) is shown in Figure 10.

Using this panel of mutant constructs, the domains involved in protein-protein interactions, subcellular localization of the protein as well as the domain involved in association with mPER2 and with BMAL1 in the CT of mCRY1 were identified (Chaves et al., 2006). The interaction domain is represented by a coiled coil (CC), embedded in the CT, which is also present in *Xenopus* CRY (Van der Schalie et al., 2007), but absent in *Drosophila* and plant CRYs. The C-terminus was also shown to harbour a putative bi-partite nuclear localization signal (NLSc) (Tamanini F. 2005; Chaves et al., 2006). The core domain of mCRY1 contains a second NLS (NLSn), previously identified by Hirayama and coworkers (Hirayama et al., 2003), which however is less potent than NLSc. Interestingly, combined deletion and/or mutagenesis of the NLS domains still allows the protein to reach the nucleus in an NLS-independent manner involving the CC domain as CRY protein are co-transported into nucleus. Previously, overexpressed

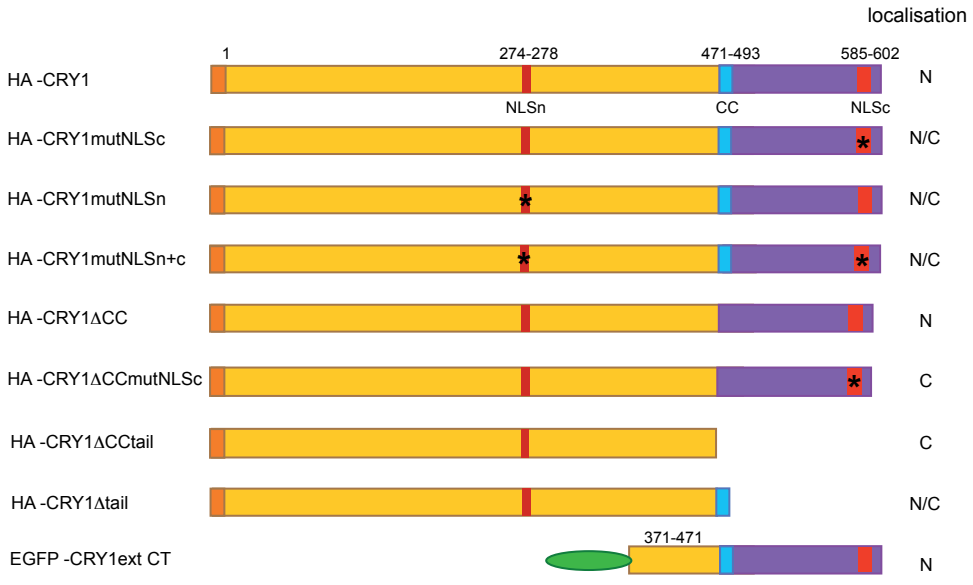

Figure 10. Schematic representation of HA-mCRY1 and mCRY1 mutants
The mutants shown are: HA-CRY1mutNLSc, HA-CRY1mutNLSn, HA-CRY1mutNLSn+c, HA-CRY1ΔCC, HA-CRY1ΔCCmutNLSc, HA-CRY1ΔCCtail, HA-CRY1Δtail, EGFP-CRY1extCT. Mutations in NLSc and NLSn are indicated by a star. The core domain is shown in yellow, the C-terminal extension of the mammalian cryptochrome 1 in magenta, the nuclear localisation signal at the N-terminal extension (NLS_n) in dark red, the nuclear localisation signal at the C-terminal extension (NLSc) in red, the predicted coiled-coil domain in cobalt, the HA-tag in orange and the EGFP-tag in light green.

mPER2 was shown to shuttle between the nucleus and cytoplasm through the combined action of NLS and Nuclear Export Sequences (NES) (Vielhaber et al., 2001; Yagita et al., 2002), while co-expression with mCRY1 (or mCRY2) caused complete nuclear localization of mPER2 (Kume et al., 1999). Following our observation that mPER2 and BMAL1 can competitively bind to the CC of mCRY1 and facilitate nuclear localization of mCRY1mutNLSc, it was proposed that the balance between the activities exerted by nuclear import and export signals harbored by CRY and PER2 determine the final subcellular localization of the complex (Chaves et al., 2006).

Since the key function of mCRY proteins is to inhibit CLOCK/BMAL1 mediated transcription, the performance of the various mutant mCRY1 proteins in *in vitro* CLOCK/BMAL1 transcription assays was analysed. Although deletion of either CC, or tail, or NLSc did not affect the CLOCK/BMAL1 inhibitory capacity of mCRY1, complete removal of the C-terminal extension (as in HA-CRY1ΔCCtail) rendered the protein inactive (Chaves

et al., 2006). These data suggest that the "gain" of the unique C-terminal extension during evolution has been critical in conferring core oscillator function to the core domain. In line with this, mutagenesis of conserved tryptophanes (involved in intraprotein electron transport during photoactivation) abolishes the photoreceptor function of *Drosophila* and butterfly CRY, while it does not affect the CLOCK/BMAL1 transcription inhibitory capacity of mouse CRY1 (Zhu et al., 2005; Song et al., 2007; Chaves et al., 2006).

Since the core domain of mCRY1 shares a high degree of homology with photolyase it was therefore interesting to examine whether the CT of mCRY1 could convert a photolyase into cryptochrome. Fusion of the CT of mCRY1 (aa 471-606) to *Arabidopsis thaliana* (6-4)PP photolyase did not result in a protein with CLOCK/BMAL1 inhibitory capacity. However, additional substitution of the last 100 amino acids of the photolyase core domain with that of the CRY1 core (aa 371-470) allowed the chimeric protein to inhibit CLOCK/BMAL1 driven transcription. When fused to EGFP, the extended CT (aa 371-606) of mCRY1 was not able to inhibit CLOCK/BMAL1. This led to the suggestion that the remainder of the photolyase/cryptochrome core domain is very important for the clock function of cryptochrome proteins, likely through a complex network of interactions and intrinsic (core domain like) structural requirements for proper transcription inhibition (Chaves et al., 2006). Indeed it was shown that the CTs of animal and plant CRYs are intrinsically unstructured and that a stable tertiary structure is achieved via intramolecular interaction with the core domain (Özgür and Sancar, 2003). In a comparable series of experiments, Van der Schalie et al. (2007) showed that *Xenopus* CRY photolyase fusion proteins maintain CLOCK/BMAL1 inhibitory capacity, further underlining the need for proper structural folding.

IV Future directions in the analysis of mammalian cryptochromes

The mCRY1 and mCRY2 proteins are indispensable components of the circadian core oscillator (Van der Horst et al., 1999) and act as the most potent repressors of CLOCK/BMAL1 transcription (Kume et al., 1999; Griffin et al., 1999). Notably, analysis of the phenotype of *mCry* mutant mice revealed that the *mCry1* gene drives long period circadian clockworks, whereas the *mCry2* gene confers short periodicity to the circadian oscillator (Okamura et al., 1999; Van der Horst et al., 1999). Although it is possible that the two genes/proteins could be expressed in different circadian time windows, CRY1 and CRY2 proteins reach the nuclear compartment in a synchronous manner (Field et al., 2000; Lee et al., 2001). Therefore, it was proposed that the two proteins have opposing circadian effects because of sequence differences translating in different activities in and/or posttranslational regulation of the clock. Vanselow et al.

(2006) proposed the interesting hypothesis that the short- and long-period phenotypes of *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mutant mice could be the result of alterations in the phosphorylation patterns of mPER proteins. The lack of mCRY1 or mCRY2 would differentially influence the phosphorylation state of mPER proteins and thus could modulate the circadian period in opposite directions. In line with this idea, phosphorylation of mPER2 at different serines can have opposite effects on clock speed. The important role of phosphorylation in determining the circadian period length was further confirmed by the finding that FASPS syndrome is due to a mutation in the hPER2 phosphorylation site that influences hPER2 protein stability and eventually leads to hPER2 protein degradation (Toh et al., 2001). Therefore it was concluded that the PER proteins are primarily responsible for a proper timing of the mammalian circadian oscillator. In this light it is worth to investigate the role of CRY proteins in regulating proper timing of the mammalian circadian clock.

Deletion analysis has shown that the putative CC domain in the CT of mCRY1 is required for interacting with mPER1/2 and BMAL1 (Chaves et al., 2006). The CT further contains domains involved in nuclear import (Chaves et al., 2006) and domains required at least for mCRY2 phosphorylation (Sanada et al., 2004; Harada et al., 2005). It is likely that, in addition to the already identified proteins, other factors are interacting with the CT, such as kinases and phosphatases, including FXBL3, which is the E3 ubiquitin-ligase recently shown to target mCRY1 and mCRY2 for degradation through the 26S-proteasome (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007; Dardente 2008). We believe that the identification of protein partners will elucidate the function of CRY proteins in the mammalian clock, but may also disclose new functions of these proteins.

Recently, an illuminating report showed that homodimerization of the core domain of *At*CRY1 was mandatory for subsequent light activation of its C-terminus, CCT1 (*Arabidopsis* CRY1 C-terminal domain) (Sang et al., 2005). The activated CCT1 would then bind and inhibit the E3 ubiquitin-ligase COP1 (COnstitutive Photomotphogenic 1), thereby allowing the accumulation of a set of transcription factors [e.g. HY5; (long HYpocotyl 5)] that initiate the photomorphogenic program (Wang et al., 2001; Yang et al., 2001). Moreover, interaction between the core domain of *At*CRY1 and mCRY2 with their corresponding C-termini has been shown to be an important event in establishing the stable tertiary structure of these proteins, suggesting a common biochemical mode of action for cryptochromes (Partch et al., 2005). It is tempting to speculate that the necessity for homo- or heterodimerization restricted to the photoreceptor function of plant CRY may also be required for nuclear accumulation and CLOCK/BMAL1 inhibition in mammals.

The inhibition of the CLOCK/BMAL1 heterodimer is independent of light and is triggered at relatively low doses of CRY. While the mechanism underlying CLOCK/BMAL1 repression is not known, some ideas have been proposed. One hypothesis is that CRY proteins repress CLOCK/BMAL1 by reducing CLOCK/BMAL1's affinity for the E box. Another idea is that CRYs inhibit CLOCK/BMAL1 by interacting with

the heterodimer and then recruiting histone deacetylases or inhibiting histone acetylases (Etchegaray et al., 2003). Surely, an area of exploration would be the study of the impact of mCRYs on the posttranslational properties of CLOCK and BMAL1 (Dardente et al., 2007).

Mammalian cryptochromes are members of the photolyase/cryptochrome family of flavoproteins that share an extraordinarily conserved core domain structure, while the presence of a C-terminal extension (CT) is limited to the cryptochromes. Photolyases are DNA repair enzymes that remove UV-light induced lesions (Mehl and Begley, 1999; Carell et al., 2001; Sancar, 2003). Cryptochromes of plants and *Drosophila* act as circadian photoreceptors (Cashmore, 2003; Stanewsky et al., 1998), involved in light-entrainment of the biological clock whereas mammalian cryptochromes (mCRY1 and mCRY2) are essential components of the clock machinery (Van der Horst et al., 1999). The intriguing question whether in evolution there was a common ancestor with both functions, and whether there is a naturally occurring photolyase that can (partially) function in the circadian clock remains to be answered.

The aims of this thesis are to gain insight into the molecular mechanism underlying cryptochrome function and to study the evolutionary link between cryptochromes and photolyases.

Cryptochromes and photolyases share evolutionary conserved sequence similarities within their core domain. **Chapter 2** is focusing on two novel photolyase (*phr*) genes identified in the plusiine-infecting group II nucleopolyhedroviruses (NPVs), *Chrysodeixis chalcites* (*Chch*NPV), respectively called *Cc-phr1* and *Cc-phr2*. In this study we overexpressed, purified and analysed *Cc*-phr2 photolyase.

Another approach to learn more about the function of cryptochromes and evolutionary link between cryptochromes and photolyases is to study new mouse models. We examined the circadian behaviour of earlier generated and described transgenic mice expressing the *Arabidopsis thaliana* (6-4)PP-photolyase and/or the *Potorous tridactylus* (rat kangaroo) CPD-photolyase from the ubiquitous β-actin or keratinocyte-specific K14 promoter (Schul et al., 2002). The circadian analysis of these mice is presented in **Chapter 3.**

To further investigate the function of mammalian cryptochromes (**Chapter 4**) using biochemical analyses available to us, we try to answer the question whether homo- or heterodimerization is required for proper function of cryptochromes (mCRY1 and mCRY2). We also generated *CRY1*^{ACC} mutant knock-in mice. The circadian behavioural analyses of those mice are described in **Chapter 5**.

Finally, in the study presented in **Chapter 6** we used amylose-bead immobilized purified MBP-extCRY1CT and MBP-extCRY2CT domains to probe mouse tissue (kidney lysates) coupled with MALDI-TOF mass spectrometry to identify proteins interacting with the CTs of mCRY1 and mCRY2. We believe that identification of a new CRY partner(s) will broaden our current knowledge about cryptochrome functions.

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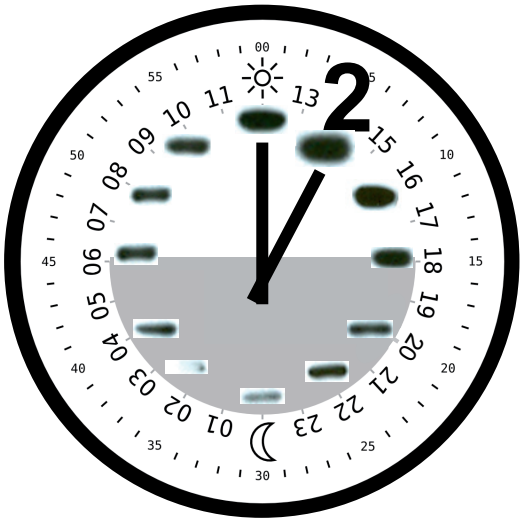
Active DNA photolyase encoded by a baculovirus from the insect *Chrysodeixis chalcites*



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Active DNA photolyase encoded by a baculovirus from the insect *Chrysodeixis chalcites*

Monique M. van Oers[a,*], *Margit H. Lampen*[a], *Monika I. Bajek*[b], *Just M. Vlak*[a], *André P.M. Eker*[b]

[a] *Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands*
[b] *MGC-CBG Department of Cell Biology and Genetics, Erasmus University Medical Centre, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands*

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ABSTRACT

The genome of *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) contains two open reading frames, Cc-*phr1* and Cc-*phr2*, which encode putative class II CPD-DNA photolyases. CPD-photolyases repair UV-induced pyrimidine cyclobutane dimers using visible light as an energy source. Expression of Cc-*phr2* provided photolyase deficient *Escherichia coli* cells with photoreactivating activity indicating that Cc-*phr2* encodes an active photolyase. In contrast, Cc-*phr1* did not rescue the photolyase deficiency. Cc-*phr2* was overexpressed in *E. coli* and the resulting photolyase was purified till apparent homogeneity. Spectral measurements indicated the presence of FAD, but a second chromophore appeared to be absent. Recombinant Cc-phr2 photolyase was found to bind specifically F0 (8-hydroxy-7,8-didemethyl-5-deazariboflavin), which is an antenna chromophore present in various photolyases.. After reconstitution, FAD and F0 were present in approximately equimolar amounts. In reconstituted photolyase the F0 chromophore is functionally active as judged from the increase in the *in vitro* repair activity. This study demonstrates for the first time that a functional photolyase is encoded by an insect virus, which may have implications for the design of a new generation of baculoviruses with improved performance in insect pest control.

1. Introduction

Baculoviruses form a family of large double stranded DNA viruses, which mainly infect phytophagous insects. These viruses are widely used as biocontrol agents of insect pests and as vectors for the large-scale expression of foreign proteins [1,2]. Progeny virus is spread over the plant foliage upon death and liquefaction of the infected insect larvae to await a new cohort of insects. On foliage UV exposure has been reported to rapidly inactivate baculoviruses [3–5]. This UV-sensitivity forms a major limitation in large-scale application of baculoviruses as biocontrol agents of insect pests in agriculture and forestry, as expensive UV-protective chemicals have to be added to baculovirus formulations (see e.g., [6–8]). The purpose of the research presented here is to determine whether putative photolyases encoded by the baculovirus *Chrysodeixis chalcites* NPV [9], are active enzymes that have the potential to protect baculovirus DNA from UV damage. If so, this would

Abbreviations: 8-HDF, 8-hydroxy-7,8-didemethyl-5-deazaflavin; F0, 8-hydroxy-7,8-didemethyl-5-deazariboflavin; MTHF, 5,10-methenyltetrahydrofolate.

* *Corresponding author*. Tel.: +31 317 485082; fax: +31 317 4848420.
E-mail address: monique.vanoers@wur.nl (M.M. van Oers).

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predict prolonged survival in the field of baculoviruses carrying such a gene, with clear implications for their application in insect biocontrol.

When organisms are exposed to UV light in the range of 250–320 nm two types of DNA lesions are introduced: cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts ((6-4)PPs). CPDs form the majority of UV-lesions [10–12]. Specific photolyases, which are able to repair these lesions with the help of blue or near-UV light, occur in most organisms except placental mammals [13,14]. Besides photoreactivation other DNA repair systems exist, such as base excision and nucleotide excision repair, as recently reviewed (e.g., [15,16]). Based on amino acid sequence divergence, photolyases can be divided into two classes [14,15]. Class I is very heterogeneous, comprising CPD-photolyases, mainly from bacteria and microbial eukaryotes, (6-4)PP-photolyases from plants and animals, and cryptochromes. The latter are repair-deficient photolyase homologues involved in the regulation of the biological clock [17]. Class II CPD-photolyases are found mainly but not exclusively in plants and animals. CPD-photolyases play an important role in the protection of organisms and some viruses against UV light as demonstrated in for instance plants [16] and fowlpox virus [18]. Introduction of a marsupial CPD-photolyase transgene in mice that lack endogenous photolyases resulted in enhanced UV resistance [19] and reduced incidence of skin cancer [20].

All known CPD-photolyases contain flavine adenine dinucleotide (FAD) as a catalytic co-factor. Many photolyases contain, besides FAD, a light-harvesting co-factor or antenna chromophore, which is either 5,10-methenyltetrahydrofolate (MTHF) or an 8-hydroxy-7,8-didemethyl-5-deazaflavin (8-HDF) [10,21]. Photolyases bind to UV-induced lesions in DNA in a light-independent step. Upon absorption of light by the light-harvesting chromophore, excitation energy is transferred to the catalytic co-factor (reduced FAD), which in turn transfers an electron to the CPD, which is then split into the constituent pyrimidines [22].

Class II photolyases have been discovered in viruses and parasites; several (entomo-) pox viruses [23–26] as well as a microsporidian intracellular insect parasite [27] carry a *phr* gene. Putative *phr* genes have also been found in the genome of the baculoviruses *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV), *Trichoplusia ni* (Tn) SNPV and recently *Spodoptera litura* granulovirus (SpliGV) [GenBank DQ288858]. There is evidence that some other NPVs also carry a *phr* gene [28]. ChchNPV even contains two ORFs with homology to class II CPD-photolyases [9,29]. These ORFs, Cc-*phr1* (ORF68) and Cc-*phr2* (ORF72), share 48% identity on the amino acid level. In TnSNPV one *phr* ORF was identified [30], which is closely related to Cc-*phr*1 (77% amino acid identity).

In this study, the two class II CPD-photolyases of ChchNPV were analyzed in rescue experiments in a photolyase deficient *E. coli* strain. Subsequently, Cc-phr2 photolyase was overexpressed in *E. coli*, purified and further characterized with respect to biological activity and co-factor content. These experiments showed that the Cc-*phr*2 gene encodes a functional DNA photolyase.

2. Materials and methods

2.1. Bacterial strains and virus

The *E. coli* KY29 strain [31] is deficient in photolyase activity and was used with kind permission of Dr. Kazuo Yamamoto of the Tohoku University, Japan. This strain carries a chloramphenicol resistance marker and is compatible with the inducible p_{tac} promoter present in the pKK233-3 vector (Amersham Pharmacia Biotech). The baculovirus *Chrysodeixis chalcites* NPV (ChchNPV) was isolated from a Dutch greenhouse in 2002 and has been sequenced entirely [9].

2.2. Plasmid constructs

The ChchNPV ORFs *phr1* (ORF68) and *phr2* (ORF72) were amplified with EXPAND polymerase (Roche) using ChchNPV genomic DNA as the template. *Eco*RI and *Pst*I restriction sites were added up- and downstream of the ORFs, respectively. For *phr1* (nucleotide 65290–66828) this was done in a two-step process in order to remove an *Eco*RI site present within the *phr1* ORF (nt 65408). The first PCR was performed with the mutagenesis primer phr1 MUT F (5′-GTGATCCTAAACGCATCCTA AGTTGAATC-3′) in combination with the downstream primer phr1 R (5′-GGACTGCAGTGA CGGCAATCTATTCAAACATG-3′) to modify the internal *Eco*RI, but leaving the encoded amino acid sequence unaltered. The product of the first PCR was used as a primer together with primer phr1 F (5′-GGAGAATTCATGTCGAAAGTCAACTAC-3′) to amplify a full length engineered *phr1* ORF. The *phr2* ORF was amplified using the primers phr2 F (5′-GGAGAATTCATGGCAGCAAACGATCGATTG-3′) and phr2 R (5′-GGACTGCAGCAAGGATGATGCGAATAAAAC-3′). The amplified ORFs were cloned into the pGemT-Easy vector (Promega) and, after verification by sequence analysis, recloned into the *Eco*RI site of pKK223-3 (Amersham Pharmacia Biotech) using the upstream *Eco*RI restriction site introduced by the forward PCR primer (underlined) and a downstream *Eco*RI site obtained from the pGemT-Easy multiple cloning site. The *Pst*I site introduced downstream of the ORF was used to verify the orientation of the inserts in pKK223-3 (not shown). The sequence of the final constructs was verified again, to exclude possible insertion of additional sequences derived from pGemT easy between the Shine and Dalgarno element in the pKK223-3 vector and the start codon of the *phr* ORFs. Following these cloning steps, which were performed in the *E. coli* strain DH5α, the plasmid pKK223-3 as well as its derivatives pKK-Ccphr1 and pKK-Ccphr2 were introduced into *E. coli* KY29 cells.

2.3. In vivo photoreactivation assay

E. coli KY29 cells containing plasmid pKK223-3, pKK-Ccphr1 or pKK-Ccphr2, were grown in LB with 200 mg/l ampicillin and 34 mg/l chloramphenicol for 1.5 h at 37 °C. IPTG (40 mg/l) was added and after 3 h of induction 1 ml samples were centrifuged for 5 min at 5000 rpm. Cell pellets

were washed and suspended in 1 ml M9 salt solution (6.8 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl). Ten ml of cell suspension diluted 100-fold in M9 salt solution was UV-irradiated (0.285 J/m^2 s at 254 nm) in a 9 cm glass Petri dish. Duplicate samples of 400 μl were collected in 24-well plates. One plate was illuminated with 4 white fluorescent lamps (Philips 71D18/W54) for 30 min at 30 °C while the other plate was incubated in the dark. An 8 mm window glass filter was used to remove short wavelength radiation. Suitably diluted 100 μl samples were plated on LB-agar with ampicillin (200 mg/l) and chloramphenicol (34 mg/ml). Colonies were counted after overnight incubation.

2.4. Purification of photolyase

E. coli KY29 cells harboring pKK-Ccphr2 were grown at 37 °C in 10 L phosphate buffered LB containing 4% glucose, 200 mg/l ampicillin and 34 mg/l chloramphenicol. The pH was maintained between 6.8 and 7.3 by addition of a sterile Na_3PO_4 solution. When an A_{600} of 0.7–0.8 was reached, IPTG (40 mg/l) was added and growth was allowed to continue for 3 h at 27 °C. Cells were harvested by centrifugation, washed with buffer A (100 mM NaCl, 10 mM K-phosphate pH 7.0) and stored frozen in buffer A with 15% v/v glycerol at −80 °C. To isolate photolyase, cells were sonicated in the presence of 10 mM 2-mercaptoethanol. Cell debris was removed by centrifugation and photolyase was purified from the supernatant by chromatography (twice) on a heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) column eluted with a gradient of 0–1 M NaCl in buffer B (buffer A with 10 mM 2-mercaptoethanol) followed by chromatography on a SP-Sepharose fast flow (Amersham Pharmacia Biotech) column eluted with a linear gradient of 0.1–0.65 M NaCl in buffer B. Photolyase containing fractions were identified by their absorption spectrum After addition of 20% v/v glycerol fractions were frozen quickly in liquid nitrogen and stored at −80 °C. All purification steps were done at 4 °C.

2.5. Spectral measurements

Absorption spectra were measured with a Cary 100 Bio spectrophotometer (Varian). Fluorescence spectra were measured with a LS50B luminescence spectrometer (PerkinElmer). Emission spectra were not corrected. Phosphodiesterase I from *Crotalus atrox* was obtained from Sigma.

2.6. Assay of photoreactivating activity

A solution of pdT18 (Sigma-Genosys) containing 11% v/v acetone (Fluke, for UV spectroscopy) was purged with Argon gas and irradiated with 312 nm UV-light (VL-6M, Vilber Lourmat) for 6 h. Acetone was removed by vacuum treatment. Assay mixtures (300 μl) containing UV–pdT18, purified Cc-phr2 photolyase and buffer B were illuminated at 25 °C with light of a 6 W white fluorescent lamp (Philips TL6W/33) with a 5 mm window glass cut-off filter. The conversion of thymine dimer into thymine was measured as the increase in absorbance at 265 nm.

2.7. Reconstitution

Cc-phr2 photolyase in buffer B was incubated with F0 (8-hydroxy-7,8-didemethyl-5-deazariboflavin) or riboflavin for 20 min at 0 °C [32]. Excess F0/riboflavin was removed with a small spin column (BioRad Micro Bio-Spin 6) according to manufacturer's instructions. The absorption spectrum of reconstituted photolyase was measured before and after heat denaturation for 5 min at 80 °C to release the chromophores bound to photolyase.

3. Results

3.1. Cloning and in vivo photoreactivating activity

To test whether the ChchNPV Cc-*phr1* and Cc-*phr2* genes encode active photolyases their ORFs were cloned into the bacterial vector pKK223-3 downstream of an IPTG inducible P_{tac} promoter and expressed in photolyase deficient *E. coli* KY29 cells. After induction, cells were exposed to different doses of UV light followed by visible light or dark treatment (Fig. 1). With the empty vector the number of surviving cells decreased with increasing UV-dose, irrespective of whether the cells were exposed to visible light or were kept in the dark. Cells carrying pKK-Ccphr2 showed a much higher survival when treated with visible light after UV irradiation. At a dose of 2.85 J/m^2 the survival increased from 0.1 to 77%. When kept in the dark the survival of pKK-Ccphr2 containing cells was strongly reduced compared to bacteria carrying the empty vector. Similar experiments with the pKK-Ccphr1 construct did not show any light-dependent increase of survival compared with the empty vector. Together these results indicate that the Cc-*phr2* gene complements the photolyase

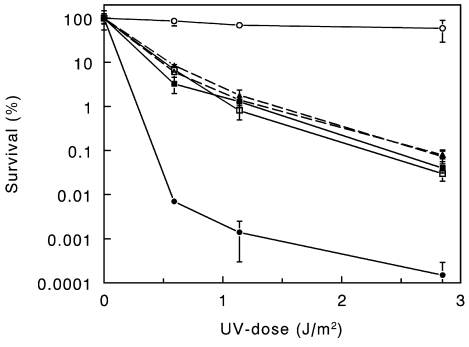

Fig. 1 – Photoreactivation of UV-induced damage in *phr*-deficient *E. coli* KY29 cells harbouring plasmids pKK-Ccphr1 (squares), pKK-Ccphr2 (circles) or empty pKK233-3 vector (triangles, dashed lines). After UV irradiation, cells were kept in the dark (closed symbols) or illuminated with white light (open symbols) for 30 min. Survival is indicated as percentage in relation to non-treated cells. Error bars represent the standard deviation of the mean.

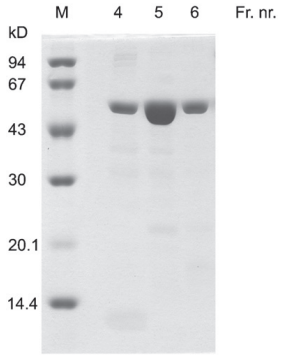

Fig. 2 – Coomassie-blue stained SDS-PAGE gel of Cc-phr2 photolyase purified from *E. coli* KY29. Successive elution fractions (2 μl/lane) of the final SP-Sepharose column were loaded (fraction 4–6). Lane M contains marker proteins with indicated molecular mass.

deficiency of *E. coli* KY29 cells and hence encodes an active photolyase. No activity was found for the Cc-*phr1* gene under these conditions.

3.2. *Purification of Cc-phr2 photolyase*

Cc-phr2 photolyase was isolated from photolyase deficient *E. coli* KY29 cells transformed with plasmid pKK-Ccphr2. Cells were disintegrated by sonication. After centrifugation to remove cell debris photolyase was purified from the supernatant by chromatography on heparin-Sepharose (twice) and SP-Sepharose columns. The final preparation was estimated to be >98% pure on SDS-PAGE (Fig. 2). A molecular mass of 58 kD was obtained, which compares well with the calculated value of 57.3 kD.

3.3. *Chromophores in Cc-phr2 photolyase*

Spectrophotometrical analysis of purified Cc-phr2 photolyase shows, in addition to a broad band around 372 nm, a well-resolved absorption spectrum in the visible region (Fig. 3A). The spectrum has maxima at 442 and 467 nm and a shoulder at 424 nm, pointing to the presence of a flavin chromophore in a hydrophobic environment. After heat denaturation, the fine structure is lost and the absorption spectrum becomes comparable to that of a flavin in aqueous solution (Fig. 3B). The fluorescence spectrum of heat denatured Cc-phr2 photolyase is characteristic for a flavin, with an emission maximum at 521 nm and maxima in the excitation spectrum at 450 and 378 nm. On incubation with phosphodiesterase I, the fluorescence intensity increased by a factor 9.8 (Fig. 4). Phosphodiesterase I cuts the phosphodiester bond which is present in FAD, but not in riboflavin or flavin mono nucleotide (FMN). This result identifies the flavin in Cc-phr2 photolyase as FAD.

Many photolyases contain, in addition to FAD, a second chromophore which is mostly MTHF or 8-HDF. Heat denaturation will release a possible 8-HDF chromophore giving rise to an absorption band at 420 nm [33], which is evidently absent in the spectrum of heat denatured Cc-phr2 photolyase (Fig. 3A). Released MTHF is not stable at neutral pH and has a half-life in the order of half an hour at room temperature. It will convert into products which do not absorb light in the near-UV region [34,35]. Therefore, MTHF will not be found after heat denaturation due to its rapid conversion at elevated temperature. Two additional denaturation experiments were done to confirm the absence of a MTHF chromophore: lithium-dodecyl sulfate was added to purified Cc-phr2 photolyase at room temperature (Fig. 5A) or HCl was added until pH 2 (Fig. 5B), since free MTHF is stable at low pH. In both cases an FAD absorption spectrum was obtained without any indication of the presence of MTHF in the 340–380 nm region. A possible explanation could be that MTHF was lost during purification. *E. coli* pho-

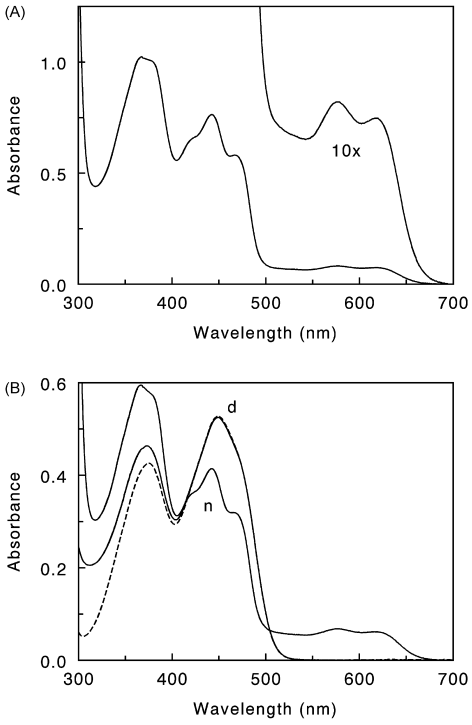

Fig. 3 – Absorption spectrum of purified Cc-phr2 photolyase. The absorption spectrum was determined of freshly prepared Cc-phr2 photolyase (panel A). Part of this spectrum is also shown at a 10-fold enhanced sensitivity. Cc-phr2 photolyase was denatured by heat treatment (5 min at 75 °C, panel B), n denotes native, d denatured photolyase. For comparison the absorption spectrum of FAD is also shown (- - -).

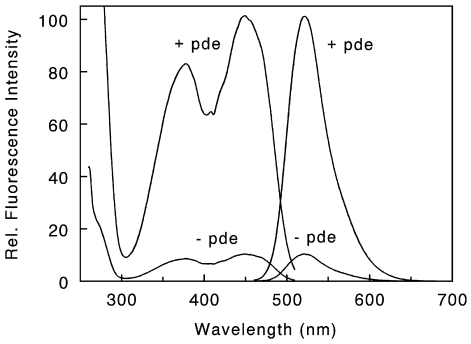

Fig. 4 – Fluorescence spectra of heat denatured Cc-phr2 photolyase treated with and without phosphodiesterase I (pde). In the left part of the figure the excitation spectra are given at the fixed emission wavelength of 522 nm. The right part of the figure shows the emission spectra with excitation at 447 nm.

tolyase for instance releases part of its MTHF chromophore during extensive purification [36]. Comparison of absorption spectra at various stages of purification of Cc-phr2 photolyase indicated no significant changes in the near-UV/visible region (not shown). It is concluded, therefore, that Cc-phr2 photolyase expressed in *E. coli* contains only a FAD chromophore.

3.4. *Photoreduction*

Purified Cc-phr2 photolyase has a long wavelength absorption band with maxima at 577 and 618 nm (Fig. 3A). This is characteristic of the presence of a neutral semiquinone flavin radical ($FADH^{\cdot}$), indicating that FAD is present in various redox states. It is known, however, that only the fully reduced form ($FADH^{-}$), which can be obtained by photoreduction (photoactivation) of oxidized or half-reduced FAD, is active as catalytic co-factor in the photochemical splitting of pyrimidine dimers [22]. When illuminated with white light, both semiquinone and oxidized FAD rapidly disappeared (Fig. 6). Exposure to air in the dark resulted in the reappearance of both forms due to oxidation of fully reduced FAD.

3.5. *Reconstitution*

Because of the apparent lack of an antenna chromophore in recombinant Cc-phr2 photolyase attempts were made to reconstitute purified photolyase with synthetic F0, the natural antenna chromophore in photolyase of the green alga *Scenedesmus acutus* and the cyanobacterium *Anacystis nidulans* [33,37]. After incubation and removal of excess free F0 on a small size-exclusion column, a new band with a maximum at 436 nm was found in the absorption spectrum (Fig. 7), suggesting that F0 spontaneously binds to Cc-phr2 photolyase. Heat denaturation shifted the band to 420 nm which is characteristic of free F0 [33]. The denatured spectrum compares well with that of a 1:1 mixture of FAD and F0, indicating that both chromophores are present in approximately equimolar amounts in reconstituted photolyase. Reconstitution with riboflavin did not induce a significant change in the absorption spectrum (not shown).

Recombinant Cc-phr2 photolyase showed *in vitro* biological activity in the UV-pdT_{18} assay (Fig. 8) as expected from the presence of the catalytic FAD co-factor. After addition of F0 the photoreactivating activity was increased by a factor 6.6, showing unequivocally that F0 acts as an antenna chromophore. Since the molar extinction coefficient of F0 is much higher than that of reduced FAD [33], the efficiency of photon absorption is enhanced. The observed increase in photoreactivating activity also suggests an efficient energy transfer between the two chromophores. Incubation of recombinant photolyase with riboflavin or FMN did not increase the biological activity.

4. Discussion

The analysis performed in this study clearly demonstrates that Cc-*phr2* encodes an active photolyase, which is able to complement a photolyase deficiency in *E. coli* and has *in vitro* photolyase activity. Under light conditions, UV-treated Cc-*phr2*

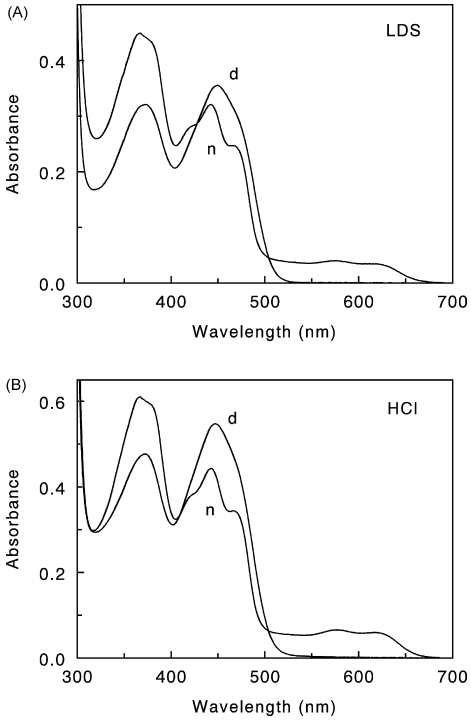

Fig. 5 – Absorption spectrum of purified Cc-phr2 photolyase denatured with (A) lithium-dodecyl sulfate (0.05%) or (B) HCl to pH 2. n denotes native, d denatured photolyase.

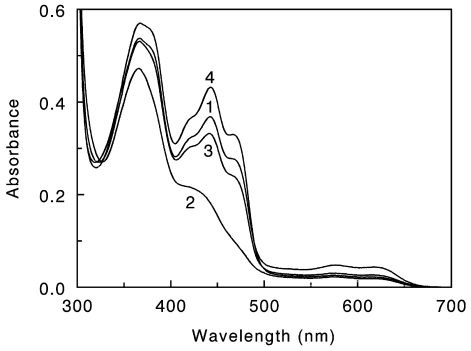

Fig. 6 – Photoreduction of Cc-phr2 photolyase. Highly purified Cc-phr2 photolyase (1) was illuminated in the presence of 25 mM 2-mercaptoethanol with white light for 15 min (2) and subsequently kept in the dark for 30 (3) and 60 (4) min.

expressing cells showed a much higher survival than cells carrying the empty plasmid (Fig. 1). Conversely, the survival of Cc-*phr2* carrying cells was strongly reduced when kept in the dark after UV irradiation, when compared to cells without photolyase (Fig. 1). Photolyases are known to influence the excision repair system in *E. coli* [38] and *Saccharomyces cerevisiae* [39]. Overexpression of a homologous photolyase stimulates excision repair. When a foreign photolyase is overexpressed, however, excision repair is largely inhibited. This causes enhanced UV-sensitivity of cells incubated in the dark compared to cells without photolyase, as was found for Cc-*phr2*.

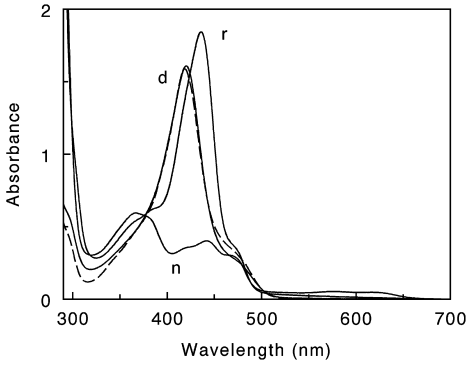

Fig. 7 – Reconstitution of Cc-phr2 photolyase. Native recombinant photolyase (n) was incubated with synthetic F0 (8-OH-7,8-didemethyl-5-dezariboflavin). After removal of excess F0 a new absorption band at 437 nm is visible (r), which shifts to 420 nm after heat denaturation (d). For comparison the spectrum of an equimolar mixture of FAD and F0 is also shown (- - -).

In contrast to Cc-*phr2*, Cc-*phr1* did not provide photolyase activity when tested in *E. coli*. It is possible that Cc-phr1 photolyase was not expressed in *E. coli*, although the nucleotide sequence of the construct was verified thoroughly. The alignment of the Cc-phr2 amino acid sequence against a representative set of class II photolyases (including 2 animal virus photolyases) with established photoreactivating activity is consistent with the class II consensus sequence (Fig. 9). For Cc-phr1 several differences are observed in strictly conserved areas. Many of these deviations from the photolyase consensus sequence are also found in TnSNPV photolyase [30]. Whether TnSNPV encodes an active photolyase is not known but it seems unlikely considering its strong resemblance to Cc-phr1.

In Cc-phr2 photolyase semiquinone and oxidized FAD were rapidly reduced on illumination with visible light (Fig. 6). A similar phenomenon is observed in class I photolyases. An electron transfer mechanism has been proposed where an electron is transferred from a photoreductor in the solvent through a chain of 3 tryptophans to the FAD co-factor [40]. Cc-phr2 photolyase contains several conserved tryptophans (Fig. 9) which may be involved in such electron transfer. Cc-phr1 lacks two of these tryptophans. Inspection of a putative Cc-phr2 photolyase structure obtained by modeling suggests that W355 could be the equivalent of W306 in *E. coli* photolyase at the end of the triple tryptophan electron transfer chain. This tryptophan is, however, absent in Cc-phr1 (and in TnSNPV phr) which may point to an impaired electron transport in Cc-phr1 protein.

This study demonstrates for the first time that a functional photolyase enzyme is encoded by an insect virus. Although photolyase genes have been found in only a few baculoviruses (although over 40 complete genomes have been sequenced

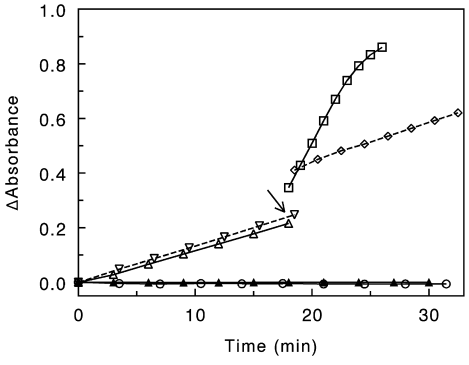

Fig. 8 – *In vitro* biological activity of Cc-phr2 photolyase. A mixture of UV-pT18 and Cc-phr2 photolyase was kept in the dark (closed triangles) or illuminated with white light (open triangles). F0 (full line) or riboflavin (dashed line) was added (arrow) and after 7 min of incubation in the dark illumination was resumed (open squares for F0, diamonds for riboflavin). As a control UV-pT18 alone was illuminated (open circles). CPD repair was measured as the absorbance increase at 265 nm.

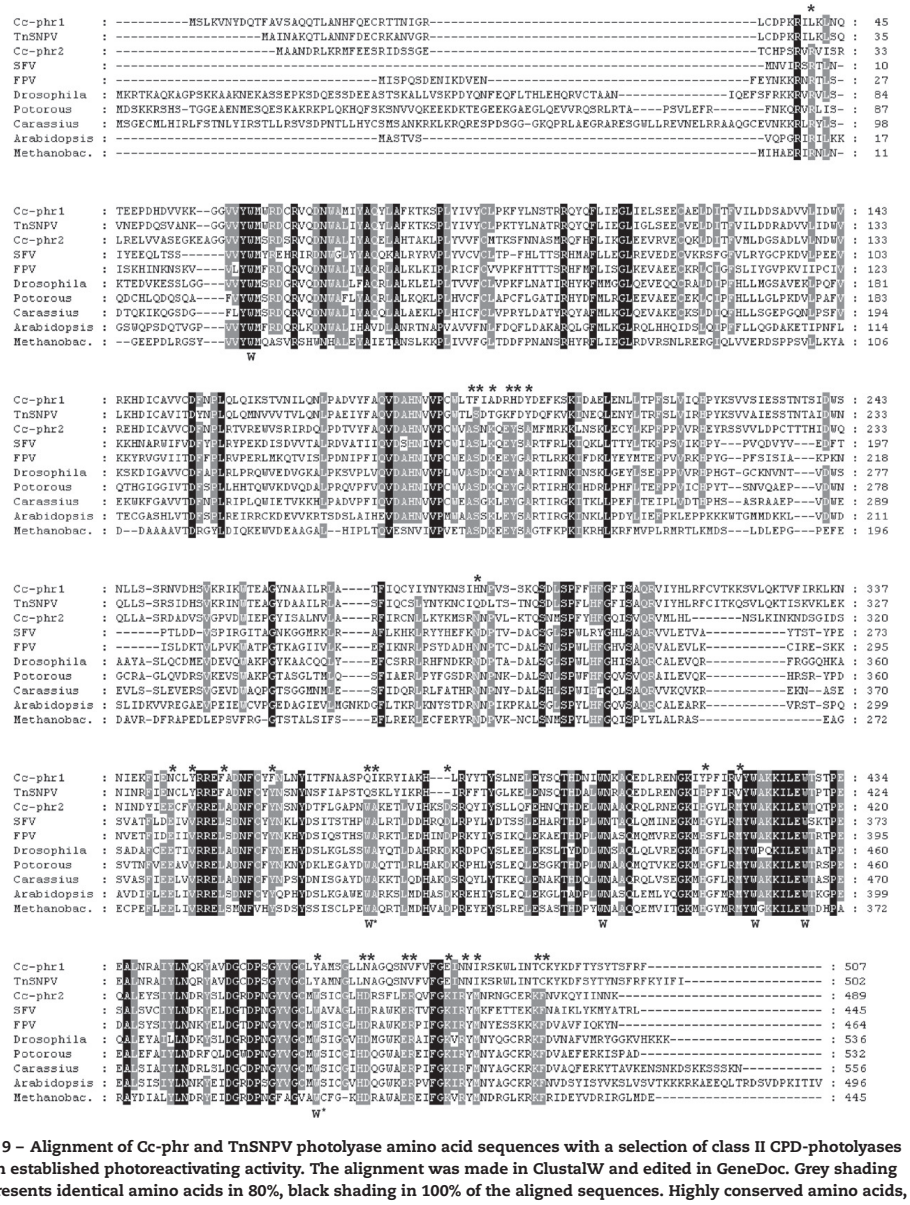

Fig. 9 – Alignment of Cc-phr and TnSNPV photolyase amino acid sequences with a selection of class II CPD-photolyases with established photoreactivating activity. The alignment was made in ClustalW and edited in GeneDoc. Grey shading represents identical amino acids in 80%, black shading in 100% of the aligned sequences. Highly conserved amino acids, which are not present in Cc-phr1, are indicated with asterisks. Conserved tryptophans are indicated by W, tryptophans not conserved in Cc-phr1 by W*. Photolyase sequences were retrieved from literature or GenBank: Cc-phr1 and Cc-phr2 [9]; Trichoplusia niSNPV [30], Fowlpox virus (FPV) [54], Rabbit (shope) fibroma virus (SFV) [25], *Drosophila melanogaster* [BAA05042], *Potorous tridactylis* [14], *Carassius auratus* [BAA01987], *Arabidopsis thaliana* [T52112] and *Methanothermobacter thermautotrophicus* (Methanobac.) [14].

already [41]), an active photolyase could be ecologically beneficial for a baculovirus. Baculovirus infection changes the behavior of host larvae, as these larvae become hypermobile and move, prior to death, towards the top of the plant canopy on which they feed [42,43]. In this way progeny virus, in the form of viral occlusion bodies, is spread efficiently over the foliage upon rupture of the cadaver's cuticle, increasing the chance of virus transmission to other individuals in the insect population. A trade-off most likely exists between effective virus spread and increased exposure to UV light. In addition, to allow transmission from insect to insect, baculovirus occlusion bodies may have to persist in the field for long periods, awaiting a new cohort of host insects. During this time baculoviruses are inactivated quickly by UV light [5]. Therefore, a functional photolyase is likely to increase the lifetime of baculoviruses.

Spectral measurements (Fig. 3) indicated the presence of a flavin chromophore in a hydrophobic environment in Cc-phr2 photolyase. The 9.2-fold increase of fluorescence intensity after treatment of the released chromophore with phosphodiesterase I (Fig. 4) compares well with the 8.2-fold increase found for FAD [37]. It identifies the flavin as FAD and rules out the presence of other flavins like FMN or riboflavin. Denaturation experiments (Fig. 5) revealed the absence of MTHF, suggesting that recombinant Cc-phr2 photolyase does not carry an antenna chromophore.

In general the antenna chromophore site is very selective in accepting a chromophore. For instance *E. coli* photolyase lacking its MTHF antenna chromophore will not accept F0 [44,45] and conversely *A. nidulans* photolyase lacking F0 will not accept MTHF [46]. Recently a "promiscuous" chromophore site in *Thermus thermophilus* photolyase was described which binds, after removal of its natural FMN chromophore, riboflavin, F0 and 8-iodo-8-demethyl-riboflavin [47]. *A. nidulans* photolyase on the other hand reconstitutes only with its natural F0 chromophore and not with riboflavin or FAD [46]. The result of reconstitution experiments indicates that the antenna chromophore site in Cc-phr2 photolyase is not promiscuous but restrictive like the site in *A. nidulans* photolyase.

The successful reconstitution with F0 (Figs. 7 and 8) suggests that the Cc-phr2 is an 8-HDF type photolyase. Little is known about the natural antenna chromophores of class II photolyases, mainly because of the common practice to express cloned *phr* genes in a heterologous host (e.g., *E. coli*), which for instance does not synthesize 8-HDF [48]. *Methanobacterium* (*Methanothermobacter*) *thermoautotrophicum* photolyase was isolated from its natural source [49] and is an example of an 8-HDF type photolyase belonging to class II [14], establishing that 8-HDF type photolyases do occur in class II.

The question may arise how Cc-phr2 photolyase could acquire a F0 chromophore. Obvious candidate sources include the *Chrysodeixis* viral genome and the insect host. Up to now 8-HDF has been found in four groups of organisms: *Archae*, *Actinobacteria*, *Cyanobacteria* and unicellular algae [50]. However, no data are available on the occurrence of 8-HDF in other organisms like insects, plants or viruses. A key step in the biosynthesis of 8-hydroxy-5-deazaflavins is the formation of F0 from precursors by F0-synthase [51], which is encoded by cofG/cofH genes or by the closely related fbiC gene [52]. No homolog of these genes is present amongst the 151 ORFs of the ChchNPV genome [9]. A Blast search of the presently known insect genomes also did not reveal such a homolog.

It is conceivable that F0 could be obtained from microorganisms present in the digestive tract of the host larvae. The presence of members of the *Actinobacterium* group, which are known to synthesize 8-HDF, in the midgut of gypsy moth larvae has been reported [53]. Alternatively Cc-phr2 photolyase might work without an antenna chromophore, since the recombinant apophotolyase exhibits considerable activity in *in vivo* experiments (Fig. 1). Further studies will reveal whether this functional DNA photolyase can confer or enhance UV resistance in baculoviruses which lack a natural photorepair system and whether it can provide an increased survival of these viruses under natural conditions.

Conflict of interest

None.

Acknowledgments

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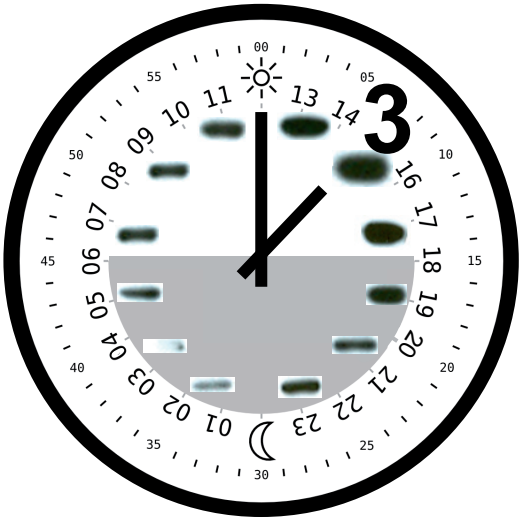
Potorous tridactylus CPD photolyase: a DNA repair protein with unexpected circadian clock function



Manuscript in preparation

Potorous tridactylus CPD photolyase: a DNA repair protein with unexpected circadian clock function

Monika I. Bajek, Karl Brand, Sander Barnhoorn, André P.M. Eker, Gijsbertus T.J. van der Horst and Inês Chaves

Department of Genetics, Chronobiology and Health Research Group, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

Abstract

Photolyases are DNA repair enzymes which use visible light to repair UV-induced DNA damage, whereas cryptochromes were identified in plants as blue-light photoreceptors, but also function as circadian photoreceptor (e.g. *Drosophila*) and core circadian clock proteins (e.g. mammals). Despite the sequence and structural conservation between cryptochromes and photolyases, their functions are divergent. However, functional domains are interchangeable as we and others have shown that photolyase/cryptochrome chimeric proteins can act in the molecular clock.

We asked whether in evolution there was a common ancestor with both functions and whether there is a naturally occurring photolyase that can (partially) function in the circadian clock. To address this question we investigated the effect of two different photolyases on the circadian oscillator, both *in vitro* and *in vivo*. The proteins studied were the (6-4)PP-photolyase from *Arabidopsis thaliana* and the CPD-photolyase from *Potorous tridactylus,* close and distant relatives of mammalian cryptochromes, respectively. Our results indicate that the CPD-photolyase can function in the molecular oscillator, as shown by an altered free-running period of locomotor activity of transgenic mice carrying this protein. Furthermore, the CPD-photolyase affects the amplitude of circadian oscillation in cultured cells and is capable of inhibiting CLOCK/BMAL1 driven transcription. This is an unexpected finding, which sheds light on the functional evolution of the photolyases/cryptochrome protein family.

Introduction

Life on earth is subject to the 24-hour rotation cycle, which imposes rhythmic changes in day/night and temperature. In order to anticipate and adjust behaviour, physiology and metabolism to these changes, most organisms have developed a circadian clock, which has a periodicity of approximately 24 hours and needs to be re-set everyday to keep pace

with the day/night cycles. The most predictable environmental cue is light, which is the strongest *zeitgeber* (from the German, "time giver"). The mammalian master clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, which receives light re-setting signals transmitted from the retina (Rusak et al., 1989). In turn, this master clock sends humoral and neuronal signals that will synchronise the peripheral oscillators, located in virtually every cell or tissue (Balsalobre et al., 1998; McNamara et al., 2001; Pando et al., 2002; Yoo et al., 2004).

At the molecular level the circadian oscillator is composed of transcription/translation-based feedback loops. The CLOCK/BMAL1 heterodimer activates transcription of (clock) genes containing an E-box promoter, and the clock gene-products mCRY1 and 2 and mPER1 and 2 will repress their own transcription, as well as that of other clock controlled genes (Reppert and Weaver, 2001; Young and Kay, 2001). A second loop is formed by REV-ERBα that feeds back on the *Bmal1* promoter repressing it (Preitner et al., 2002; Etchegaray et al., 2003). *Bmal1* transcription is further modulated by the RORs nuclear receptors (Sato et al., 2004). Adding to the transcription/translation feedback, there is an intricate network of posttranslation modifications that fine-tune the oscillator: phosphorylation, (de)acetylation, sumoylation and ubiquitylation of clock proteins are essential for the robustness and persistence of the oscillations (Toh et al., 2001; Yagita et al., 2002; Asher et al., 2008; Nakahata et al., 2008).

At the heart of the molecular oscillator are the mammalian cryptochromes CRY1 and CRY2, members of the photolyase/cryptochrome family of flavoproteins, which act as potent inhibitors of CLOCK/BMAL1 (Kume et al., 1999). Mammalian *Cry* genes were initially identified as homologues of photolyases (Van der Spek et al., 1996; Todo et al., 1997). However, studies with knockout mice for either *mCry1* or *mCry2* showed that the two mouse cryptochromes are part of the molecular circadian system. These two mutant mice have opposing circadian phenotypes, $mCry1^{-/-}$ mice with a short period and $mCry2^{-/-}$ mice with a long period, while the *mCry1/2* double knockout mice show a complete loss of the circadian clock, indicating that mammalian cryptochromes are core clock proteins (Van der Horst et al., 1999; Vitaterna et al., 1999). This was the first time that a cryptochrome was shown to be a component of the core oscillator, and more recently more cryptochromes were identified that play a role as core clock components, such as in *Xenopus* and Zebrafish (reviewed in Tamanini et al., 2007). Historically, cryptochromes were identified in plants, as blue-light receptors, and later in *Drosophila,* where they are circadian photoreceptors. The functional diversity of cryptochromes goes even further, as they sense magnetic fields and play a role in compass navigation (Zhu et al., 2008).

Photolyases, the other members of the photolyase/cryptochrome protein family, are DNA repair enzymes that use visible light to specifically reverse cyclobutane pyrimidine dimers (CPDs) or pyrimidine (6-4) pyrimidine photoproducts [(6-4)PPs] formed in DNA by UV-light (Sancar, 2003; Essen and Klar, 2006; Klar et al., 2006, 2007). The reaction mechanism of photolyases is called photoreactivation. The first step is light-

independent binding of one single photolyase molecule to the DNA lesion, followed by light-dependent dimer splitting and reversal of the lesion. Interestingly, placental mammals have lost photolyase genes during evolution (Yasui et al., 1994). Previously, we generated CPD and (6-4)PP photolyase transgenic mice to unravel the deleterious biological effects of the individual class of photolesions (Schul et al., 2002; Jans et al., 2005, 2006). Our findings indicate that CPDs, rather than the (6-4)PP photolesions, are the major cause of sunburn, mutation induction and skin cancer development.

Photolyases and cryptochromes are present in many organisms, from prokaryotes to higher eukaryotes. Cryptochromes and photolyases belong to the same family of flavoproteins, (Figure 1A).

The photolyase/cryptochrome protein family is divided in two subgroups, of which the first one is composed of Class I CPD photolyases, (6-4)PP photolyases and crypochromes. The second group is composed solely of class II CPD photolyases. It is accepted that all members of the photolyase/cryptochrome protein family evolved from a common ancestor CPD photolyase by multiple gene duplications. Cryptochromes and photolyases share a common backbone (core domain) which binds two chromophores but differing in their N- and C-termini (Figure 1B). Photolyases have N-terminal extensions containing nuclear and mitochondrial localization signals and cryptochromes have a unique and variable C-terminal extension (extCT). It is likely that the functional diversity among cryptochromes is due to the differences in length and sequence composition of the CT, and how the core domain and CT interact. The structure of the core domain of several class I photolyases is known and conserved, but no structure information is available for class II photolyases. Among the solved structures are the *E. coli* CPD photolyase (Park et al., 1995) and the *A. nidulans* CPD photolyase (Tamada et al., 1997). Furthermore, the structure of the core domain of *Arabidopsis thaliana* CRY1 (Brautigam et al., 2004) and *At*CRY3 (Huang et al., 2006) and recently of (6-4)PP photolyase from *Arabidopsis* were determined (Hitomi et al., 2009), as well as the structure of *Drosophila* (6-4)PP photolyase (Maul et al., 2008).

We and others have shown that the C-terminus of CRY is necessary but not sufficient for repression of CLOCK/BMAL1 driven transcription, and that interaction between the core domain and the CT is important (Chaves et al., 2006; van der Schalie et al., 2007). Furthermore, analysis of fusion proteins between different domains of CRYs and photolyases suggests that domains can be interchanged. In previous work (Chaves et al., 2006) we observed that it is possible to fuse the core domain of the *Arabidopsis* 6-4 photolyase to the last ~220 amino acids of mCRY1 and obtain a chimeric protein that can inhibit CLOCK/BMAL1.

To further understand the functional evolution of cryptochromes and photolyases, we focused on the 6-4 photolyase from *Arabidopsis thaliana* [*At*(6-4) PhL] and the CPD-photolyase from *Potorous tridactylus* (*Pt*CPD PhL) and tried to identify the residual core oscillator function. By studying these proteins both *in vitro* and *in vivo*,

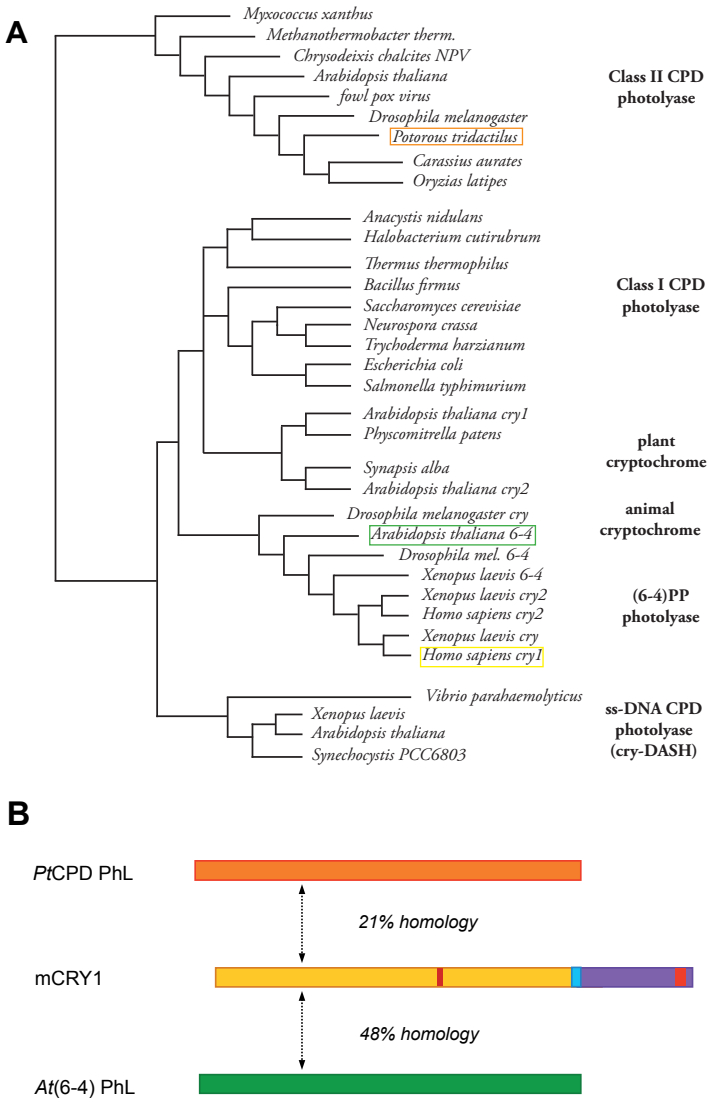


Figure 1. The cryptochrome/photolyase protein family

A Unrooted phylogenetic tree of the photolyase/cryptochrome family. Amino acid sequences were aligned with ClustalX and a tree was obtained for the core region with the neighbour-joining method (PHYLIP package vs. 3.67 by J. Felsenstein) using the Jones-Taylor-Thornton matrix. Figure adapted from Eker et al., 2009, *Cell Mol Life Sci* **66:** 968-80.

B Schematic representation of *Potorous tridactylus* CPD photolyase (orange), *Arabidopsis thaliana* (6-4) PP photolyase (green) and mammalian CRY1 (yellow: the core domain; cobalt: the predicted coiled-coil domain; red – nuclear localisation signals (NLSn and NLSc); magenta: the C-terminal tail. The percentages indicate the homology at the amino acid level.

we found that the CPD photolyase from *Potorous tridactylus,* although more divergent from mCRYs, can function in the circadian clock. Thus far, functional analysis of cryptochromes was focussed on the C-terminal extension, and little is known about the core domain. Here we use transgenic mice to show that the photolyase core domain affects circadian rhythmicity, suggesting that the core might interact with clock proteins resulting in a dominant negative effect.

Materials and Methods

Mouse lines and monitoring of circadian locomotor-activity rhythm

β-actin::*At*(6-4)PP (Jans et al., 2006), β-actin::*Pt*CPD (Schul et al., 2002) and *mPer2::luciferase* transgenic mice (generation will be described elsewhere) were in a C57BL6/J background and were housed under standard conditions and fed *ad libitum*.

For the monitoring of locomotor activity rhythms, mice of both sexes and of different ages (8-16 weeks) were used. They were individually housed in a light-proof chamber in cages (30 x 45 cm) equipped with a running wheel (11 cm in diameter) and a sensor system to detect wheel rotations. Animals were maintained in a cycle of 12 h light (150 lux) and 12 h complete darkness (LD) or in continuous complete darkness (DD) in constant ambient temperature with water and food available ad libitum. Voluntary wheel-running (wheel turns per unit of time) was continuously recorded by an online computer using the ERS program. Activity records were plotted as actograms and the period of locomotor activity was determined by the chi-square method. Unpaired Student's t-tests were used to make statistical comparisons between the different genotypes.

Plasmids, cells, culture conditions and transient transfection

The following plasmids were used pcDNA-HA-mCry1, pcDNA-*Pt*CPD, pcDNA-*At*(6-4)PP, pcDNA-Bmal1 and pcDNA-Clock, all having pcDNA3 as backbone (Invitrogen). pcDNA-*Pt*CPD and pcDNA-*At*(6-4)PP) were based on the constructs used to generate the transgenic mice (Schul et al., 2002; Jans et al., 2006).

COS7 cells, NIH 3T3 cells and wild-type, mCry1$^{-/-}$ and CPD mouse dermal fibroblasts (MDFs) were cultured in Dulbecco's modified Eagle's medium-F10 supplemented with 10% fetal calf serum, penicillin/streptomycin and transfected with Fugene reagent (Boehringer) according to the manufacturer's instructions. To generate MDFs, mice were sacrificed by cervical dislocation, and a small piece of back skin of the mouse was removed and cut into pieces with a razor blade. Skin pieces were washed in ethanol, rinsed in phosphate-buffered saline, and incubated overnight in medium supplemented with 1.6 mg/ml collagenase type II. Single cells were obtained by

passing through a cell strainer, and collected by centrifugation for 5 min at 1,000 rpm, resuspended in cultured medium, and seeded onto a 10-cm dish. MDFs were cultured in a low-oxygen incubator (5% CO_2, 3% O_2).

RNA isolation and quantitative PCR

Total RNA from cultured cells was isolated from microdissected SCN cryosections (K. Brand, manuscript in preparation). First strand cDNA was synthesized from 1 µg of total RNA using oligo-dT primers and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative PCR for the determination of (6-4)PP and CPD mRNA levels was performed in triplicate using as iCyclerIQ system (BioRad), SYBR-green and primers generating intron-spanning products of 150-300 bp. Expression levels were normalised to *Hprt* (Hypoxanthine guanine phosphoribosyl transferase) mRNA levels. The generation of specific products was confirmed by melting curve analysis, and primer pairs were tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate primer pair efficiencies.

Real time bioluminescence

To monitor circadian oscillations in cultured cells in real time, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of confluent cultures with forskolin (dissolved in 100% ethanol, added to the culture medium at a final concentration of 30 µM) bioluminescence was recorded for 7 days (75 s measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C.

Luciferase reporter assay

To determine the capacity of CPD-PhL and (6-4)PP-PhL to inhibit CLOCK/BMAL1 driven transcription, a luciferase reporter assay was carried out as described previously (Chaves et al., 2006). COS7 cells were transfected with 200 ng of m*Per1*::*luciferase* reporter gene and 15 ng of null-*renilla luciferase*, which was used as an internal control. 150 ng of *Clock, Bmal1,* m*Cry1*, CPD and (6-4)PP plasmids were added as indicated below each graph (Figure 6). The total amount of DNA transfected was kept constant at 2 µg by supplementing with empty pcDNA3.1 vector (Invitrogen). Transcriptional activity was assessed with the Dual-Luciferase 10 Reporter Assay System (Promega) by measuring a ratio of firefly luciferase activity to *Renilla* luciferase activity in each cellular lysate.

Results

Transgenic mice carrying the *Potorous tridactylus* CPD photolyase have a short tau

To investigate the effect of photolyases on the circadian clock, we used transgenic mice carrying either the *Potorous tridactylus* CPD photolyase (hereafter named CPD) and of the *Arabidopsis thaliana* (6-4)PP photolyase (hereafter named (6-4)PP. These mice were previously generated in our group; therefore it was a logic step to analyze their circadian behaviour. However, before doing so, we tested the presence of the CPD and the (6-4)PP mRNA in the SCN by quantitative PCR (see Materials and Methods for details). Material from SCN slices was laser-microdissected and RNA was isolated and amplified. As shown in Figure 2, both mRNAs are present in the SCN, at comparable levels to the *Hprt* gene.

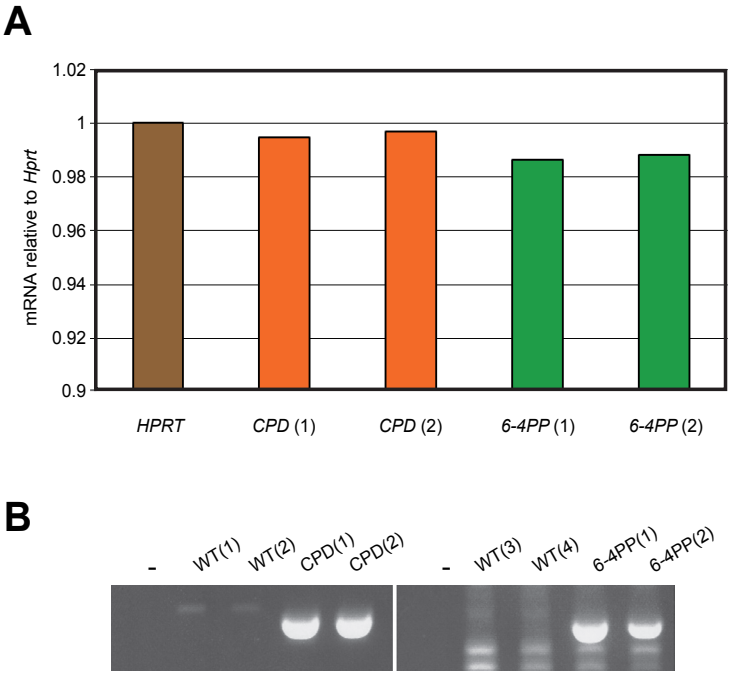


Figure 2. mRNA expression levels of the PtCPD and At(6-4)PP photolyases in the SCN of transgenic mice

A Graphic representation of qPCR amplification data (see Materials and Methods for experimental details). The Y-axis represents the mRNA levels of CPD and (6-4)PP relative to *Hprt*.

B Ethidium bromide stained gel of PCR amplified cDNA. The mouse line from which the mRNA was derived is indicated above each lane. The numbers in brackets refer to independent mice.

To determine the circadian behaviour, the photolyase transgenic mice were housed in cages provided with a running wheel and activity was recorded and analyzed (Figure 3 and supplementary Figures S1.A,B; described in Materials and Methods). The phenotype of transgenic mice was compared to that of wild type littermates. The tau of (6-4)PP transgenic mice does not deviate from that of their wild type littermates, both in constant darkness (Figure 3A) and in constant light (Supplementary Figure S1.A), suggesting that this photolyase does not affect the circadian clock. Interestingly, the analysis of the CPD transgenic mice gave a different result. The free-running period of the transgenic mice is approximately 20 minutes shorter than that of wild type (Figure 3B), and this difference is significant, although small. In addition, the tau of the CPD mice has a larger standard deviation than that of littermates, suggesting a variable effect and a less robust clock. The activity of these mice was also analyzed in constant light, but here we found no difference (Supplementary figure S1.B). We show that the *Pt*CPD photolyase can affect the mouse circadian clock, making it less robust and ticking slightly faster.

Mouse dermal fibroblasts from CPD transgenic mice mimic the behavioural phenotype

We have shown that the *Pt*CPD photolyase in mice leads to a short free-running period, and proceeded to confirm the tau changes in cultured mouse dermal fibroblasts (MDFs). The CPD mice were interbred with *mPer2::luciferase* transgenic mice in order to obtain a mouse line that could be used for real time imaging. Primary MDFs were isolated and brought in culture. To analyse the oscillatory capacity, primary MDF cultures were synchronized with forskolin and luminescence was recorded in real time (Figure 4). Analysis of independent cell lines shows that the behavioural phenotype can be reproduced in cultured fibroblasts, as we observe that the period of the oscillations is 23.3 hours, approximately 45 minutes shorter than that of fibroblasts that do not carry the CPD transgene (24.1 hours).

Cells overexpressing CPD-PhL oscillate with lower amplitude

To further study the effect of the *Pt*CPD photolyase on the molecular oscillator and understand at what level it is acting, we analyzed the effect of overexpression in NIH 3T3 cells. We used real-time monitoring of the *luciferase* gene under control of the *mPer2* promoter, in synchronized NIH 3T3 cells. Cells were transected with the reporter construct and the construct to be tested, and 72 hours later were synchronized with forskolin and luminescence was measured. The results obtained are shown in Figure 5. Cells transfected with the empty vector, pcDNA3, oscillate with a period of 25.2 hours. We observed that expression of mCry1 abolishes the oscillation, which is in agreement with its strong repressor capacity. Expression of the CPD reduces the baseline and the

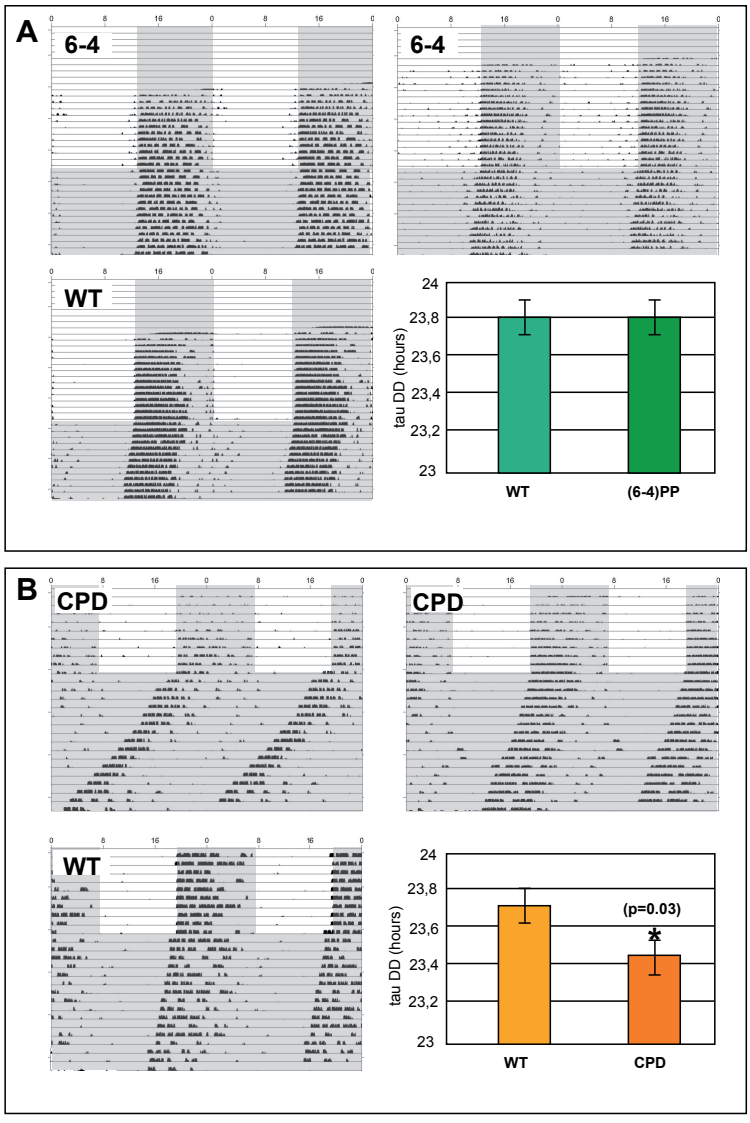


Figure 3. Circadian behaviour of photolyase transgenic mice

Each panel includes representative examples of the 24h double-plotted actograms obtained for each transgenic line, two of transgenic mice and one of wt littermates, and a graphic representation of the free-running period in constant darkness (the error bars represent the standard deviation, the asterisk indicates significance). The grey shade in the actograms indicates lights off.

A β-actin:(6-4)PP transgenic mice (light and dark green).

B β-actin::CPD transgenic mice (light and dark orange).

Shading indicates darkness and the black vertical lines represent animal activity.

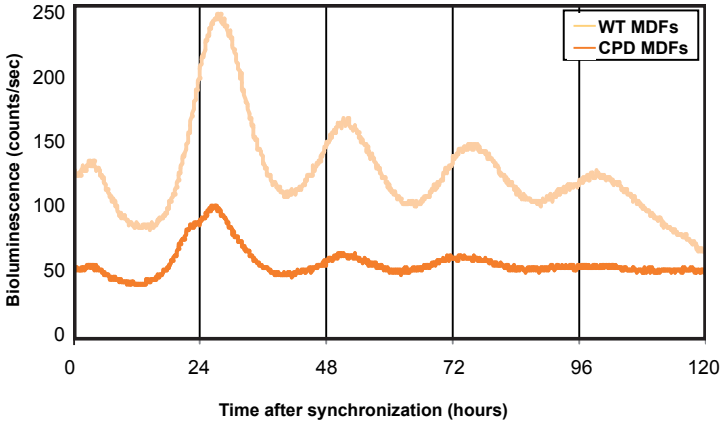


Figure 4. Circadian oscillation of forskolin synchronized mouse dermal fibroblasts
Representative example (n=3) of bioluminescence rhythms in MDFs derived from CPD transgenic mice in an *mPer2::luciferase* background. Wild type MDFs are shown in light orange and CPD MDFs are shown in dark orange. The X-axis indicates the time after forskolin synchronization. The Y-axis indicates bioluminescence in counts/second.

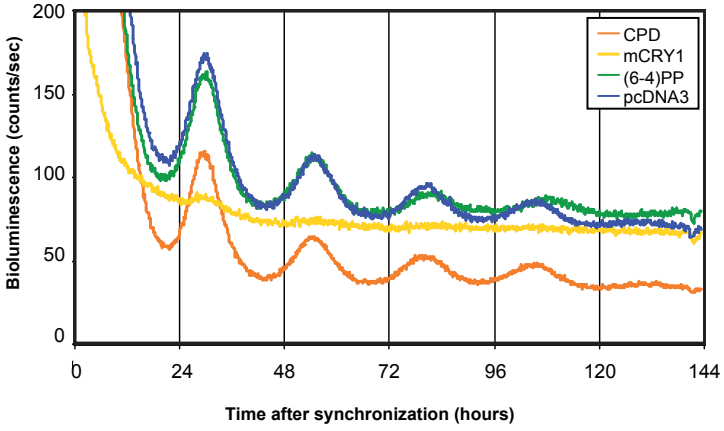


Figure 5. PtCPD alters the circadian oscillation of synchronized NIH 3T3 cells
Representative example (n=3) of bioluminescence rhythms in NIH 3T3 cells transfected with mPer2::luciferase and either pcDNA3 (blue), mCry1 (yellow) or *Pt*CPD (orange). Additionally the (6-4)PP shown in green, which has no effect on the circadian oscillation. The X-axis indicates time after forskolin synchronization.

amplitude of the oscillation, suggesting that the CPD-PhL is possibly acting at the transcriptional level. We have also analyzed the *At*(6-4) photolyase, which, according to the mouse behavioural data, did not have an effect on the circadian oscillation (Figure 5).

The *Potorous tridactylus* CPD photolyase can inhibit CLOCK/BMAL1 driven transcription

Having shown that CPD photolyase interferes with the clock, possibly at the transcriptional level, we next investigated in more detail the possible mode of action. Making use of a luciferase reporter gene under control of an E-box containing promoter it is possible to study the capacity of a protein to inhibit the transcription activation mediated by the CLOCK/BMAL1 heterodimer (Kume et al., 1999). We have used this system to analyze the ability of the CPD and (6-4)PP photolyases to inhibit the CLOCK/BMAL1 driven transcription of the *mPer1* promoter. In the presence of CLOCK and BMAL1 the luciferase gene is expressed, and transcription is strongly repressed in the presence of mCRY1. This was not the case for either the CPD or the (6-4)PP photolyase, suggesting the absence of inhibitory capacity (Figure 6A). Furthermore, by mixing each of the two photolyases with mCRY1 in different ratios, we show that they do not compete with mCRY1 or inhibit its function.

This was an unexpected result, as we have shown that the CPD photolyase can affect the circadian clock both *in vivo* and in cultured cells. However, as mCRY1 is a very strong repressor, it is possible that small effects would be missed. We therefore have performed the same assay but using increasing amounts of DNA (up to 300 ng, in case of the photolyases). We now observe that the CPD photolyase, but not the (6-4)PP photolyase, is capable of partially inhibiting CLOCK/BMAL1 in a dose dependent fashion (Figure 6B). Considering that mCRY1 is twofold homologous to the (6-4)PP photolyase than to the CPD photolyase, we suggest that it is not the amino acid sequence per se, but more the overall structure, that is important for repressing CLOCK/BMAL1.

Discussion

We have previously shown that fusion of the extCT of mCRY1 (aa 371-606) to *Arabidopsis thaliana* (6-4)PP photolyase resulted in a chimeric protein which is able to inhibit CLOCK/BMAL1 driven transcription, whereas the extCT of mCRY1 fused to EGFP was not (Chaves et al., 2006). This result led us to suggest that an interaction between the CT and the core domain is very important for the clock function of cryptochromes, possibly directed by structure. C. Green and co-workers (Van der Schalie et al., 2007) have also used CRY-PhL chimeras to demonstrate that the C-terminal region of the

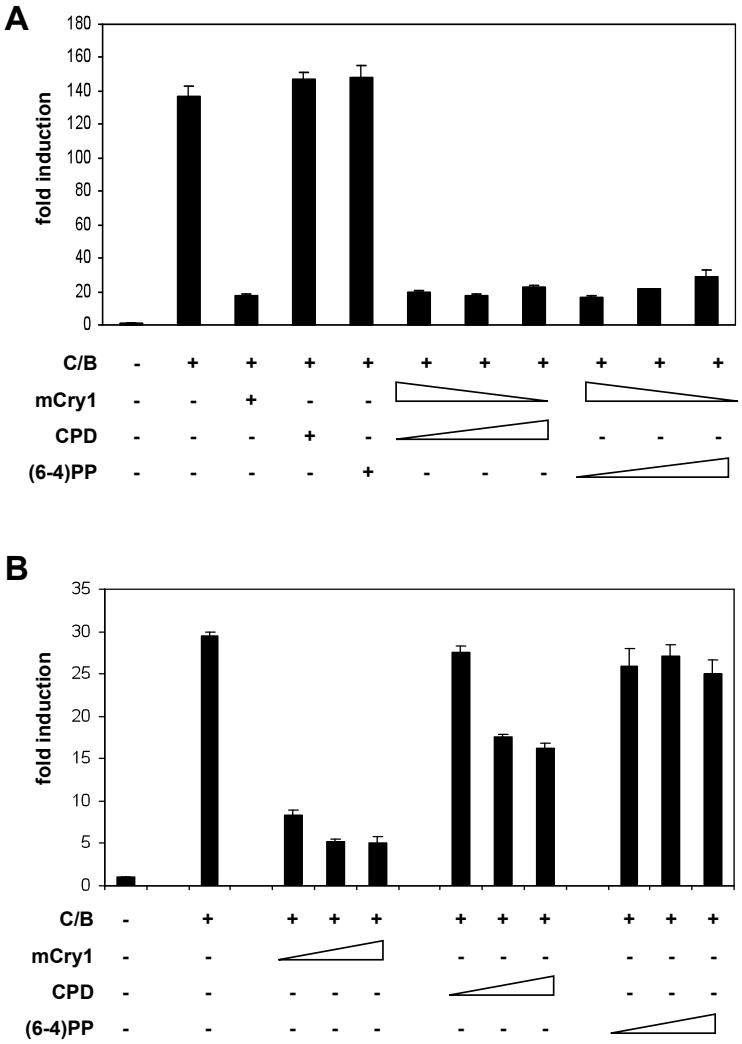


Figure 6. Effect of the PtCPD photolyase on CLOCK/BMAL1 driven transcription

CLOCK/BMAL1 transcription assay using an *mPer2* E-box promoter-luciferase reporter construct. Luminescence, shown as fold induction from the basal level, is indicated on the *y* axis. pcDNA3, pRL-CMV, and the *mPer2::luc* were added in all conditions. The presence or absence of the other expression plasmids is indicated. The values are relative to pcDNA alone, set to 1. Mean and standard deviation of triplicate samples are shown.

A Experiment including a single amount of each tested plasmid, as well as mixing mCry1 with either *Pt*CPD or *At*(6-4)PP, in different ratios.

B Experiment including increasing amounts of each tested DNA (mCry1: 10-100 ng; *Pt*CPD: 100-300 ng; *At*(6-4)PP: 100-300 ng).

core domain is crucial for transcription repression. Taken together, these data show that although photolyases do not have a clock function, upon domain swapping with cryptochromes it is possible to obtain chimeric proteins which are (partially) functional as clock proteins.

In the present study we take a step further in understanding the functional diversity between Cryptochromes and Photolyases, and how nature uses the same core sequence for completely different functions (e.g. photoreactivation by photolyases vs clock function of cryptochromes). We analyzed the capacity of two different photolyases to influence the circadian clock, both *in vivo* and *in vitro*: the CPD photolyase from *Potorous tridactylus*, which belongs to class II of photolyases and is a distant relative of mCRY1, and the (6-4) PP photolyase from *Arabidopsis thaliana*, which belongs to class I and is closely related to mCRY1. Surprisingly, our results suggest that the *Pt*CPD can change the free-running period of transgenic mice, and does so by inhibiting CLOCK/BMAL1 driven-transcription (Figure 7). Because the data are obtained from a single mouse transgenic line available, we cannot exclude that the effect we observe is a result from the integration of the transgene. Analysis of the integration of the transgenic locus did not reveal any neighbouring gene known to regulate circadian rhythms (data not shown). Furthermore, we have supporting *in vitro* data suggesting a role of the *Pt*CPD in transcription repression. A conclusive answer will come from knockdown experiments performed in cells derived from the CPD transgenic mice, where we should observe a correction of the phenotype.

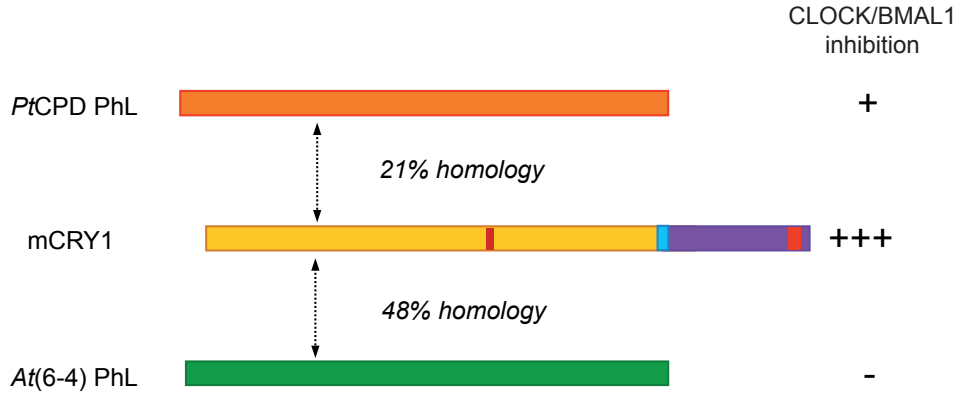
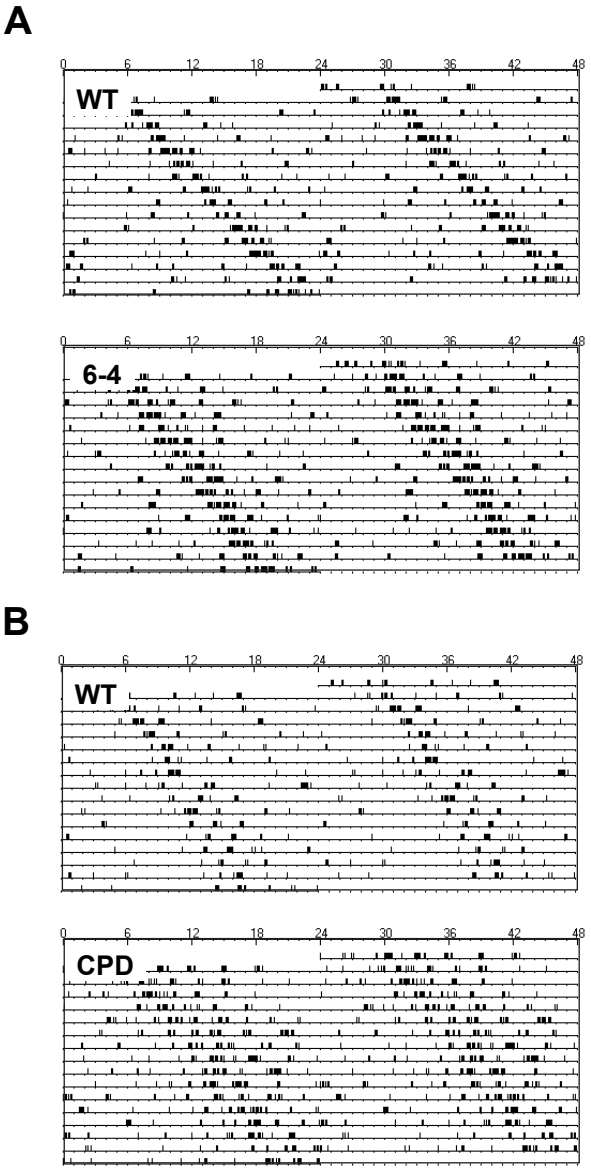


Figure 7. Schematic representation of *Potorous tridactylus* CPD photolyase (orange), *Arabidopsis thaliana*, (6-4)PP photolyase (green) and mammalian CRY1 (yellow: the core domain; light blue: the predicted coiled-coil domain; red: nuclear localisation signals (NLSn and NLSc); magenta: the C-terminal tail and their capacity to inhibit CLOCK/BMAL1.

The recent finding of Coesel et al., (2009) reinforces our data, as it shows that it is possible to have photoreactivating and clock repressor function in the same protein. In this study the authors show that the (6-4)PP photolyase from a marine diatom, in addition to its photoreactivating function, can function in the mammalian circadian clock. This protein belongs to class I and is likely a missing link in evolution, as gene duplication events have likely separated the two functions. We now show that a marsupial photolyase, from class II, has partial capacity to inhibit CLOCK/BMAL1, an effect on the circadian clock that is also detectable *in vivo*. Whether the *Pt*CPD has a residual function from an ancestor protein or it plays a circadian role in *Potorous tridactylus* remains to be studied. Further analysis of CPD photolyases from class II will be needed to answer this question. Additionally, studying the circadian properties of cells from the *Potorous tridactylus* may shed new light on understanding the functional evolution of the cryptochrome/photolyase family.

Analysis of genome sequence data reveals that marsupials have genes that are homologous to mammalian cryptochromes, but so far nothing is known about how their molecular clock functions. Although we are not able to use this animal as a model, we can make use of available cell lines and study its circadian clock and a possible role of the CPD photolyase. Experiments with the *Potorous tridactylus* kidney cell line PtK2 (rat kangaroo kidney epithelial cell line) are ongoing, and so far we have been able to show that these cells can be synchronized and display circadian oscillation of a luciferase reporter under control of the mPer2 promoter (data not shown).



Supplementary figure S1. Circadian behaviour of (6-4)PP (A) and CPD (B) photolyase transgenic mice in constant light

Each panel includes representative examples of the 24h double-plotted actograms obtained for each transgenic line, on the top one of transgenic (6-4)PP *(A)* and *CPD (B)* mice and on the bottom one of wt littermates. The black vertical lines represent animal activity.

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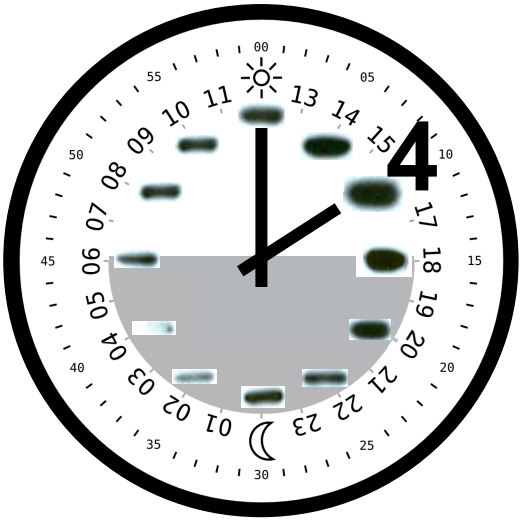
Mammalian Cryptochrome 1 and 2 do not homo- or heterodimerize



Manuscript in preparation

Mammalian Cryptochrome 1 and 2 do not homo- or heterodimerize

Sander Barnhoorn, Monika I. Bajek, Inês Chaves, Gijsbertus T.J. van der Horst and Filippo Tamanini

Department of Genetics, Chronobiology and Health Research Group, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

Abstract

Cryptochromes (CRYs) are circadian clock proteins that share structural similarity with the DNA repair enzyme photolyase. CRYs differ from photolyases by the presence of a unique C-terminal extension (extCT), and their functional diversity in organisms ranging from plants to mammals has been related thus far to these protein regions. Recently, it has been shown that the core domain of *Arabidopsis thaliana* CRY1 (*At*CRY1) undergoes homodimerization and that this biochemical change is primary to the light activation of its C-terminus. In mammals, mCRY1 and mCRY2 are integral component of the clock machinery, in which they perform transcriptional and post-translational light-independent functions (e.g. inhibition of CLOCK/BMAL1 mediated transcription). In this study we aimed to address whether mCRY1 homodimerization occurs as for its plant homologue. For that differentially tagged mCRYs molecules were co-expressed in COS7 cells and their interactions were tested in multiple assays (co-immunoprecipitation, native gel filtration, nuclear co-translocation). We show that mCRYs do not homo- or heterodimerize, which suggests that such complex formations of mCRYs are not required for their biological function. This suggests that the core domain of plant and mammalian CRYs may have a different mechanism of action.

Introduction

In mammals, circadian rhythms in behaviour, physiology and metabolism are generated by a self-sustaining molecular oscillator that drives gene expression through interconnected positive and negative transcription/translation feedback loops (Reppert and Weaver, 2001; Young and Kay, 2001). In the positive limb of the mammalian feedback loop, transcription of the *Period* (*mPer1*, *mPer2* and *mPer3*), *Cryptochrome* (*mCry1* and *mCRY2*) and *Rev-Erbα* clock genes is daily activated by a heterodimer of the two helix-loop-helix (bHLH)/PAS-domain transcription factors CLOCK and BMAL1 that act via CANNTG E-box enhancer elements in the promoters of these genes (Gekakis et al., 1998; Cermakian

and Sassone-Corsi, 2000; Ripperger et al., 2000; Reppert and Weaver, 2001; Young and Kay, 2001; Preitner et al., 2002). The mammalian CRY1 and CRY2 proteins (mCRYs) are central components of the negative limb of the clock (Okamura et al., 1999; Van der Horst et al., 1999; Vitaterna et al., 1999). As their protein concentration rises in the nucleus, they strongly inhibit CLOCK/BMAL1 mediated transcription (Griffin et al., 1999; Kume et al., 1999; Okamura et al., 1999), and thus shut down their own expression.

Co-immunoprecipitation studies with transiently expressed proteins and yeast two-hybrid experiments have uncovered direct interactions between mCRY proteins and various other core oscillator components (i.e. mPER2, mPER1, CLOCK, and BMAL1) (Griffin et al., 1999; Kume et al., 1999; Shearman et al., 2000). In subsequent immunohistochemical and biochemical studies mCRY and mPER proteins were shown to undergo synchronous circadian patterns of abundance, phosphorylation status, and nuclear localization (Kume et al., 1999; Field et al., 2000; Lee et al., 2001). mPER2 is an unstable protein that is protected from ubiquitylation and subsequent fast degradation (by the 26S proteasome) by association with mCRY1 or mCRY2 (Yagita et al., 2002). So far, all interactions and functions of mCRY within the mammalian clock are light-independent.

Cryptochromes (CRYs) were initially identified as putative photoreceptors because of the high degree of homology to the DNA repair enzyme photolyase (Van der Spek et al., 1996; Todo et al., 1997), which removes ultraviolet light induced DNA damage using visible light as an energy source [reviewed by (Sancar, 2003)]. The analysis of the resolved crystal structures of *E. coli* and *Anacystis nidulans* photolyases, as well as the core domain of *Arabidopsis thaliana* cryptochrome 1 (*At*CRY1), has revealed an almost absolute conservation of their tertiary structures (Park et al., 1995; Tamada et al., 1997; Brautigam et al., 2004), which contains a N-terminal α/β domain and a C-terminal α-helical domain that are connected by a long interdomain loop. However, despite the predicted similarity in folding of the core domain within the cryptochrome family, CRY proteins from *Arabidopsis thaliana*, *Drosophila melanogaster*, *Xenopus laevis*, and mammals are functionally and structurally distinct from photolyase for the lack of DNA repair activity and the presence of a unique C-terminal extension (Van der Spek et al., 1996; Todo et al., 1997). Recently, we have provided evidence that the acquirement of different (species-specific) C-termini during evolution not only functionally separated cryptochromes from photolyase, but also caused diversity within the cryptochrome family, allowing mCRYs to act as circadian core oscillator proteins (Chaves et al., 2006).

A recent report shows that light-independent homodimerization of *At*CRY1 through its core domain is mandatory for light activation of the C-terminus (CCT1) of this photoreceptor protein (Sang et al., 2005). The CCT1 binds and inhibits the function of the E3 ubiquitin ligase COP1, thereby allowing the accumulation of a set of transcription factors (e.g. HY5) that initiate the photomorphogenic program (Wang et al., 2001; Yang et al., 2001). In *Drosophila*, dCRY functions both as circadian photoreceptor (Stanewsky et al., 1998; Emery et al., 2000) and integral component of the

clock machinery in peripheral tissues (Krishnan et al., 2001). The core domain of dCRY is essential and sufficient for light detection, while the C-terminus of dCRY is involved in the degradation of dTIM in response to light (Busza et al., 2004; Dissel et al., 2004). It is unknown whether homodimerization occurs in dCRY and/or mCRY proteins.

Here, we report that mCRY1 and mCRY2 do not physically interact when expressed in cultured cells. This finding suggests, that mCRY1 and mCRY2 homo- or heterodimerization is not required for nuclear accumulation and CLOCK/BMAL1 inhibition.

Material and Methods

Cell culture, Immunofluorescence and Immunoprecipitation

COS7 cells were cultured in DMEM/F10 (1:1) (Gibco) medium, supplemented with 10% fetal calf serum and 50 µg/ml penicillin and streptomycin (Gibco) and transfected with Fugene (Boehringer) using the protocol provided by the manufacturer. The generation of mCRY and mPER expression constructs has been described (Yagita et al., 2002; Chaves et al., 2006). Immunofluorescence studies were performed as described (Yagita et al., 2002). Briefly, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton. To detect the various proteins, we employed rabbit anti-HA (ECL Oregon 1:1000) and mouse anti-V5 (Sigma 1:500) antibodies. As a fluorescent secondary antibody, we used anti-rabbit Alexa 594 (1:1000) and anti-mouse Alexa 488 (1:1000). The subcellular localization of the proteins was analysed 24 hours after transfection using a confocal microscope Zeiss Meta 729. Pictures were acquired with a 400 x lens (at 0.7 magnifications) and subsequently enlarged to show the details of the cells. For immunoprecipitation, we used anti-GFP (Clontech) and anti-V5 antibodies (Sigma). Total protein contents (5% of the lysate) and precipitated proteins (50% of the precipitate) were separated by SDS-PAGE and subjected to Western blot analysis. As primary antibodies in the immunoblot analysis we used the same antibodies (1:1000 dilution) with the addition of rat anti-HA to detect HA-mCRY1. As secondary antibodies we used horseradish peroxidase-conjugated anti-rabbit IgG (Biosource Int.) and anti-rat IgG (DAKO) at a 1:1000 dilution, visualized using the ECL system (Pharmacia Biotech).

Gel filtration

Size fractionation studies were performed using a Precision Column PC 3.2/30, pre-packed with Superdex 200, running on a SMART system (Pharmacia). Cell homogenates were obtained by resuspension of pelleted COS7 cells, transiently expressing HA-mCRY1 or YFP-mCRY1 and mPER2-GFP, in phosphate buffered saline (PBS) containing the detergent NP40 (0.05% final concentration) and a complete

cocktail of inhibitors (Roche). After incubation on ice for 10 minutes, cells were broken by vortexing and the insoluble material was spun down at 4 °C in a table centrifuge at 10.000 rpm. The resulting supernatant was applied to the column. The optimal range for separation of globular proteins in a Superdex 200 column is 1-600 kDa, with an exclusion of 1300 kDa. In order to calibrate the column and to determine the molecular weight of the eluent, ferritin (440 kDa) and albumin (68 kDa) protein markers were run in aforementioned homogenization buffer. Columns were run at a flow rate of 50 µl/min and protein profiles were monitored by detecting absorbance at 280 nm. Fractions (50 µl each) were collected in 2x Laemli Sample buffer.

Results

Immunoprecipitation of differentially tagged CRY1 and CRY2 proteins shows no homo- or heterodimerization

As dimerization constitutes a mandatory step in the photoreceptor activity of *Arabidopsis thaliana* CRY1 (*At*CRY1) (Sang et al., 2005) and 3D-computer modelling allows highly significant superposition of the core domain of mammalian CRYs (mCRY1 and mCRY2) on *At*CRY1 (Tamada et al., 1997; Özgür and Sancar, 2003; Brautigam et al., 2004), we addressed the question whether mCRY1 could homodimerize and/or heterodimerize with mCRY2 in mammalian cells. To this end, we generated a panel of constructs to transiently express differentially tagged mCRY proteins (i.e. mCRY1-YFP, YFP-mCRY1, HA-mCRY1-GFP, V5-mCRY2) in COS7 cells. In all cases, individually expressed exogenous mCRY proteins accumulated in the nucleus (data not shown).

We next expressed combinations of mCRY proteins (i.e. HA-mCRY1-GFP + HA-mCRY1, V5-mCRY2 + HA-mCRY1 and V5-mCRY2 + YFP-mCRY1), as well as the combination mPER2-GFP + HA-mCRY1 (control on co-immunoprecipitation conditions), and investigated whether immunoprecipitation of one component (e.g. with GFP antibodies) resulted in co-precipitation of the other (e.g. detected using HA-specific antibodies). Since mCRY1 and mCRY2 interact equally well with multiple components of the clock machinery *in vivo* (mPER1, mPER2, CLOCK, BMAL1), the absence of detectable amounts of endogenous clock proteins makes COS7 cells an ideal system for detection of direct interactions between CRY proteins in a cellular context. As expected from previous studies (Yagita et al., 2002), immunoprecipitation with anti-GFP antibodies shows that GFP-mPER2 is in physical contact with HA-mCRY1, as the latter protein is efficiently co-immunoprecipitated (Figure1A). In contrast, immunoprecipitation with anti-GFP antibodies pulls down HA-mCRY1-GFP but fails to coprecipitate HA-mCRY1 (Figure 1A). Similarly, immunoprecipitation of V5-mCRY2 does not result in co-immunoprecipitation of co-expressed HA-mCRY1, or YFP-mCRY1 (Figure 1B).

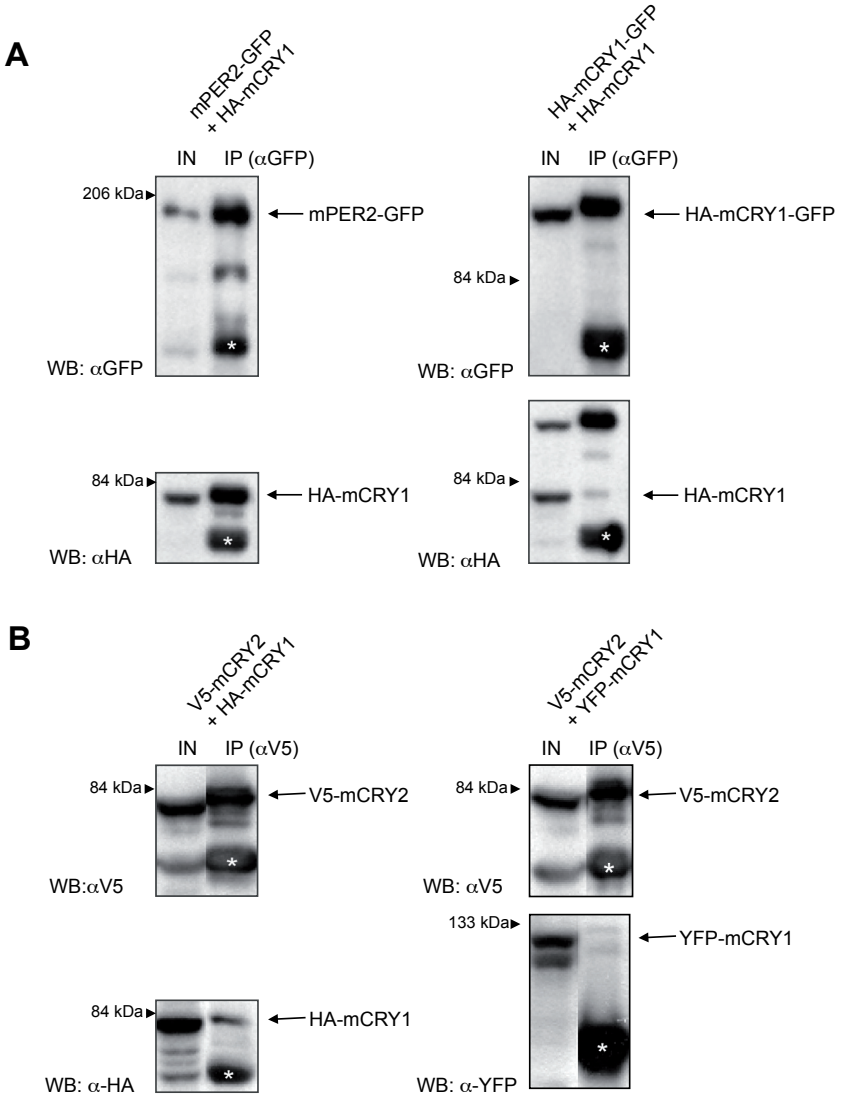


Figure 1. Immunoprecipitation of differentially tagged mCRY1 and mCRY2

A Western blot analysis of an immunoprecipitate from COS7 cells expressing HA-mCRY1 and either mPER2-EGFP (left), or HA-mCRY1-GFP (right). Proteins were precipitated with anti-GFP antibodies and visualized on Western blots using anti-GFP (top panels) and anti-HA antibodies (lower panels).

B Western blot analysis of an immunoprecipitate from COS cells transfected with V5-mCRY2 and either HA-mCRY1 (left), or mCRY1-YFP (right). Proteins were precipitated with anti-V5 antibodies and visualized on Western blots using anti-V5 antibodies (top panels) and anti-HA, or anti-GFP antibodies (lower panel). Asterisks represent the IgG band, while arrows indicate the various proteins on the filter.

IN – input, 5% of the total extract, IP- Immunoprecipitation, 50%

These results strongly suggest that mCRY1 and mCRY2 are not forming homo- or heterodimers in cultured cells.

Over-expressed mCRY1 is a monomer in COS7 cells

To further confirm the lack of homo- and heterodmerization of mammalian CRY proteins, we performed gel filtration studies in order to determine the native molecular weight of mCRY1 within the cellular homogenate.

After gel filtration of homogenates from COS7 cells that co-express HA-mCRY1 and mPER2, we found HA-mCRY1 to elute in two distinct fractions, corresponding to a molecular weight slightly larger than 450 kDa (fraction 12) and smaller than 70 kDa (fraction 24-26) (Figure 2). Since mPER2 can form homodimers (~400 kDa) (Yagita et al., 2000), the larger complex likely represents a complex composed of two HA-mCRY1 and two mPER2 molecules. Fraction 24-26 likely contains free HA-mCRY1. Importantly, size fractionation of a homogenate from COS7 cells that only express HA-mCRY1 revealed that this protein elutes as a monomer (Figure. 2) rather than a homodimer (expected molecular weight ~140 kDa), that would eventually elute in earlier fractions. This result is in line with the lack of mCRY1 homodimerization observed in the co-immunoprecipitations studies.

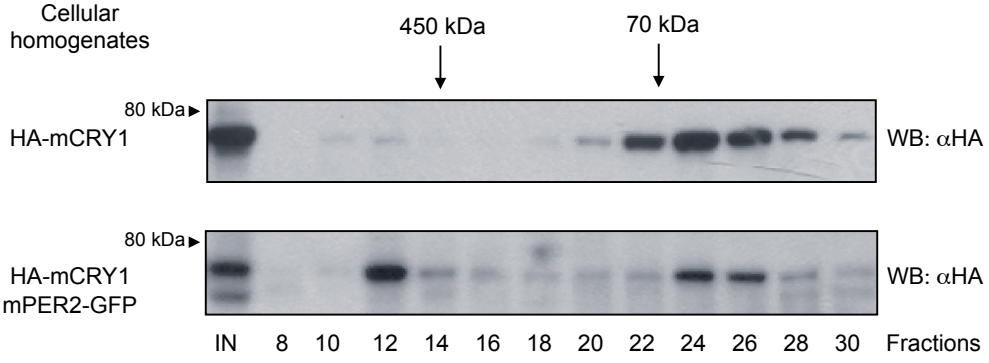


Figure 2. Size fractionation of protein extracts from COS7 cells transfected with HA-mCRY1 (top panel) or HA-mCRY1 and mPER2-GFP (lower panel) on a Superdex 200 column. Fractions of eluted proteins were subjected to immunoblot analysis using anti-HA antibodies for the detection of HA-mCRY1. The position of the molecular weight standards are shown on the top. The fraction numbers are shown on the bottom. The left lane on each panel represent the input (IN - 5% of the total extract).

A direct nuclear co-translocation assay fails to detect mCRY homo- and heterodimerization

Biochemical studies as applied above (i.e. immunoprecipitation, gel filtration of cellular homogenates) deal with artificial buffer conditions that may disrupt delicate protein-protein interactions. Particularly, this problem has been encounter in the characterization of *At*CRY1, where dimerization could only be visualized after chemical cross-linking with formaldehyde (Sang et al., 2005).

To investigate whether mCRY1 homo- or heterodimerizes in the living cell, we made use of our previous observations that; i) complexed mCRY1 and mPER2 can shuttle between cytoplasm and nucleus; ii) mCRY1 with a mutation in the dominant bipartite NLS (NLSc) located in the unique C-terminus (HA-mCRY1mutNLSc) has a nucleo-cytoplasmic subcellular distribution (see Figure 3); iii) the cellular distribution of HA-mCRY1mutNLSc can be fully shifted towards the nucleus by co-translocation with associating protein partners such as mPER2 and BMAL1 (Yagita et al., 2002; Chaves et al., 2006). Thus, in case of homo- or heterodimerization, wild type mCRY1 or mCRY2 (exclusively nuclear when transiently expressed in COS7 cells) should be able to drag HA-mCRY1mutNLSc into the nucleus. To illustrate the principle, GFP-mPER2 (596-1257), a protein that localizes in the nucleus as a result of deletion of dominant

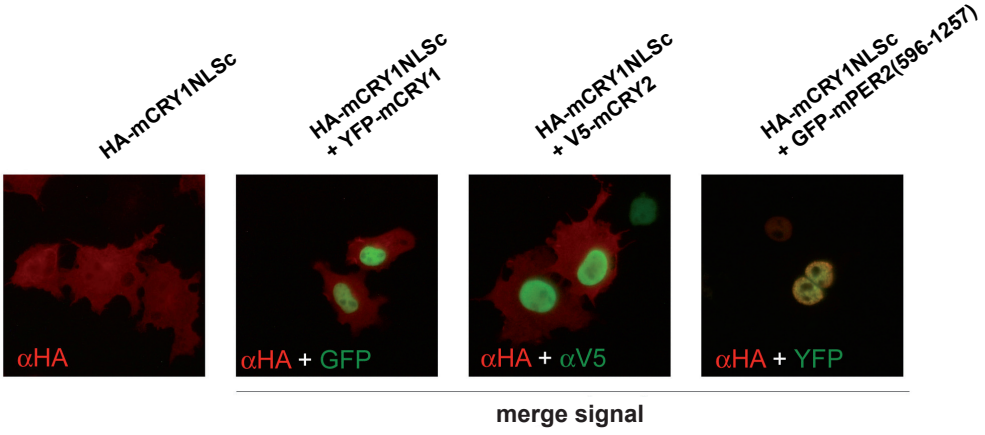


Figure 3. Immunofluorescence photographs of cells expressing only HA-mCRY1mutNLSc (first picture), or HA-mCRY1mutNLSc in combination with YFP-mCRY1, V5-mCRY2 (middle pictures), or HA-mCRY1mutNLSc with GFP-mPER2 (596-1257) (last picture). HA-mCRY1mutNLSc was visualized with anti-HA antibodies (red), whereas the other proteins were visualized by anti-V5 (green) or GFP and YFP fluorescence. In case of co-expression of various proteins pictures represent merged signals. Co-localization of HA-mCRY1NLSc and GFP-mPER2 (596-1257) served as an internal control.

dominant nuclear export sequences (Yagita et al., 2002) is shown to promote complete nuclear accumulation of HA-mCRY1mutNLSc (Figure 3). In marked contrast, neither YFP-mCRY1, nor V5-mCRY2 were able to promote nuclear entry of HA-mCRY1mutNLSc (Figure 3), thereby providing strong evidence that mCRY1 neither homodimerizes, nor heterodimerizes with mCRY2 under physiological conditions.

Discussion

Cryptochromes (CRYs) are components of the circadian system that act as a photoreceptor (e.g. in *Arabidopsis thaliana*), or a photoreceptor and core oscillator protein (e.g. in *Drosophila melanogaster* and Zebrafish), or a pure core oscillator protein (e.g. in *Xenopus laevis* and mammals). Recently, homodimerization of the core domain of *At*CRY1 has been shown to constitute an important requirement for photoreceptor activity of the protein (Sang et al., 2005). This prompted us to investigate whether homo- or heterodimerization of mammalian cryptochromes is an essential event in core oscillator function. In this manuscript we provide data in support of a lack of mCRY1 homodimerization and mCRY1/mCRY2 heterodimerization in cultured mammalian cells.

Neither co-immunoprecipitation experiments with overexpressed differentially tagged mCRY1 and mCRY2 proteins in COS7 cells (Figure 1), nor a gel filtration assay of cellular lysates containing HA-mCRY1 (Figure 2), nor (immuno)fluorescent subcellular localization experiments in mCRY-dependent nuclear translocation assays (Figure 3), provided evidence for the occurrence of homo- or heterodimerization. In particular, the direct immunofluorescence assay, in which both V5-mCRY2 and YFP-mCRY1 efficiently translocate mPER2 to the nucleus (data not shown), but fail to do so with HA-mCRY1mutNLSc (a cytoplasmic protein that contains a point mutation in the C-terminal NLS) (Figure 3), is very robust and indicative for the absence of mCRY/mCRY interactions under physiological conditions.

These results are somewhat surprising as comparison of the amino acid sequences of the core domain of the members of the cryptochrome/photolyase family, as well as molecular modelling of the 3D structures indicate that all cryptochromes (CRYs) have an almost identical folding of their core domain. Moreover, interaction between the core domain of the *At*CRY1 photoreceptor and mCRY2 core oscillator protein with their respective C-termini (CCT1 in *At*CRY1) has recently been reported to constitute an important event in establishing a stable tertiary structure (Partch et al., 2005), suggesting similarity in the biochemical mode of action for the two proteins.

It would be interesting to study whether mCRY homo- or heterodimerization occurs under more physiological conditions, particularly in tissue explants, or even (through *in vivo* imaging techniques) in the intact mouse. This would require the generation of transgenic or knock-in mice, expressing differentially tagged fluorescent

mCRY proteins from endogenous promoters. With the use of Fluorescence Resonance Electron Transfer (FRET) technology, the analysis of this biochemical property is within reach and answers can be provided on the question whether CRY proteins in retinal photoreceptor or ganglion cells would perhaps behave differently from their counterparts in other cell types.

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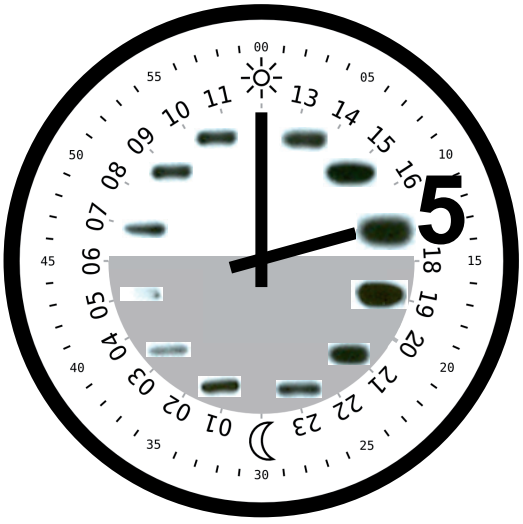
Uncoupling the circadian functions of mammalian cryptochrome 1



Manuscript in preparation

Uncoupling the circadian functions of mammalian cryptochrome 1

Monika I. Bajek, Sander Barnhoorn, Gijsbertus T.J. van der Horst and Inês Chaves

Department of Genetics, Chronobiology and Health Research Group, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

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Abstract

Circadian rhythms are generated by a molecular oscillator which is generated by interconnected feedback loops of transcription and translation, and are further modulated by a network of post-translational modifications. Mammalian cryptochromes have been shown to be key components of the circadian clock, and they function both in the transcription/translation feedback by repressing CLOCK/BMAL1 driven transcription and in the posttranslational regulation by interacting with PER2 and regulating its sub-cellular localization and stability. We have previously identified a predicted coiled-coil domain in the C-terminus of CRY1 which is necessary for robust transcription inhibition and complex formation with the PER proteins. Here we analyse the *in vivo* effect of a CRY1 mutant which lacks the coiled-coil domain. Our findings suggest that, although the CRY1-ΔCC protein is not sufficient to drive sustained circadian oscillations, it is capable of maintaining peripheral oscillators synchronized in culture conditions. We speculate that by deleting the coiled-coil domain it is possible to uncouple two distinct functions of CRY1 in regulating circadian rhythms.

Introduction

Most if not all organisms have evolved a circadian clock in order to adjust to and anticipate the daily cycles of light/dark and temperature, among others, imposed by the 24 hour rotation of the earth around its axis. The circadian clock governs rhythms of physiology (temperature, heart rate, metabolism) and behaviour (alertness, sleep/wake cycles) with a periodicity of approximately 24 hours, and is entrained every day by external stimuli, the strongest and most predictable of which is light. In mammals, the master clock is located in the brain, more specifically in the suprachiasmatic nuclei of the hypothalamus (SCN). The SCN receives light input from the retina via the retinohypothalamic tract (Ralph and Menaker, 1989; Rusak et al., 1989) and in turn synchronises the peripheral oscillators located in virtually every cell or tissue (Balsalobre et al., 1998; McNamara et al., 2001; Pando et al., 2002; Yoo et al., 2004).

Cryptochromes belong to the photolyase/cryptochrome family of flavoproteins (Sancar, 2003), which includes proteins that share a common core domain but differ in the presence of either N-terminal extensions harbouring nuclear and mitochondrial localization signals (photolyases), or unique C-terminal extensions involved in function modulation (cryptochromes). Mammalian cryptochromes were initially identified as homologues of photolyases, enzymes that repair UV-induced DNA damage (Van der Spek et al., 1996; Todo et al., 1997). Based on their sequence similarity to the plant blue-light receptors, they were called cryptochromes (CRY). Mammals have two cryptochrome encoding genes, *mCry1* and *mCry2*, which share a high degree of homology in their core domain but have divergent C-terminal extensions (CT). Despite their sequence homology to photolyases, mammalian CRYs do not have any DNA repair function. Instead, we and others have shown that they are integral components of the circadian clock (Van der Horst et al., 1999; Vitaterna et al., 1999; Albus 2002). Mice that are deficient for both *mCry* genes lose their circadian clock, as shown by their behavioural and biochemical arrhythmicity (Van der Horst et al., 1999). On the other hand, mice that are deficient for either *mCry1* or *mCry2* have opposing circadian phenotypes, a fast and a slow ticking clock, respectively.

Mammalian cryptochromes play a central role in the molecular circadian clockwork, which is driven by interconnected positive and negative feedback loops of transcription and translation (Reppert and Weaver, 2001; Young and Kay, 2001). The CLOCK/BMAL1 heterodimer positively regulates transcription of (clock) genes with an E-box containing promoter, after which the CRY1, CRY2 and PER1, PER2 protein products repress their own transcription and that of other clock controlled genes. In addition, transcription of the *Bmal1* gene is negatively regulated by Rev-erbα (Preitner et al., 2002; Etchegaray et al., 2003) and is further modulated by the ROR nuclear receptors (Sato et al., 2004). The complexity of the molecular clock goes beyond the transcription/translation feedback, as an intricate network of posttranslational modifications such as phosphorylation, ubiquitylation, sumoylation and (de)acetylation is required for the robustness and persistence of the oscillations (Toh et al., 2001; Cardone et al., 2005; Lee et al., 2008). The mammalian CRY proteins are differentially regulated by ubiquitylation and phosphorylation, as only CRY2 is a target of the kinase GSK3β (Sanada et al., 2004; Harada et al., 2005), which could explain the opposite effect they have on the length of the circadian cycle. Furthermore, we have recently shown that also (de)acetylation differentially regulates the function of the CRY proteins (Bajek et al., manuscript in preparation).

CRY1 has been extensively studied, and new findings add to the behavioural phenotype observed in *mCry1*-deficent (*mCry1*$^{-/-}$) mice. Although *mCry1* is rhythmically expressed and is an indispensable component of the core oscillator, it was shown that constitutive expression of *mCry1* does not affect the circadian clock (Okano et al., 2009). Furthermore, it was shown that CRY1 is required for sustained rhythmicity of peripheral

tissues in organotypical culture (Liu et al., 2007). Although the SCN of *mCry1*-deficient mice shows sustained circadian oscillations in culture, cells and peripheral tissues rapidly lose synchrony once removed from the organism.

Mutation analysis and domain swap experiments have given further insight into the function of CRY1 in the molecular clockwork (Zhu et al., 2003; Chaves et al., 2006; van der Schalie et al., 2007). The C-terminal extension of CRY1 contains a bi-partite nuclear localisation signal (NLS) and a predicted coiled-coil domain (CC) whereas functional analysis revealed that the CT is necessary for correct nuclear localization, transcription inhibition and protein-protein interaction (Chaves et al., 2006). By deleting the CC, we have shown that this is an important functional domain of CRY1, which interacts with both PER1, PER2 and BMAL1, mediates NLS-independent nuclear import, and is necessary for complete repression of CLOCK/BMAL1 driven transcription. Complex formation with PER2 via the CC regulates the stability and the nucleo/cytoplasmic shuttling of PER2, and interaction with BMAL1 assures robust transcription inhibition. To further understand the functional impairment of the mCRY1-ΔCC mutant and how the function a CRY1 is affected *in vivo* in the absence of the CC domain, we generated *mCry1* mutant mice with a deletion of the CC domain. Our data suggest that although the CC is required to generate oscillations with a correct period and maintain rhythmicity in a *mCry2*-deficient (*mCry2*$^{-/-}$) background, it is not necessary to maintain peripheral oscillators synchronised *ex vivo*.

Materials and Methods

Targeting construct, embryonic stem cell transfection and blastocyst injection

Isogenic mouse genomic DNA was obtained by amplification of 129Ola-derived IB10 ES cell DNA (Takara, LA-PCR; IB10 is a subclone from the original E14 line, provided by A. Berns, Netherlands Cancer Institute) using primer sets designed on the basis of the mouse *Cry1* complementary DNA sequence (GenBank accession nos 000777) (described in Van der Horst et al., 1999). Genomic clones containing exons 3 to 7 and exons 8 to 10 were used as 5′and 3′homology regions, respectively. To generate the *mCry1*$^{\Delta CCneo}$ targeting vector (see Figure 2A), the mCry1-ΔCC cDNA (base pair 1059 to stop codon) was cloned using a BamHI restriction site in exon 7. A LoxP site flanked PGK::neo cassette, preceded by the human β-globin 3′-UTR, was cloned in a HindIII restriction site in intron 7. The backbone is pBlueScriptKS^{-} (Stratagene).

ES cell line IB10 was maintained on gelatin-coated dishes in 50% BRL conditioned DMEM/ 50% fresh DMEM medium, supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 2 mM glutamine, 50 mg/ml penicillin and streptomycin, 1,000 U/ml leukemia inhibitory factor (all components purchased from Gibco) and 0.1 mM 2-mercaptoethanol. Linearized targeting vector DNA (15mg) was

transfected into IB10 cells (10^7 cells in 400 ml PBS) by electrophoration for 10 ms at 1,200 mF and 117 V, using a Progenitor II Gene Pulser (Hoeffer). Electroporated cells were reseeded onto 10-cm dishes and subjected to neomycin selection by addition of 200 mg/ml G418 (Geneticin, Gibco) the following day. Counterselection against randomly integrated DNA was obtained by including 0.2 mM Fialuridine in the selection medium. After nine days colonies were randomly picked and expanded in 24-well dishes. Duplicate dishes were used for cryopreservation and genotyping (Southern blot analysis), respectively. We obtained a targeting frequency of 5%.

Gene-targeted ES cells, checked for proper chromosome composition by karyotyping, were injected into C57BL6/J blastocysts by standard procedures. Chimeric male mice were mated with C57BL6/J females and transmission of IB10-derived germ cells was identified by an agouti coat color in the offspring. Heterozygous male and female mice were interbred to generate *mCry1*$^{+/\Delta CCneo}$ mice. Excision of the neo-cassette was achieved by interbreeding the *mCry1*$^{+/\Delta CCneo}$ with cag::CRE transgenic mice (*mCry1*$^{+/\Delta CCneo}$).

The following mouse lines were used, in addition to the above described *mCry1*$^{+/\Delta CCneo}$ and *mCry1*$^{+/\Delta CC}$: *mPer2::luciferase* (generation to be described elsewhere) and cag::CRE (Sakai and Miyazaki, 1997).

Cells, culture conditions and transient transfection

NIH 3T3 cells and wild type (wt), *mCry1*$^{-/-}$, *mCry1*$^{\Delta CCneo/\Delta CCneo}$ and *mCry1*$^{\Delta CC/\Delta CC}$ mouse dermal fibroblasts (MDFs) were cultured in Dulbecco's modified Eagle's medium-F10 supplemented with 10% fetal calf serum, penicillin/streptomycin and transfected with Fugene (Boehringer) according to the manufacturer's instructions. To generate MDFs, mice were killed by cervical dislocation, and a small piece of back skin of the mouse was removed and cut into pieces with a razor blade. Skin pieces were washed in ethanol, rinsed in phosphate-buffered saline, and incubated overnight in medium supplemented with 1.6 mg/ml Collagenase type II. Single cells were obtained by passing through a cell strainer, and collected by centrifugation for 5 min at 1,000 rpm, resuspended in cultured medium, and seeded onto a 10-cm dish. MDFs were cultured in a low-oxygen incubator (5% CO_2, 3% O_2).

Analysis of mouse-derived DNA

Genotyping of ES cell DNA was performed by Southern blot analysis and Long-Range PCR. Primers used: (A) 5′-GGAGGTTAACAATGGTATTGAACG-3' and (B) 5′-ATCTGCATCAAGCAGTAACTCTTC-3'. Genotyping of mouse dermal fibroblast (MDF) or tail DNA was performed by PCR. Primers used: (a) 5′-CACATCT-GAAATCCGAGCAGCAG-3', (b) 5′-CAAGCTCTTCCCCACCTCACTC-3' and (c) 5′-CGCATCGCCTTCTATCGCCT-3'. The location of the primers is shown in

Figure 2B, PCR results in Figure 2C and E. For southern blot analysis (Figure 2D), NcoI digested DNA was probed with a 560-bp NcoI-XbaI fragment, located outside the targeting construct.

Quantitative Real-time PCR (qPCR)

Total RNA from cultured cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 µg of total RNA using oligo-dT primers and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative PCR for the determination of mCry1 and mCry1-ΔCC mRNA levels was performed in triplicate using as iCyclerIQ system (BioRad), SYBR-green and primers generating intron-spanning products of 150-300 bp. Expression levels were normalised to *Hprt* (hypoxanthine guanine phosphoribosyl transferase) mRNA levels. The generation of specific products was confirmed by melting curve analysis, and primer pairs were tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate primer pair efficiencies.

Western blot analysis

Liver protein extracts were obtained from wt, *mCry1*$^{\Delta CCneo/\Delta CCneo}$, *mCry1*$^{\Delta CC/\Delta CC}$ and *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mice sacrificed at ZT 20, when liver *mCry1* mRNA levels reach a maximum. Protein concentrations were determined with the Bradford method. For each time point, 50µg of protein was subjected to SDS-PAGE on premade gradient gels (Invitrogen), followed by western blot analysis. Endogenous CRY1, CRY1-ΔCC and CRY2 were detected using polyclonal anti-mCRY1 and anti-mCRY2 antibodies kindly provided by J. Ripperger.

Monitoring of circadian locomotor activity rhythms

For the monitoring of locomotor activity rhythms, mice of both sexes and of different ages (8-16 weeks) were used. They were individually housed in a light-proof chamber in cages (30 x 45 cm) equipped with a running wheel (11 cm in diameter) and a system to detect wheel rotations. Animals were maintained in a cycle of 12 h light (150 lux) and 12 h complete darkness (LD) or in continuous complete darkness (DD) in constant ambient temperature with water and food available *ad libitum*. Voluntary wheel-running (wheel turns per unit of time) was continuously recorded by an online computer using a modified version of the ERS program. Activity records were plotted as actograms and the period of locomotor activity was determined by the chi-square method. Unpaired Student's t-tests were used to make statistical comparisons between the different genotypes.

Real time bioluminescence
To monitor circadian oscillations in cultured cells in real time, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of confluent cultures with forskolin (dissolved in 100% ethanol, added to the culture medium at a final concentration of 30 μM) bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C.

Results and Discussion

Overexpression of mCry1-ΔCC reduces the amplitude of circadian oscillation
Using a luciferase reporter assay we have previously shown that the CRY1-ΔCC mutant protein can still inhibit CLOCK/BMAL1 driven transcription, although to a lesser extent than CRY1 (Chaves et al., 2006). In addition, we observed that this mutant protein no longer can interact with PER2 or BMAL1. To better understand the effect of this mutant CRY1 protein with partially impaired functions, we transiently overexpressed

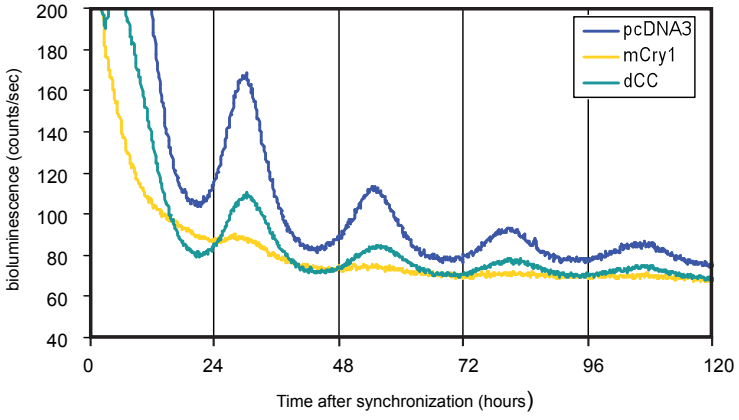

Figure 1. mCRY1-ΔCC reduces the amplitude of circadian oscillations in cultured cells
Representative example (n=3) of bioluminescence rhythms in NIH 3T3 cells transfected with *mPer2::luciferase* and either empty pcDNA3 (blue), mCry1 (yellow) or mCry1-ΔCC (teal). The X-axis represents time after forskolin synchronization.

the mCRY1-ΔCC protein in NIH 3T3 cells and analyzed the oscillation of a luciferase reporter gene under control of either the *mPer2* or the *Bmal1* promoter. Cells were synchronized with forskolin and luminescence was measured in time.

As shown in Figure 1, cells transfected with the empty pcDNA3 vector (used as a negative control), oscillate with a period of approximately 25.2 hours. In line with their function as potent repressor of CLOCK/BMAL1 transcription (Kume et al., 1999), overexpressed mCRY1 abolishes circadian oscillations. In contrast, cells transfected with an mCRY1-ΔCC expression construct are capable of oscillating, although with lower amplitude and a period of 26.0 hours (Figure 1). This result suggests that CRY1-ΔCC is partially impaired in its function, which is in agreement with our previous *in vitro* data. Interestingly, the increase in the period of the oscillation upon overexpression of CRY1-ΔCC is consistent with the decrease in the period of locomotor activity in *mCry1*-deficient mice. Here we show that CRY1-ΔCC can inhibit CLOCK/BMAL1, but not completely, and this effect is visible in synchronized cells.

Generation of a knock-in mCry1ΔCC mutant mouse model

The results obtained thus far were derived from experiments performed *in vitro* or in tissue culture, and in overexpression conditions. In order to analyze the effect of the CRY1-ΔCC mutant protein *in vivo* and under physiological conditions, we have generated knock-in mice which carry the ΔCC mutation in the *mCry1* genomic locus (see Figure 2A) in which exon 7 was fused in frame to the mouse *Cry1* cDNA, containing the remaining coding sequence, but lacking the CC domain. The cDNA sequence was followed by a cassette containing the neomycin selection marker gene, preceded by the human β-globin 3′-UTR and polyadenylation signal for transcription termination. This cassette was flanked by LoxP sites to allow Cre-recombinase-mediated excision of the β-globin/neomycin sequences, allowing transcription to terminate at the endogenous 3′-UTR of the *mCry1* locus. The composition of the wild type *mCry1* locus and that of the targeted ($mCry1^{\Delta CCneo}$) and Cre-recombined ($mCry1^{\Delta CC}$) alleles is shown in Figure 2B.

The targeting construct (Figure 2A) was transfected into mouse embryonic stem cells (ES cells) by electroporation, followed by selection of clones with a targeted *mCry1* genomic locus (Figure 2B, C and D). Correct homologous recombination was detected using long-range PCR (Figure 2C) and Southern blot analysis with 5' and 3' probes (Figure 2D) (see Materials and Methods). Targeted ES cell clones with a correct karyotype were injected into blastocyts, which were subsequently implanted in pseudopregnant female recipient mice (see Materials and Methods for a detailed description). We obtained two chimeric males which were further crossed with C57BL6/J mice and which both transmitted the targeted allele, yielding heterozygous $mCry1^{\Delta CCneo}$ mice. To remove the β-globin/neomycin cassette, $mCry1^{+/\Delta CCneo}$ mice were crossed with *Cag-Cre* transgenic mice, expressing the Cre-recombinase in the oocyte (Sakai and Miyazaki, 1997).

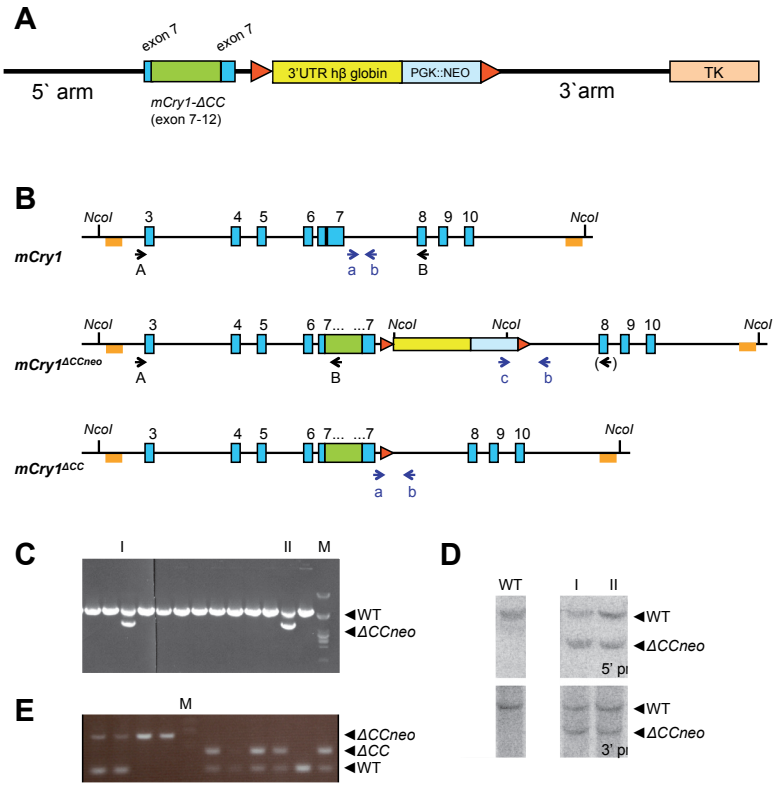

Figure 2. Generation of mutant mice carrying the mCry1-ΔCC mutation

A Schematic representation of the targeting vector. 5′- and 3′-indicate the homology regions, TK (in beige) is a negative selection cassette with the timidine kinase gene under control of the PGK promoter. The red triangles indicate LoxP recombination sites, the neo cassette is indicated in ultramarine, the human βglobin 3′-UTR in yellow, exon 7 of the mCry1 locus in cobalt, and the mCry1-ΔCC cDNA in lime (base pair 1059 to stop codon).

B Representation of part of the genomic locus of the mCry1 (top), mCry1-ΔCCneo (middle) and mCry1-ΔCC (bottom) alleles. Exons are shown in blue and numbered, primers used for genotyping by PCR are indicated below each locus by labeled arrows and probes used for southern blot are in orange. NcoI restriction sites are indicated.

C Ethidium bromide stained gel of PCR amplified ES cell DNA using primer set A-B. I an II indicate two positive clones, M is the DNA size marker (λ PstI).

D Southern blot confirming the correct 5′- and 3′-integration sites of the two positive clones identified in C.

E Ethidium bromide stained gel of PCR amplified mouse tail DNA using primer set a-b-c. On the left is shown genotyping of mCry1ΔCCneo mice and on the right of mCry1ΔCC (after CRE recombination). M is the DNA size marker (λ PstI).

Next, *mCry1*$^{+/\Delta CCneo}$ and *mCry1*$^{+/\Delta CC}$ mice were backcrossed on a C57BL6/J genetic background. Genotyping of mouse DNA was further performed by PCR (Figure 2E). Heterozygous and homozygous mutant *mCry1*$^{\Delta CCneo}$ and *mCry1*$^{\Delta CC}$ mice were born at Mendelian ratio and did not develop any overt abnormalities. Both mouse lines were analyzed further and compared to wild type littermates, as well as to the *mCry1*$^{-/-}$ knockout mouse model (Van der Horst et al., 1999).

Analysis of mRNA and protein derived from the mutant *mCry1*$^{\Delta CC}$ alleles

To analyze the expression of the *mCry1*$^{\Delta CCneo}$ and *mCry1*$^{\Delta CC}$ knock-in alleles, we compared the *mCry1* mRNA and mCRY1 protein expression levels of the wild type, *mCry1*$^{\Delta CCneo}$ and *mCry1*$^{\Delta CC}$ mice. Total mRNA was extracted from unsynchronized primary mouse dermal fibroblasts (MDFs), isolated from each of the three genotypes and the relative levels of mRNA originating from the wt, *mCry1*$^{\Delta CCneo}$, and *mCry1*$^{\Delta CC}$ loci (normalized to *Hprt* expression) were determined by quantitative polymerase chain reaction (qPCR) (Materials and Methods). Despite the presence of the neomycin and a heterologous 3'-UTR in the *mCry1*$^{\Delta CCneo}$ gene the steady state level of messenger RNA derived from it is similar to that of the wild type *mCry1* locus (Figure 3A). On the other hand, the *mCry1*$^{\Delta CC}$-derived mRNA is approximately 30% lower (Figure 3A), which could be due to the presence of an extended exon 7 and additional 60 bp in intron 7 and/or the large non-translated region (corresponding to exon 8-10 downstream of the cDNA sequence), which may destabilize this mutant *mCry1* mRNA (Figure 2B).

Protein levels were analyzed in liver lysates isolated from mice sacrificed at ZT20, the time when CRY1 protein peaks in the liver. The CRY1 and CRY1-ΔCC proteins are present at comparable levels (Figure 3B). We conclude that the targeted allele is correctly expressed and that the mCRY1-ΔCC protein is present, and at least at this time point there is more mCRY1-ΔCC than mCRY1. This difference could be derived from an increased stability of the mutant or from a shift of the oscillation. Further analysis of different time points will clarify this question. Interestingly, the levels of mCRY2 are elevated in the mutant mice (Figure 3C), which could be explained by a reduced inhibition of E-box promoters due to the lack of repression by mCRY1-ΔCC. In addition, one cannot exclude the possibility of a shifted oscillation.

***mCry1*$^{\Delta CC/\Delta CC}$ and *mCry1*$^{\Delta CCneo/\Delta CCneo}$ mutant mice have a short free-running period**

Mice deficient for CRY1 have an accelerated circadian clock, as shown by the short free-running period in constant darkness measured by voluntary wheel-running activity (Van der Horst et al., 1999). We now generated *mCry1* mutant mice that carry a partially functional protein, mCRY1-ΔCC, which, as shown in *in vitro* experiments, no longer interacts with PER1, PER2 and BMAL1, but still is partially capable of inhibiting

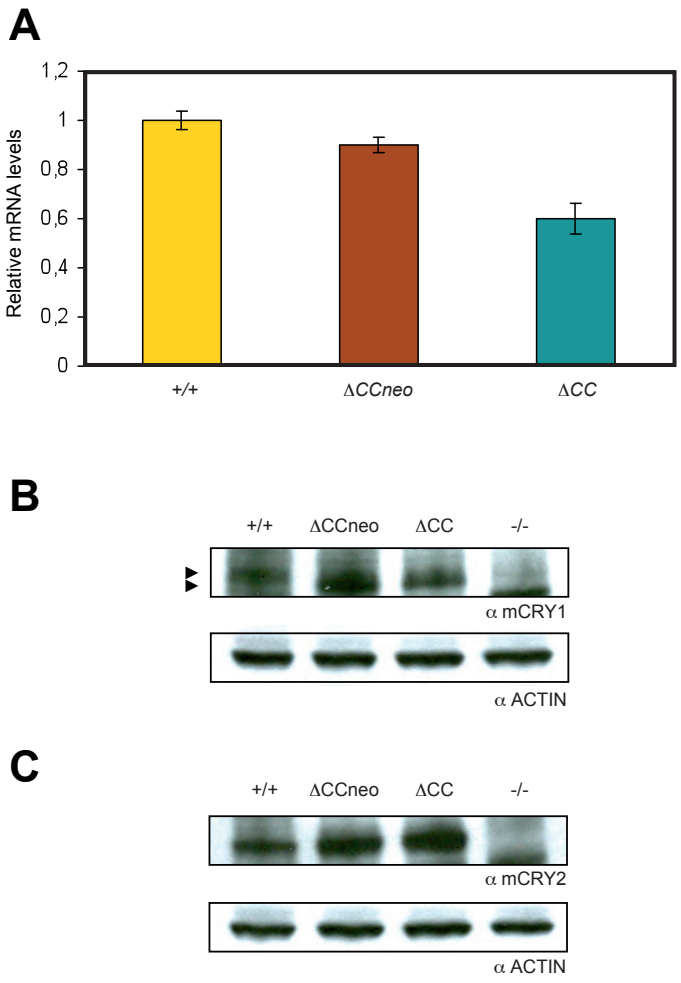

Figure 3. Expression levels of the mCry1-ΔCCneo, mCry1-ΔCC and mCry2 alleles

A Graphic representation of qPCR amplification data, mCRY1$^{-/-}$ RNA: in yellow; mCry1-ΔCCneo RNA: brown and mCry1-ΔCC RNA: teal (see Materials and Methods for experimental details). The Y-axis represents the mRNA levels, relative to the wt allele (set to 1).

B and C Western blot using an anti-mCRY1 (B) and mCRY2 (C) antibodies to detect mCRY1, mCRY1-ΔCC and mCRY2. Liver protein lysates of the following mouse lines (from left to right): mCry1$^{+/+}$, mCry1$^{\Delta CCneo/\Delta CCneo}$, mCry1$^{\Delta CC/\Delta CC}$ and mCry1$^{-/-}$mCry2$^{-/-}$. Arrow heads point to mCRY1 and mCRY1-ΔCC proteins (top and bottom, respectively). Actin was used as a loading control.

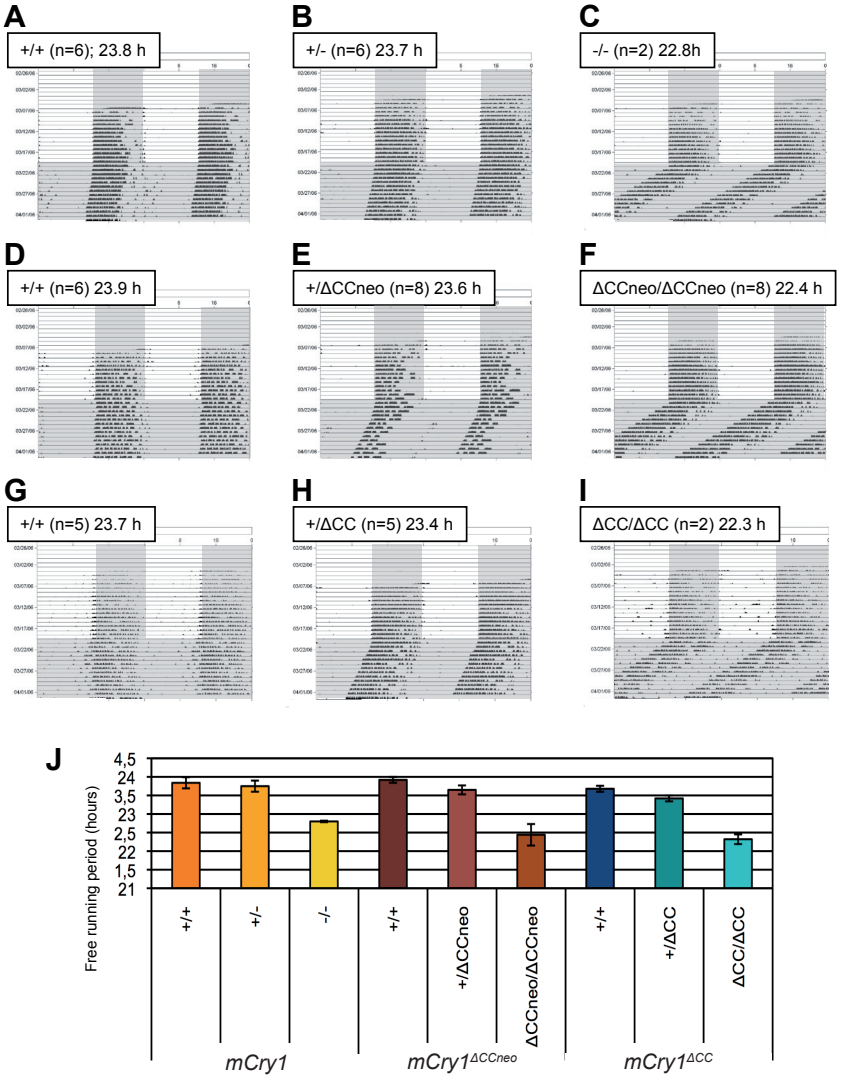

Figure 4. Circadian behaviour of mCry1$^{\Delta CC}$ mice

Representative examples of the 24h double-plotted actograms obtained for *mCry1*-deficient (A-C), *mCry1*$^{\Delta CCneo}$ (D-F) and *mCry1*$^{\Delta CC}$ (G-I) mouse lines. For each line wt, heterozygote and homozygote examples are shown. The number of animals analysed and the free-running period are indicated above each actogram. J. Graphic representation of the free-running period in constant darkness, *mCry1*-deficient in yellow and orange, *mCry1*$^{\Delta CCneo}$ in brown and *mCry1*$^{\Delta CC}$ in teal (the error bars represent the standard deviation, the asterisk indicates significance). The Shading indicates when the mice were placed in constant dark conditions and the black vertical lines represent animal activity.

CLOCK/BMAL1. To analyze the circadian behavioural phenotype of *mCry1*$^{\Delta CCneo/\Delta CCneo}$ and *mCry1*$^{\Delta CC/\Delta CC}$ mice we measured their running-wheel activity. For each line the wild-type, heterozygote and homozygote mice were analyzed. Mice were first housed in light/dark cycles of 12 hours (LD 12:12) and after two weeks were placed in constant dark conditions (DD). Representative examples of the actograms obtained for each genotype are shown in Figure 4. In line with our previous observations (Van der Horst et al., 1999), *mCry1*-deficient mice have a free-running period in constant darkness (tau DD) which is 1.3 hours shorter than that of wild type littermates, whereas the circadian behaviour of heterozygous mice appears normal (Figure 4A-C and J). The phenotypes observed for the *mCry1*$^{\Delta CCneo/\Delta CCneo}$ (Figure 4D, F and J) and *mCry1*$^{\Delta CC/\Delta CC}$ (Figure 4G, I and J) mice are comparable to that of *mCry1*$^{-/-}$ mice, suggesting that the *in vivo* effect of deletion of the CC is that of a functional null mutation. Interestingly, *mCry1*$^{+/\Delta CCneo}$ and *mCry1*$^{+/\Delta CC}$ mice, in contrast to *mCry1*$^{+/-}$ animals have a small but significant change in: the period length of their circadian clock, which is 10-20 minutes shorter than that of wt littermates (Figure 4B, E, H and J). Furthermore, the tau of the mice carrying the *ΔCC* mutation is slightly shorter than that of *mCry1*$^{-/-}$. Taken together, these data suggest that the CRY1-ΔCC protein may have a dominant negative effect. A more extensive analysis will be needed to further address this question.

CRY1-ΔCC is not sufficient for sustained rhythmicity in a *mCry2-deficient* background

To further understand the functionality of the CRY1-ΔCC protein, we interbred the *mCry1*$^{\Delta CCneo/\Delta CCneo}$ mice with *mCry2*$^{-/-}$ mice. The outcome of this experiment will tell whether the mutant protein is a true null mutant or whether it is still sufficient to maintain the circadian oscillator ticking. Previous experiments by Van der Horst et al., (1999) and Vitaterna et al., (1999) have shown that mice lacking both cryptochromes are arrhythmic in constant conditions. As shown in Figure 5, *mCry1*$^{\Delta CCneo/\Delta CCneo}$*mCry2*$^{-/-}$ mice are arrhythmic in constant darkness, as observed for *mCry1*$^{-/-}$/*mCry2*$^{-/-}$ mice. This result suggests that CRY1-ΔCC alone is not sufficient for sustained oscillations, and further indicates that, *in vivo*, the CRY1-ΔCC protein is almost a functional null mutant.

Skin fibroblasts and peripheral tissues from *mCry1*$^{\Delta CC/\Delta CC}$ mice remain rhythmic *ex vivo*

As CRY1 is required for sustained oscillation of synchronized fibroblasts and peripheral tissues in organotypic culture (Liu et al., 2007), we next asked whether CRY1-ΔCC still is functional at this level. To study the rhythmic behaviour of peripheral oscillators in real time, the *mCry1*$^{\Delta CCneo/\Delta CCneo}$ and *mCry1*$^{\Delta CC/\Delta CC}$ mice were bred with transgenic mice carrying a luciferase reporter gene under control of the *mPer2* promoter

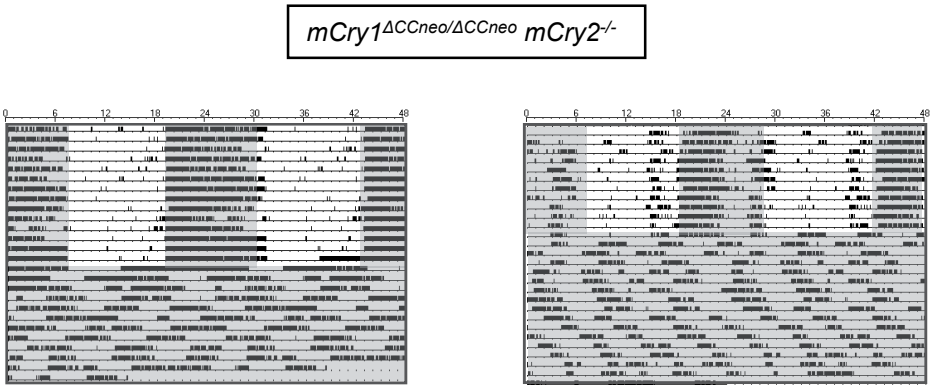

Figure 5. Circadian behaviour of mCry1$^{\Delta CCneo/\Delta CCneo}$mCry2$^{-/-}$ mice
Representative examples of the 24h double-plotted actograms. Shading indicates darkness and the black vertical lines represent animal activity. The actograms obtained were very heterogeneous, but in none was it possible to calculate a period, indicating arrhythmicity.

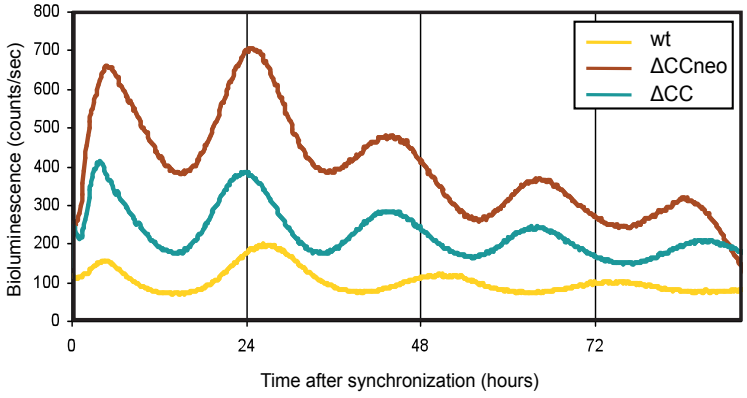

Figure 6. Circadian oscillation of forskolin synchronized mouse dermal fibroblasts
Representative example (n=3) of bioluminescence rhythms in MDFs derived from *mCry1*$^{+/+}$ (yellow), *mCry1*$^{\Delta CCneo/\Delta CCneo}$ (brown) and *mCry1*$^{\Delta CC/\Delta CC}$ (teal) mice in a *mPer2::luciferase* background. The X-axis indicates the time after forskolin synchronization. The Y-axis indicates bioluminescence in counts/second.

(*mPer2::luc* mice). As shown in Figure 6, forskolin synchronized primary skin fibroblasts from *mCry1*$^{\Delta CCneo/\Delta CCneo}$*mPer2::luc* and *mCry1*$^{\Delta CC/\Delta CC}$*mPer2::luc* mice have a robust oscillation, comparable to cells from *mCry1*$^{+/+}$*mPer2::luc* littermates. However, and in agreement with the *in vivo* phenotype, cells carrying th e CRY1-ΔCC protein oscillate with a period that is shorter than the wild type: 20.0 + (n= 3) hours versus 24.0 hours, respectively (Figure 6). The observed period difference is similar in *mCry1*$^{\Delta CCneo/\Delta CCneo}$ and *mCry1*$^{\Delta CC/\Delta CC}$ cells. These results indicate that the CRY1-ΔCC mutant protein, like the wild-type CRY1, is capable of maintaining synchrony of peripheral oscillators and that the coiled-coil domain is not required for this function.

Conclusions

Since the identification of mammalian cryptochromes as core components of the circadian clock (Van der Horst et al., 1999; Vitaterna et al., 1999), a large number of studies have focused on understanding how these proteins regulate rhythm generation and which are the functional domains that can be identified (Chaves et al., 2006; Van der Schalie et al., 2007). There are two mammalian cryptochromes, CRY1 and CRY2, which despite their homology regulate circadian rhythms in opposite directions, as shown by the short and long free-running period of locomotor activity of *mCry1*- and *mCry2*-deficient mice, respectively (Van der Horst et al., 1999). In addition, peripheral oscillators from *mCry1*$^{-/-}$ mice (but not from *mCry2*$^{-/-}$ mice) become arrhythmic in culture conditions (Liu et al., 2007), suggesting that CRY1 is a stronger regulator of circadian rhythms than CRY2. Here we focused on a deletion mutant of CRY1, CRY1-ΔCC, which lacks the predicted C-terminal CC domain. Previous analysis of this mutant *in vitro* showed that the CRY1-ΔCC protein is partially impaired in the transcriptional feedback and that it can no longer interact with either the PER proteins or BMAL1 (Chaves et al., 2006).

To allow the study of CRY1-ΔCC function *in vivo* we generated genetically modified mice that express mCRY1-ΔCC from the endogenous *mCry1* locus. Two versions have been generated, one that uses the human β-globin 3'-UTR preceding the neomycin resistance marker cassette (*mCry1*$^{\Delta CCneo}$) and another one, obtained by CRE-mediated excision of the β-globin/neomycin sequences from the targeted allele, which uses the endogenous 3'-UTR of the *mCry1* locus and (*mCry1*$^{\Delta CC}$). Analysis of the two mouse lines suggests that in both cases the mutant mRNAs and CRY1-ΔCC protein are present. Accordingly, the obtained *in vitro* and *in vivo* phenotypes are largely comparable. From the results obtained, *in vivo* and *ex vivo*, we conclude that by deleting the CC, the CRY1 protein largely loses its ability to inhibit CLOCK/BMAL1. Unexpectedly, cells and tissues derived from *mCry1*$^{\Delta CCneo/\Delta CCneo}$ or *mCry1*$^{\Delta CC/\Delta CC}$ mice show sustained oscillations, which does not occur in the absence of CRY1 (Liu et al., 2007).

This observation suggests that the CC is not required to maintain peripheral oscillators synchronized. As the CC is conserved between CRY1 and 2, and CRY2 is not required to maintain peripheral oscillators synchronized, we suggest that the domain in CRY1 that is responsible for this function is in the C-tail, which is divergent between the two proteins. It is becoming more evident that the functional differences between CRY1 and CRY2 are derived from the different C-tails, and further analysis of CRY mutants in the C-tail should answer this question.

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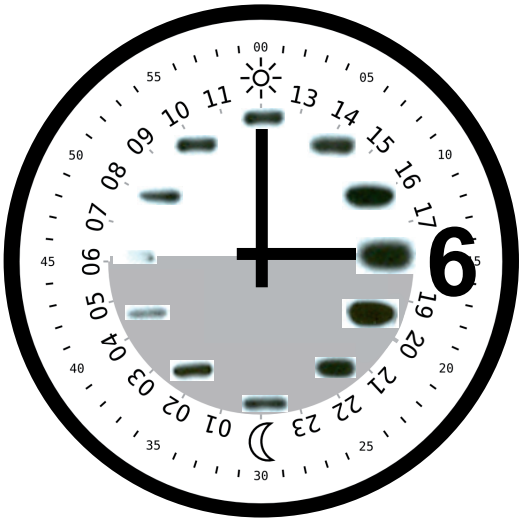
SIRT2 modulates the speed of the mammalian circadian clock via Cryptochromes



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SIRT2 modulates the speed of the mammalian circadian clock via Cryptochromes

Monika I. Bajek[1], Eugin Destici[1], Inês Chaves[1], Andre P.M. Eker[1], Fei Wang[3], Filippo Tamanini[1], Jeroen Demmers[2], Qiang Tong[3], Gijsbertus van der Horst[1]

[1] Department of Genetics, Chronobiology and Health Research Group, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands.
[2] Proteomics Center, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands.
[3] USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA.

Abstract

Circadian rhythms regulate appropriate timing of physiology, behaviour and metabolism. The molecular mechanism underlying those changes is composed of a core (post)transcription/translation feedback loop and additional interlocking loops. Cryptochromes are essential components of the core molecular oscillator. Mammals contain two cryptochrome genes (*mCry1* and *mCry2*). Here we show that SIRT2, an NAD^+-dependent protein deacetylase, forms a heterocomplex with CRY1 and CRY2 and reduces their acetylation level. SIRT2 deacetylation activity attenuates repressor activity of CRY1 but not of CRY2. Reducing endogenous SIRT2 levels in cultured fibroblasts shortens the period of the circadian clock, while increasing SIRT2 by exogenous overexpression lengthens the period. Importantly, we demonstrate that *Sirt2* knockout mice exhibit a shorter rhythm in constant darkness, which demonstrates SIRT2 contribution to the circadian regulation *in vivo*. Interestingly, the deacetylation activity of SIRT2 appears to be under circadian control, suggesting that SIRT2-dependent processes may be affected by the circadian system.

Introduction

The circadian clock is a biochemical timing mechanism that drives daily rhythms in physiology (e.g. blood pressure, body temperature and hormonal levels), behaviour (e.g. alertness, sleep cycle) and metabolism with an intrinsic periodicity of approximately 24 hours (Reppert and Weaver, 2001; Gachon et al., 2004; Lowrey and Takahashi, 2004). In mammals, the central circadian clock is located in the neurons of suprachiasmatic nuclei (SCN) of the hypothalamus. In order to keep pace with the daily light/dark cycle,

the SCN clock receives photic information from the retina that is transmitted to the SCN as electrical signals through the retinohypothalamic tract (Ralph and Menaker, 1989; Rusak et al., 1989). As the retina and the master clock in SCN are the only structures entrained by light in mammals, the peripheral oscillators, which are molecular clocks operating in the peripheral tissues (Zylka et al., 1998), are synchronized via neuronal and humoral signals from the SCN (Balsalobre A., 1998; McNamara P., 2001; Pando M.P., 2002). Nevertheless, recent findings have revealed that peripheral tissues and cultured cells express self-sustained and self-autonomous circadian oscillations, as those found in the SCN neurons (Yoo et al., 2004, Nagoshi et al., 2004).

The molecular mechanism regulating mammalian circadian rhythms is composed of ingeniously designed auto-regulatory negative and positive transcription/translation feedback loops (Reppert and Weaver, 2001; Shearman et al., 2000). The cycle is initiated once two transcription factors CLOCK and BMAL1 form a heterocomplex and activate a number of circadian controlled genes (CCG) including two cryptochrome genes (*mCry1*, *mCry2*) and three Period genes (*mPer1*, *mPer2* and *mPer3*) by binding to their E-box containing promoters. Products of these genes negatively regulate their own expression by inhibiting CLOCK/BMAL1 activity (Reppert and Weaver, 2001; Shearman et al., 2000; Gekakis et al., 1998). Additionally, the CLOCK and BMAL1 heterocomplex activates two orphan nuclear receptors, REV-ERBα and RORα which form additional interlocking loops. REV-ERBα acts as a repressor of CLOCK and BMAL1 mediated transcription (Preitner et al., 2002; Ueda et al., 2002; Yin and Lazar, 2005) whereas RORα acts as an activator of BMAL1 (Sato et al., 2004, Guillaumond et al., 2005, Akashi and Takumi, 2005). In the core clock mechanism REV-ERBα plays a more prominent role than REV-ERBβ (Liu et al., 2008).

The mCRY1 and mCRY2 proteins are indispensable components of the circadian core oscillator (Van der Horst et al., 1999) and act as the most potent repressors of CLOCK/BMAL1 transcription (Kume et al., 1999; Griffin et al., 1999). Notably, *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ knockout mice display accelerated and delayed free running periodicity of locomotor activity, respectively (Van der Horst et al., 1999; Vitaterna et al., 1999), suggesting that CRY proteins are important for determination of the circadian periodicity in mammals. In Drosophila, the clock's time keeping mechanism is directly connected to PER stability (Edery, 1994). In humans a mutation in *Per2* gene is associated with familial advanced sleep phase syndrome (FASPS), an autosomal dominant condition with early morning awakening and early sleep times (Toh et al., 2001). It was shown that FASPS *hPer2* mutation causes changes in hPER2 stability as hPER2 becomes hypophosphorylated by casein kinase I (CKI) (Xu et al., 2005). How CRY proteins regulate circadian periodicity in mammals has not been fully established, yet.

For maintaining the correct circadian period length in mammals the time of nuclear entry and length of time core clock proteins spent in the nucleus, prior to degradation, are essential (Tamanini et al., 2005). The evidence that these processes

are highly regulated in mammals has been provided using *in vitro* systems (Yagita et al., 2002). It was demonstrated that mPER2 protein shuttles between nucleus and cytoplasm using the functional nuclear localisation sequence (NLS) and the nuclear export sequence (NES) (Yagita et al., 2002). Nevertheless the subcellular localisation and stability of mPER2 are specifically controlled by mCRY proteins: mCRY1 protein binds specifically to the C-terminal region of mPER2 (thereby keeping mPER2 in the nucleus) and it inhibits mPER2 ubiquitylation and proteasomal degradation of the mCRY/mPER complex (Yagita et al., 2002). Further study revealed that mPER2 binds specifically to the C-terminal extension of mCRY1 (mCRY1extCT) which contains a nuclear localization signal and a putative coiled-coil domain (CC) and therefore drives nuclear localization and shuttling of the mCRY1/mPER2 complex. The C-terminal extension of mCRY1 is necessary but not sufficient for inhibition of mCLOCK/mBMAL1 mediated transcription (Chaves et al., 2006).

The identification of the posttranslational modifications of the clock proteins has revealed new levels of control in maintaining correct timing of the mammalian circadian clock (Lee et al., 2001; Dardente and Cermakian, 2007; Gallego and Virshup, 2007). The posttranslational modifications are induced upon formation of heterodimers or larger protein complexes and they control intracellular shuttling of clock proteins, their functionality and finally their degradation. Mammalian CRY proteins undergo posttranslational modifications, namely ubiquitylation and phosphorylation. F-box and leucine-rich repeat protein 3 and 21 (Fbxl3 and Fbxl21), ubiquitin ligases, control the oscillations of the circadian clock as they alter the inhibitory effect of mCRY by regulating its stability via ubiquitylation and degradation (Busino et al., 2007; Dardente et al., 2008). CKIε phosphorylates mCRY in an mPER dependent manner (Eide et al., 2002) and additionally mCRY2 but not mCRY1 is phosphorylated in a circadian manner by glycogen synthase kinase 3β (GSK-3β), subjecting phosphorylated mCRY2 for ubiquitylation and proteasomal degradation (Harada et al., 2005).

Other posttranslational modifications like sumoylation (Cardone et al., 2005) and histone acetylation (Hirayama et al., 2007) play a critical role in circadian gene expression. It was proposed that mBMAL1 acetylation, mediated by its partner mCLOCK, might be involved in modulating mCRY1 mediated repression (Hirayama et al., 2007). Recently, one of the members of the mammalian sirtuins SIRT1, the NAD^+-dependent protein deacetylase, was shown to regulate the circadian clockwork by deacetylating BMAL1 (Nakahata et al., 2008) and PER2 (Asher, et al, 2008). SIRT1 together with the metabolite nicotinamide adenine dinucleotide (NAD^+) and nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme involved in the NAD^+ biosynthesis, were newly proposed to form a negative feedback loop of the biological clock (Nakahata et al., 2009; Ramsey et al., 2009).

Sirtuins belong to a family of proteins that exhibits NAD^+-dependent deacetylase activity (Frye, 1999). In mammals there are seven members of sirtuins named mSIRT1

through mSIRT7. The mSIRT2 protein is mainly found in the cytoplasm (Michishita et al., 2005; North et al., 2003; Yang et al., 2000; Perrod et al., 2001, Afshar and Murnane, 1999) where mainly co-localizes with microtubules and deacetylates α-tubulin at the lysine 40 (North et al., 2003). Nevertheless mSIRT2 can also be found in nucleus as it was shown that SIRT2 can transiently migrate to the nucleus in the G2/M transition where mSIRT2 deacetylates histone H4 at the lysine 16 (H4K16) (Vaquero et al., 2006) and that SIRT2 constantly shuttles between the nucleus and cytoplasm during interphase (North and Verdin, 2007). Additionally, upon DNA damage induced conditions resulting in acetylation on histone H3 at lysine 56 (H3K56), mSIRT2 deacetylates H3K56 (Das et al., 2009). Recently our studies have shown that mSIRT2 deacetylates members of the FOXO family of transcription factors: FOXO3a (Forkhead box O3a) and FOXO1 (Forkhead box O1) (Wang et al., 2007; Wang and Tong, 2009).

In the present study we identified SIRT2 as a novel mCRY1 binding partner by probing mouse tissues with amylose beads immobilized purified MBP-mCRY1extCT and MBP-mCRY2extCT, [extended C-terminal domains (CT)] along with MBP used as a negative control, as we were able to purified those MBP fusion proteins at large scale. To the contrary, our attempts to purify full length mammalian CRY proteins have remained unsuccessful. We later showed *in vitro* that physical interaction of SIRT2 is not only restricted to mCRY1 since SIRT2 also physically associates with mCRY2. Since SIRT2 is a deactylase we tested whether mCRY proteins are acetylated and whether this can be modulated by SIRT2. Our findings reveal that CRY proteins undergo acetylation/deacetylation. We found that SIRT2 controls the period of the circadian clock *in vitro* and *in vivo*. Furthermore, we show that the deacetylation activity of SIRT2 is under circadian control.

Material and Methods

Plasmids

To generate expression constructs for MBP-fusion proteins containing the extended C-terminal tail of mCRY1 and mCRY2 (mCRY1extCT and MBP-mCRY2extCT, respectively), we used a PCR-based approach. The HA-mCRY1 (Chaves et al., 2006) and V5-mCRY2 (kindly provided by P. Szendro), cloned pcDNA3.1 expression constructs were used to amplify the tail regions of the *mCry1* (encoding aa 396-606) and *mCry2* (encoding aa 396-601). The following forward and reverse primers (extended with restriction sites to facilitate cloning) were used: mCry1exCT-F: 5'-ATG GAT CCT GGG TCA GCT GG-3'; mCry2exCT-F: 5'-ATG GAT CCC AGC TGG GAG AG-3' (BamH1 restriction sites are underlined), mCry1exCT-R: 5'-ATT TCG AA**G AT**G TTA CTG CTC TG-3', and mCry2exCT-R: 5'-ATT TCG AA**A GT**G GAG TCC TTG C-3' (HindIII restriction sites are underlined, stop codons are presented in bold).

The resulting BamH1/HindIII fragments were subsequently cloned in frame into the pMBP-Parallel1 vector (Sheffield P., 1999). To obtain a non-fused MBP expression construct, we introduced a stop codon at the last coding aa of MBP (123 aa) in the original pMBP-Parallel1 construct using the QuickChange mutagenesis kit (Stratagene) and forward primer 5'-GAC GCG CAG ACT AAT TCG AGC TAG AAC AAC AAC AAC AAC AAC AAC-3' (the stop codon is underlined). The reverse primer contained the reverse complementary sequence of the forward primer.

The generation of pCMV-mSIRT2 and pCMV-mSIRT2 N168A plamids has previously been described (Wang et al., 2007, Wang and Tong, 2009). FLAG-hSIRT2 cloned in pcDNA3 plasmids was kindly provided by E. Verdin ["Addgene plasmid 13813" (North, et al., 2003)].

Overproduction and purification of mCRY tails and MBP

Transformed *E. coli* BL21 pLysS cells were grown during 4 h at 37ºC in ampicillin (200 mg/l) and chloramphenicol (34 mg/l) containing medium, followed by 3 h induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 20 mg/l) at 27ºC. For purification cells from 7 liters of expression culture were suspended in buffer A (0.1 M NaCl, 10 m M K-phosphate pH 7.0). Cells were disintegrated by sonication, followed by centrifugation for 90 min at 43.000g at 4ºC. The supernatant was applied to a 2.5 x 5.4 cm amylose resin (New England BioLabs) column. After washing with buffer A MBP fusion protein was eluted with 10 mM maltose in buffer A. MBP-mCRYT1extCT, MBP-mCRY2extCT or MBP-containing fractions were pooled, diluted 4-fold with buffer B (40 mM NaCl, 10 m M K-phosphate pH 7.0) and loaded on a Q-Sepharose Fast Flow (Amersham Pharmacia Biotech AB) column (1.6 x 14.3 cm,). After washing with buffer B proteins were eluted by applying a linear gradient of 0 → 0.6 M NaCl in buffer B. Fractions containing highly purified MBP-mCRYT1extCT, MBP-mCRY2extCT or MBP (checked on SDS-PAGE gel by Coomassie Blue staining) were stored at -80ºC after addition of 1/5 volume of glycerol. Protein concentrations were measured with a Coomassie Plus Protein Assay kit (Pierce).

Pull down assay and mass spectrometry

To immobilize purified MPB-mCRY1extCT, MBP-mCRY2extCT, and MBP (used as a negative control) protein samples were added to the amylose beads (New England Biolabs) and were allowed to bind for 2 hours at 4ºC. The resin was washed with buffer A to remove non-specifically bound proteins and added to a kidney extract, obtained from wild type C57BL/6J mice sacrificed at ZT20. Following 3h incubation under continuous rotation at 4ºC and washing for three times with IP buffer, proteins were eluted with 1 M NaCl. Non-eluted proteins were removed by boiling the beads at

100°C for 3 min. Obtained protein samples were separated on a pre-made gradient (4-12%) SDS–PAGE gel (Invitrogen). Proteins were visualized by Flamingo fluorescent gel staining (Bio-Rad). In order to screen for potential new binding partners of the extCRY tails, the total SDS–PAGE gel containing only eluted protein fractions was used to perform a mass spectrometry analysis. Gel lanes were cut and subjected to in-gel digestion with trypsin (Promega), essentially as described previously (Wilm et al., 1996). Nanoflow liquid chromatography-tandem mass spectrometry was performed on an 1100 series capillary liquid chromatography system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo), as described previously (Sánchez et al., 2007). Database searches to assign proteins to the found peptide fragmentation spectra were performed using Mascot, as described previously (Sánchez et al., 2007).

Generation of *Sirt2*$^{-/-}$ mice

Sirt2$^{-/-}$ mice (kindly provided by F. Alt, generation will be described elsewhere) were in a C57BL6/J background and were housed under standard conditions and fed *ad libitum*.

For the monitoring of locomotor activity rhythms, mice of the same ages (8 weeks) were used. At this stage numbers of animal were for *Sirt2*$^{+/+}$ and *Sirt2*$^{+/-}$ n=2 and for *Sirt2*$^{-/-}$ n=1. (We are currently expanding the *Sirt2*$^{-/-}$ cohort). They were individually housed in a light-proof chamber in cages (30 x 45 cm) equipped with a running wheel (11 cm in diameter) and a sensor system to detect wheel rotations. Animals were maintained in a cycle of 12 h light (150 lux) and 12 h complete darkness (LD) or in continuous complete darkness (DD) in constant ambient temperature with water and food available *ad libitum*. Voluntary wheel-running (wheel turns per unit of time) was continuously recorded by an online computer using the ERS program. Activity records were plotted as actograms and the period of locomotor activity was determined by the chi-square method. Unpaired Student's t-tests were used to make statistical comparisons between the different genotypes.

Cell culture

HEK293T, COS7, NIH 3T3, and wild type and *Sirt2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium/F10, supplemented with P/S and 10% fetal calf serum. Cell transfections were performed with Fugene (Boehringer) according to the manufacturer's instructions.

Sirt2$^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos obtained from matings between *Sirt2*$^{+/-}$ mice (F1). Cells were grown in Dulbecco's modified Eagle's medium-F10-P/S-10% fetal calf serum.

Sirt2$^{-/-}$ immortalized MEFs were kindly provided by A.Vaquero.

Immunoprecipitation and Western blot analysis
For (co-)immunoprecipitation studies, transfected HEK293T cells (6 cm dish) were lysed in 500 µl of immunoprecipitation (IP) buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % NP-40) supplemented with a complete protease inhibitor cocktail (Roche) and incubated on ice for 15 min. After rapid sonication and centrifugation, supernatants were incubated with anti-FLAG M2 agarose affinity gel (Sigma) for 3 h at 4°C under continuous rotation. Samples were washed three times with IP buffer, boiled at 100°C for three minutes, and separated by SDS poly acrylamide gel electrophoresis on premade gradient SDS–PAGE gels (Invitrogen). Next, proteins were electro-transferred to a nitrocellulose membrane for immunoblot analysis. The following antibodies were used: rat anti-HA (Roche), mouse anti-V5 (Invitrogen), rabbit anti-SIRT2 (Santa Cruz), mouse anti-FLAG (Sigma), mouse anti-GFP (Clontech), rabbit anti-acetylated lysine (Cell Signalling), mouse anti-actin (Abcam), mouse anti-α-tubulin (Sigma), mouse anti-acetylated-tubulin (Sigma), all at a 1:1000 dilution. As secondary antibody for immunoblot analysis, we used horseradish peroxidase-conjugated (HRP) anti-rabbit IgG (Amersham), anti-mouse IgG (Amersham) and anti-rat IgG (Santa Cruz) at a 1:10000 dilution. In addition anti-FLAG-HRP (Sigma) was also used. Chemiluminescence was performed using Renaissance Western Blot Chemiluminescence Reagent Plus (NEN).

Acetylation studies
The acetylation status of mCRY proteins in HEK293T cells transfected with mSIRT2, mSIRT2 N168A, HA-mCRY1 and V5-mCRY2 (as indicated in figures 3C, D) was studied by harvesting the cells in 500 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 20 mM NaF, 1 mM Na_3VO_4, 10 mM EDTA, 1% NP-40, 10 mM nicotinamide, 1 mM TSA, 1 mM PMSF, and 5 µg/ml leupeptin). After rapid sonication and centrifugation, supernatants were incubated in the presence of anti-HA (Roche) or/and anti-V5 (Invitrogen) antibodies overnight at 4°C under continuous rotation) to immunoprecipitate HA-mCRY1 or V5-mCRY2, respectively. After washing for five times with IP buffer, samples were boiled at 100°C for three minutes, and proteins were separated by SDS-PAGE on a 10% gel. Acetylated lysins were detected by Western blot analysis, using rabbit anti-acetylated lysine antibody (Cell Signalling).

Immunofluorescence
To detect the expression and subcellular localization of various proteins in single, double, and triple transfection studies in transfected COS7 cells, cells were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS. After fixation, cells were washed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min. Cells were subsequently washed twice in PBS, blocked with 1.5% BSA in PBS containing

100 mM glycine (Sigma) for 10 min and incubated with: rat anti-HA (Roche), mouse anti-V5 (Invitrogen), rabbit anti-SIRT2 (Santa Cruz), and mouse anti-GFP (Clontech). The fluorescent secondary antibodies were Texas red-conjugated anti-rat immunoglobulin G (IgG) antibody (1:500; Jackson Immuno Research Laboratories), anti-rabbit antibody-Alexa 350 (1:250; Molecular Probes) and anti-mouse antibody-Alexa 488 (1:1,000). Microscopic images were obtained using a Zeiss Meta 729 microscope.

Luciferase reporter assay of CLOCK/BMAL1 activity

Sirt2$^{-/-}$ MEFs were seeded in 24-well plates and transfected with 100 ng of plasmid expressing an *mPer2* promoter-driven luciferase reporter gene (*mPer2::luc*), 300ng of HA-mCRY1 or V5-mCRY2, 900 ng of FLAG-hSIRT2, 900 ng of mSIRT2, 900 ng of mSIRT2 N168A and 2 ng of internal control plasmid pRL-CMV, for triplicate transfections. The total amount of DNA per transfection was adjusted by adding pcDNA3 vector. After 48 h, bioluminescence was measured using the Dual-Luciferase Reporter Assay system (Promega) following the manufacturer's protocol.

For statistical analysis we used a two-tailed *t*-test to make group comparisons and column statistic (Figure 5B).

Real-time bioluminescence

To study the effect of knock-down of SIRT2 on circadian core oscillator function, NIH 3T3 cells stably expressing an *mPer2* promoter-driven luciferase reporter gene (*mPer2::luc*) were transfected with *Sirt2* siRNA or *GFP* siRNA oligos (used as a negative control). The effect of overexpression of SIRT2 was studied in NIH 3T3 cells transiently co-transfected with a plasmid expressing a *mBmal1* promoter-driven destabilized luciferase reporter gene (*mBmal1::luc*) and plasmids expressing SIRT2 or with pcDNA3 (used as a negative control). For real-time bioluminescence monitoring of circadian rhythmicity, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of confluent cultures with forskolin (dissolved in 100% ethanol, added to the culture medium at a final concentration of 30 μM) bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C.

Quantitative Real-Time PCR (RT-PCR)

Total RNA from cultured cells was extracted using Trizol reagent (Invitrogen) as described by the manufacturer. First-strand cDNA was synthesized from 1 mg of total RNA using oligo (dT) primers and SuperScript reverse transcriptase (Invitrogen) according

to the manufacturer's protocol. Real-time quantitative RT-PCR for determination of *Rev-erbα, Dbp*, *Per1, Per2, Cry1, Cry2,* and *Bmal1* mRNA levels was performed in duplicate using an iCyclerIQ system (Bio-Rad), SYBR Green and primer pairs generating intron-spanning products of 150–300 bp. Expression levels were normalized to *Hprt* (hypoxanthine guanine phosphoribosyl transferase) mRNA levels. The generation of specific PCR products was confirmed by melting curve analysis, and each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate the primer pair efficiency.

Protein extraction

Liver protein extracts were obtained from mice (wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$) (Van der Horst et al., 1999) which were sacrificed at 4 h intervals around the clock. Protein concentrations were determined with the Bradford Protein Assay Kit (Bio-Rad) For each time point, 50 µg of protein was subjected to SDS-PAGE on premade, 4-12% gradient gels (Invitrogen), followed by western blot analysis.

Results

Expression and purification of CRY C-terminal extended tails fused to MBP in *E. coli*

Our attempts to overproduce and purify full-length mammalian cryptochrome at large scale in *E. coli* remains unsuccessful. Previously, we have shown that C-terminal extension (extCT) of mCRY1 contains a nuclear localization signal and a putative coiled-coil to which binds mPER2 protein and therefore drives nuclear localization and shuttling of the mCRY1/mPER2 complex (Chaves et al., 2006). It is also necessary but not sufficient for inhibition of CLOCK/BMAL1 mediated transcription (Chaves et al., 2006). Consequently, we decided to overproduce and purify mCRY tails in *E. coli* instead of full-length CRY to gain more insight in the function of the C-terminal domain of mCRY. mCRY1extCT and mCRY2exyCT fused to C-terminal maltose binding protein tag (MBP) were recombinantly expressed and purified from *E. coli* (Figure 1A, B, C). The described purification scheme in material and methods resulted in overall yields of 4-4.5 mg of highly purified MBP-mCRY1extCT and MBP-mCRY2extCT. We also expressed and purified MBP from *E. coli* in yield of 5 mg highly purified MBP protein (Figure 1D).

Mass spectrometry analysis revealed SIRT2 interaction with the mCRY1extCT but not mCRY2extCT

In order to search for novel binding partners of cryptochromes, we performed pull

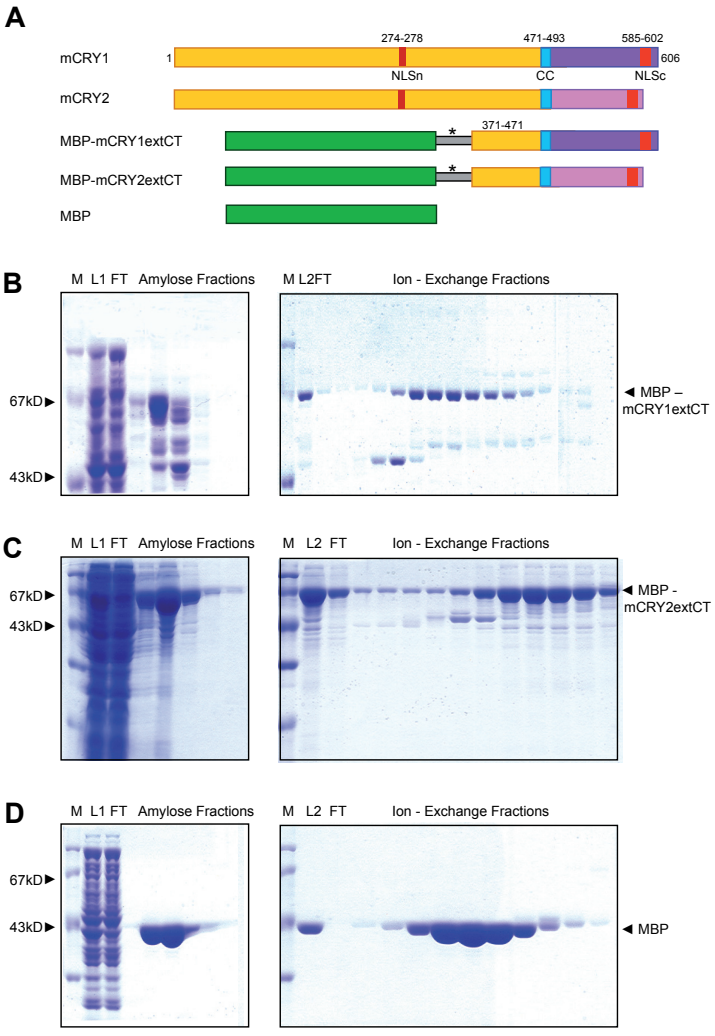

Figure 1. Expression and purification of MBP-CRYCT and MBP from E. coli BL21(DE3)pLysS cells
An *E. coli* lysate was generated from IPTG-induced cells, the clarified supernatant was applied to an amylose resin column and the protein was eluted with 10 mM maltose. Eluted fractions were followed by further purification using ion-exchange chromatography (Q Sepharose Fast Flow column).
(A) Schematic representation of MBP-mCRY1CT, MBP-mCRY2CT and MBP. The core domain of CRY proteins is shown in yellow, the C-terminus of CRY shown in magenta (CRY1) and pink (CRY2), the predicted coiled-coil (CC) domain shown in cobalt, the nuclear localisation signal at the N-terminal domain (NLSn) and nuclear localisation signal at the C-terminal domain (NLSc) shown in dark an light red, respectively, the MBP is shown in green and the linker including TEV protease restriction site (*) shown in gray. B) Coomassie-blue stained SDS-PAGE gels after the purification of MBP-mCRY1CT, (C) MBP-mCRY2CT and (D) MBP. M- marker, L1 – load 1; whole bacterial cell lysate, L2 – Load 2; amylose fractions, FT – flow trough

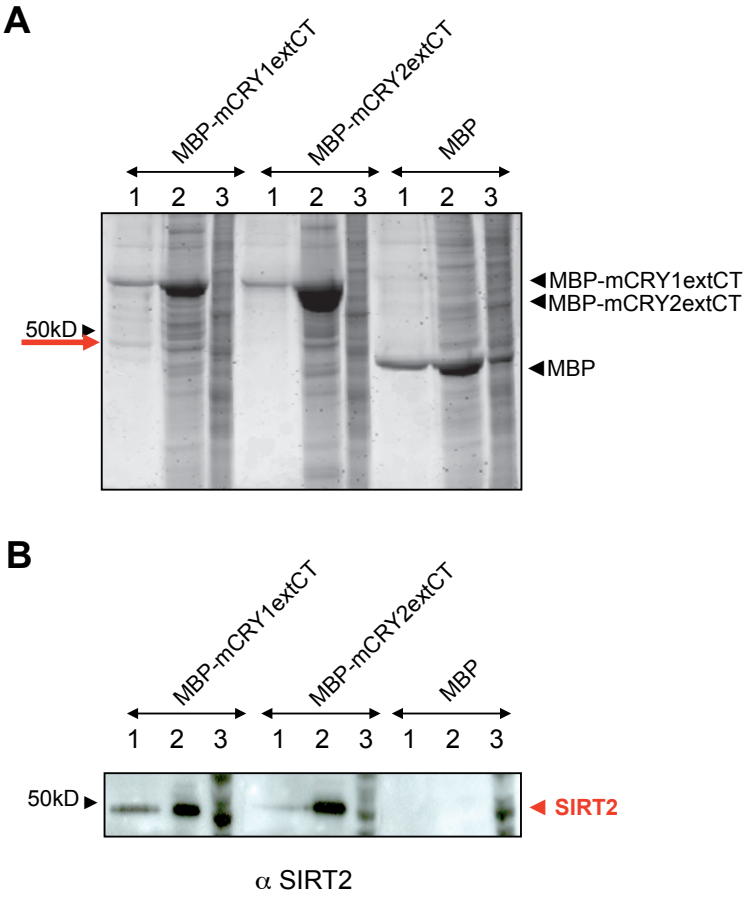

Figure 2. Flamingo staining and Western Blot analysis of SDS-PAGE gel of the pull down assay from kidney extract

A Purified MBP-mCRY1extCT, MBP-mCRY2extCT and MBP were separately immobilized to amylose resin beads. The immobilized beads were incubated with kidney extract obtained from wt C57BL6 mice (sacrificed at ZT20) for 3h at 4°C. After the beads were extensively washed the bound proteins were eluted with 1M NaCl (lane 1). After the treatment with 1M NaCl amylase resin beads were boiled at 100°C for 3 min in order to remove all not specifically bound proteins including immobilized and purified MBP-mCRY1CT, MBP-mCRY2CT and MBP (lane 2). After the incubation all unbound proteins from the kidney extract were also collected (lane 3). The samples were separated on premade, gradient 4-12% SDS-PAGE, followed by staining with Flamingo. The unique band representing the approximately 50 kDa protein is indicated by the red arrow.

B Western Blot analysis with anti-SIRT2 antibody on the same samples used for Flamingo staining.

down assays using our purified MBP-mCRY1extCT and MBP-mCRY2extCT (along with MBP alone used as a negative control) immobilized to amylase beads. For this experiment we used kidney extract obtained from wt C57BL/6J mice which were sacrificed at ZT20, when the maximal level of mCRY expression is displayed. After 3 hours of incubation all proteins were eluted by 1 M NaCl and separated on SDS-PAGE under reducing conditions. Flamingo stain analysis of a polyacrylamide gel containing the proteins specifically bound to the mCRY1extCT and mCRY2extCT identified among other major bands a band specific for MBP-mCRY1extCT running just above the 50,000-molecular-weight marker (Figure 2A). Mass spectrometry analysis revealed the presence of SIRT2 (7 unique peptides) specific for mCRY1extCT but not in the control. Indeed, by Western Blot analysis, SIRT2 could be detected in the specifically bound protein fraction to the mCRY1extCT but not mCRY2extCT as a band of approximately 45 kDa (predicted molecular weight: 43 kDa; Figure 2B). Interestingly, SIRT2 was also detected in the protein fraction remained bound to the beads with immobilized MBP-mCRY1extCT and also MBP- mCRY2extCT after elution whereas it was absent in the control (Figure 2B). This suggests that SIRT2 binds to both mCRY1extCT and mCRY2extCT although the eluent used in this assay (1 M NaCl) allowed to elute SIRT2 bound to the mCRY1extCT only. We recently have shown that SIRT2 deacetylates FOXO family of transcription factors: FOXO3a and FOXO1 (Wang et al., 2007; Wang and Tong, 2009) and FOXO was shown to regulate sensitivity of the circadian clock to the oxidative stress in flies (Zegn X., 2007). Another member of the sirtuin family, SIRT1, was also shown to regulate the mammalian circadian clock via its deacetylase activity (Nakahata et al., 2008; Asher et al., 2008) and therefore we proceeded with further analysis with SIRT2.

SIRT2 directly interacts with CRY

As mass spectrometry analysis revealed that the NAD^+-dependent deacetylase SIRT2 interacts with CRY1extCT and possibly also with CRY2extCT, we further investigated the interaction between SIRT2 and CRY. We tested whether SIRT2 binds to CRY by transiently transfecting HEK293T cells with plasmid constructs expressing full-length of the CRY and FLAG-tagged SIRT2. Cell lysates were collected and SIRT2 proteins were immunoprecipitated with an anti-FLAG antibody. Consistent with the result of MBP-pull down as shown in Figure 2, both CRY1 and CRY2 coimmunoprecipitated with SIRT2 (Figure 3A and 3B).

Since PER2 protein physically interacts with CRY (Griffin et al., 1999) through the coiled-coil domain (CC) of CRY1 (Chaves et al., 2006) we investigated whether there is competition between SIRT2 and PER2 for binding to the CC domain of CRY1. To test our hypothesis we looked at SIRT2 binding to CRY in absence or presence of PER2. We performed an immunoprecipitation experiment with transiently transfected HEK293T

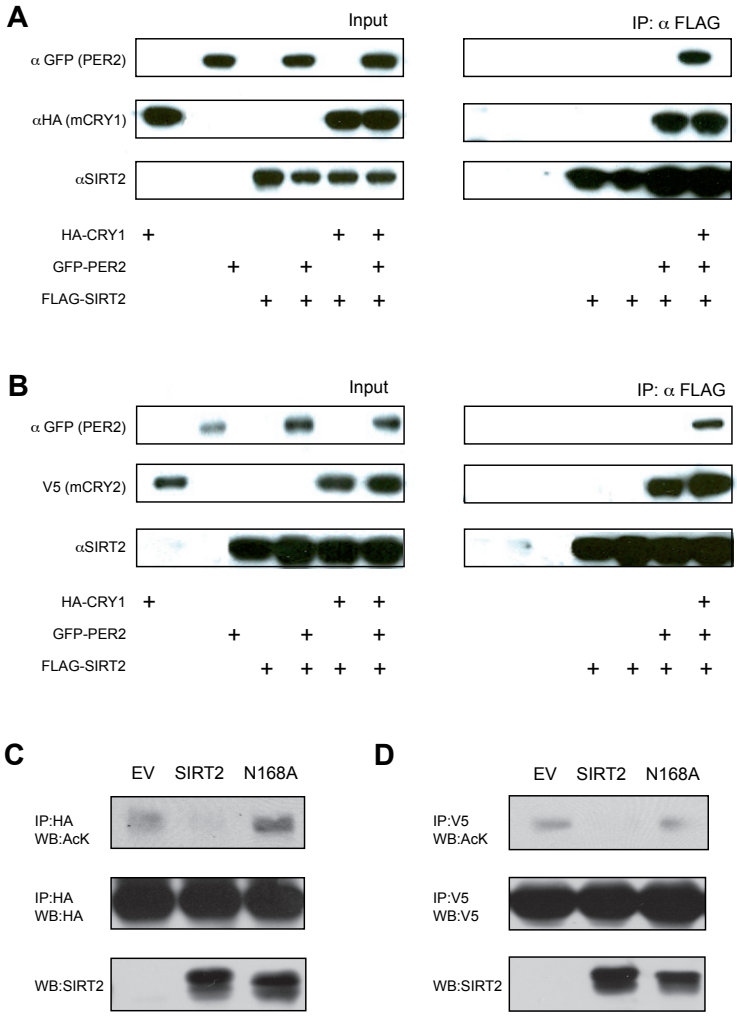

Figure 3. SIRT2 interacts with CRY and deacetylates CRY

A, B HEK293 cells were transiently transfected with CRY: CRY1 (A), CRY2 (B) and FLAG-tag SIRT2. 24 hours after transfection, cells were lysed, and FLAG-SIRT2 proteins were immunoprecipitated with an anti-FLAG M2 agarose affinity gel. The precipitated proteins were washed five times and analyzed by Western Blotting as indicated.

C, D HEK293 cells were transfected with HA-CRY1 (C) V5-CRY2 (D) SIRT2 and SIRT2 N168A mutant. Cells were treated with 1 μM tricostatin A (TSA) for 2 h before cells were harvested. HA-CRY1 or V5-CRY2 were immunoprecipitated with anti-HA or anti-V5 antibodies, respectively. Acetylated CRY was detected with anti-acetylated lysine antibodies (AcK acetylated lysine).

IP - Immunoprecipitation, WB – Western Blotting, EV – Empty Vector, N168A – SIRT2 mutant, AcK - Acetylated Lysine (AcK) antibody

cells. We co-transfected CRY, PER2 and Flag-SIRT2 and SIRT2 was immunoprecipitated using anti-Flag antibodies, as indicated in Figure 3A and 3B. We found that SIRT2 does not interact with PER2 directly but indirectly when CRY1 or CRY2 proteins are present (Figure 3A and 3B). In addition SIRT2 association with CRY is not affected by PER2 as CRY and PER2 coimmunoprecipitated with SIRT2 simultaneously (Figure 3A and 3B). We conclude that there is no competition between SIRT2 and PER2 for binding to CRY. Further analysis is required in order to identify the domains involved.

CRY proteins are modified by acetylation, which can be removed by SIRT2

As SIRT2 is a NAD^+-dependent deacetylase (Frye et al., 1999), we examinated whether CRY proteins are acetylated and are potential substrates for SIRT2 deacetylation activity. To this end, we performed an immunoprecipitation experiment with transiently transfected HEK293T cells. We co-transfected CRY with SIRT2 and the SIRT2 mutant with greatly reduced deacetylation activity (SIRT2 N168A) (Finnin et al., 2001; Borra et al., 2002). HA-CRY1 and V5-CRY2 were pulled down from cell lysates with an anti-HA or anti-V5 antibody, respectively. Acetylation of CRY was detected by an anti-acetylated lysine antibody. For the first time, we showed that CRY1 and CRY2 are acetylated proteins. CRY acetylation levels are greatly decreased in the presence of SIRT2 indicating that CRY are new substrates for SIRT2 deacetylation activity (Figure 3C). Importantly, the acetylation state of CRY was not affected by the SIRT2 N168A catalytically inactive mutant.

SIRT2 keeps portion of CRY1 but not CRY2 protein in cytoplasm

To understand the mechanism and function of the SIRT2 deacetylation of CRY proteins, we initially studied the regulatory effect of SIRT2 on nuclear localisation of CRY in presence or absence of PER2. For this aim we performed an immunofluorescence assay in COS7 cells overexpressing CRY1, CRY2, PER2 and SIRT (as indicated in Figure 4). Previously we have shown that CRY1 is mostly a nuclear protein, while PER2 is found in the cytoplasm and nucleus, and the CRY1/PER2 complex is found mainly in the nucleus (Chaves et al., 2006). SIRT2 exhibits predominantly cytoplasmic localisation (Michishita et al., 2005; North et al., 2003; Yang et al., 2000; Perrod et al., 2001, Afshar and Murnane, 1999) nevertheless it can be also found in the nucleus (Vaquero et al., 2006; North and Verdin, 2007; Das et al., 2009). Our results confirmed sub-cellular localisation of CRY, PER2, CRY1/PER2 complex and SIRT2 as was shown previously (Figure 4). Additionally, we have found that SIRT2 keeps a portion of the CRY1 protein in the cytoplasm and presence of PER2 does not alter the CRY1/SIRT2 cytoplasmic localisation (Figure 4A). In contrast, we do not observe any difference in the localisation of CRY2 in presence or absence of SIRT2 (Figure 4B).

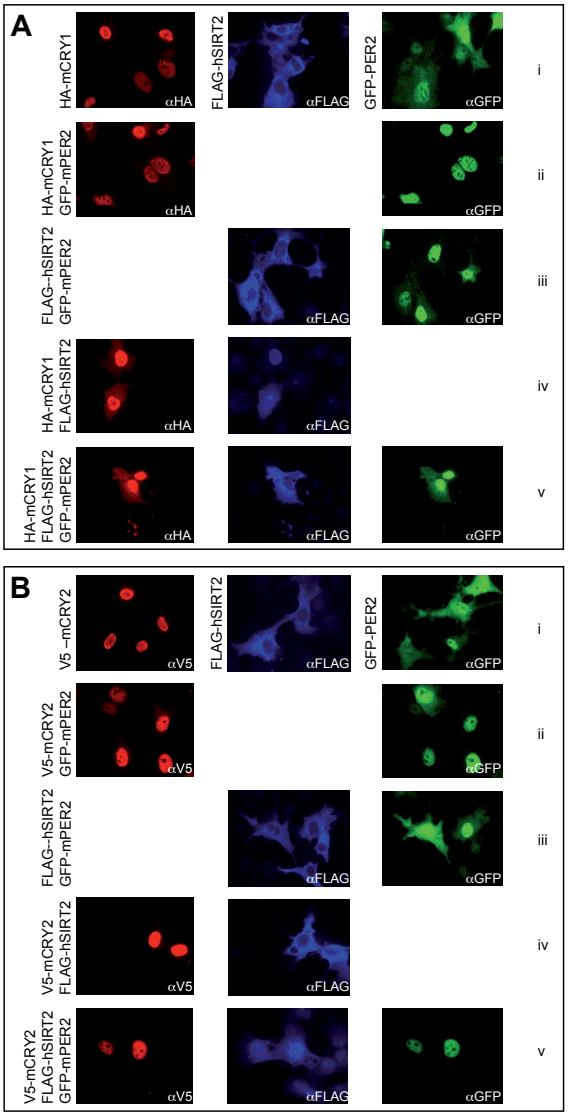

Figure 4. Subcellular localization of the CRY1/SIRT2 or the CRY2/SIRT2 complexes in absence or presence of PER2

A Immunofluorescence pictures of COS7 cells individualy expressing: HA-mCRY1, FLAG-hSIRT2 and GFP-PER2 (i), and coexpressing: HA-mCRY1 and GFP-mPER2 (ii), FLAG-hSIRT2 and GFP-mPER2 (iii), HA-mCRY1 and FLAG-hSIRT2 (iv) and HA-mCRY1, FLAG-hSIRT2 and GFP-mPER2 (v).

B Immunofluorescence pictures of COS7 cells individualy expressing: V5-mCRY2, FLAG-hSIRT2 and GFP -PER2 (i), and coexpressing: V5-mCRY2 and GFP-mPER2 (ii), FLAG-hSIRT2 and GFP-mPER2 (iii), V5-mCRY2 and FLAG-hSIRT2 (iv) and V5-mCRY2, FLAG-hSIRT2 and GFP-mPER2 (v).

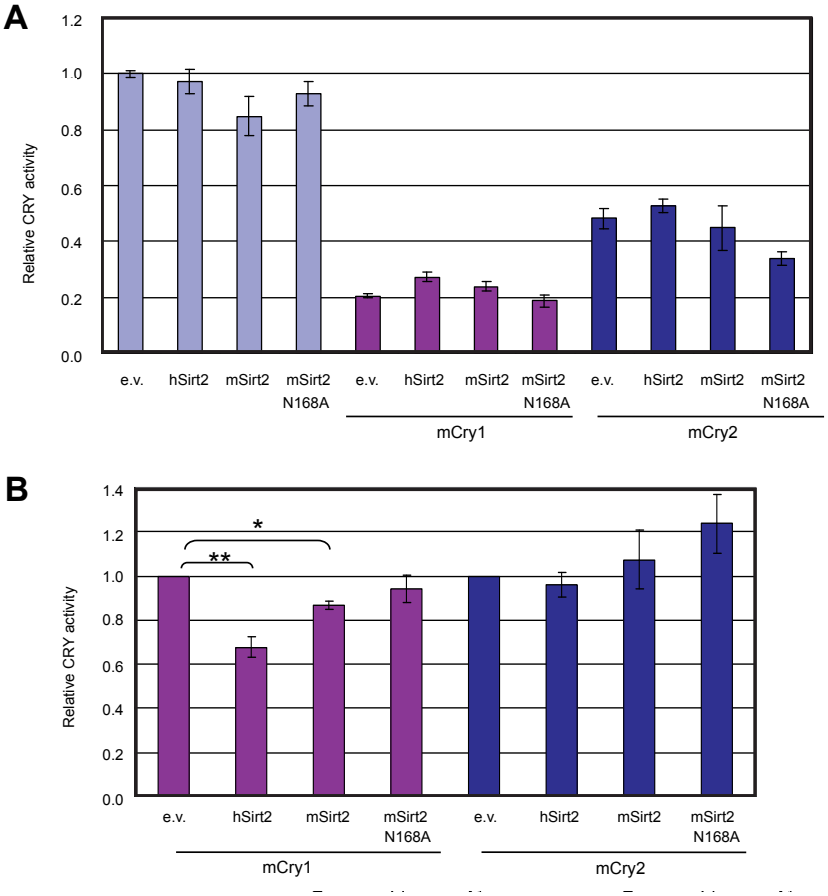

Figure 5. The role of SIRT2 on CRY mediated inhibition of CLOCK/BMAL1 driven transcription in Sirt2$^{-/-}$ *MEFs*
CLOCK/BMAL1 transcription assay using a *mPer2* E-box promoter-luciferase reporter construct. Luminescence, shown as a several fold induction from the basal level, is indicated on the *y* axis. pcDNA3, pRL-CMV, and the *mPer2::luc* were added in all conditions. The presence or absence of the other expression plasmids is indicated.

A Individual expreriment showing CLOCK/BMAL1 repression by CRY, which is slightly attenuated by SIRT2. The values are relative to pcDNA alone, set to 1. Mean and standard error of triplicate samples are shown.

B Quantification of tree independent experiments. CRY1 and CRY2 were set to 1 in each experiment and the SIRT2 samples are relative to that. * p= 0,009, ** p=0.008, using column statistic comparing to hypothetical value of 1.

SIRT2 attenuates CRY1-mediated transcription inhibition

The primary function of the CRY protein is the inhibition of transcription activation of E-box-containing promoters by the CLOCK/BMAL1 heterodimer (Kume et al., 1999; Griffin et al., 1999). To investigate whether SIRT2 dependent deacetylation of CRY influences repressor activity of CRY we performed a luciferase reporter assay using immortalized SIRT2$^{-/-}$ MEFs. Nuclear localisation of CRY is a very important aspect for this assay. Figure 5 shows that SIRT2 significantly attenuates CRY1 mediated transcription inhibition (Figure 5). Importantly, CRY1 inhibition of transcription was not affected by the SIRT2 N168A catalytically inactive mutant. While SIRT2 attenuates inhibitory activity of CRY1, we did not observe any effect of SIRT2 on CRY2. This might be linked to the lack of the SIRT2 effect on CRY2 localisation (Figure 5).

SIRT2 influences expression of endogenous core clock genes

We investigated whether SIRT2 has an effect on the expression of endogenous circadian genes, as it was shown that SIRT1 strongly influences expression of various clock transcripts (Asher, et al., 2008). We analysed the level of several transcripts in primary wild-type and SIRT2$^{-/-}$ MEFs by real-time PCR. Preliminary qPCR analysis of clock gene expression in liver suggests that SIRT2 affects clock gene expression in a gene specific manner (Supplementary Figure S1). To further confirm these data we need to analyse more animals per time point.

Knockdown of SIRT2 by siRNA causes a shorter period in cultured fibroblasts

In order to test whether SIRT2 might influence circadian gene expression, we followed real-time bioluminescence recordings of an *mBmal1-* or *mPer2-luciferase* reporter (*mBmal1::luc*, *mPer2::luc*) where expression is driven by the *mBmal1* or *mPer2* promoter, respectively. First we performed a loss-of-function experiment. NIH 3T3 cells stably expressing *mPer2::luc* reporter were transfected with *Sirt2* siRNA or *GFP* siRNA as a negative control. We found that the *Sirt2* siRNA shortened the tau by about 15 minutes ($p=0.003$) (Figure 6A). Since all the cells express the reporter, but not all the cells will be transfected with siRNA, this is probably an underestimation. In NIH 3T3 cells with stable *Sirt2* shRNA knockdown transfected with the *Bmal1-luciferase* reporter plasmid we observed a similar effect (data not shown).

Next, we examined whether overexpression of SIRT2 has an effect on circadian oscillations. Co-transfection of the SIRT2 expression vector with the *mBmal1::luc* reporter plasmid lengthened the circadian period by approximately 30 minutes ($p=0.017$) (Figure 6B).

Importantly the SIRT2 N168A mutant did not show any effect.

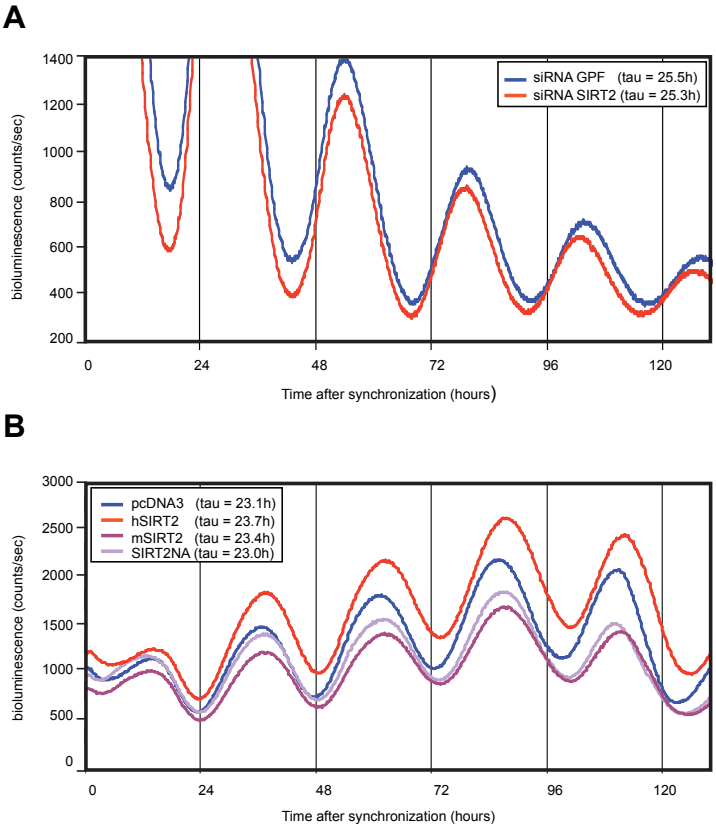

Figure 6. SIRT2 affects circadian gene expression of the Per2 *or* Bmal1-luciferase *reporter*

A Representative examples of bioluminescence rhythms in NIH 3T3 cells stably expressing an mPer2 promoter-driven luciferase reporter gene (*mPer2::luc*), transfected with: Sirt2 siRNA, 25 nM final concentration (red) or Gfp siRNA (blue), after initial synchronization with forskolin. (n=3, tau GFP=25.5 h, tau Sirt2i=25.3h, p=0.003).

B Representative examples of bioluminescence rhythms in NIH 3T3 transiently transfected with 250 ng *Bmal1-luciferase* reporter gene (*mBmal1::luc*) along with FLAG-hSIRT2 (red), mSIRT2 (plum), mSIRT2NA (lavender) pcDNA3 (blue); 750 ng of each construct, after initial synchronization with forskolin. (n=4, tau pcDNA3=23.12h, tau FLAG-hSIRT2=23.72h, mSIRT2=23.42, mSIRT2NA=23.0, p=0.017).

Sirt2$^{-/-}$ mice exhibit a shorter rhythm in constant darkness – primary data

To investigate the *in vivo* contribution of SIRT2 on the circadian clock, we analysed running-wheel behaviour of *Sirt2*$^{-/-}$, *Sirt2*$^{+/-}$ and wild-type littermates.

To determine their circadian behaviour, the mice were housed in cages provided with a running-wheel and activity was recorded and analysed (Figure 8). The preliminary analysis of the *Sirt2*$^{-/-}$ mice (n=1) shows that the free-running period of the *Sirt2*$^{-/-}$ is approximately 30 minutes shorter than that of wild type (Figure 8). In addition, the free-running period of the *Sirt2*$^{+/-}$ mice does not deviate from that of its wild type littermate (Figure 7).

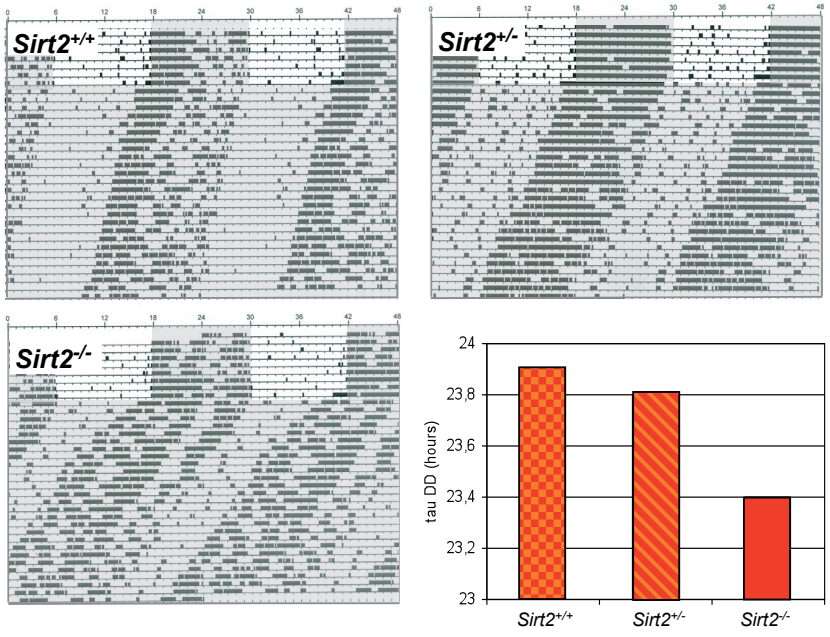

Figure 7. The double-plotted actograms obtained for Sirt2$^{+/+}$*, Sirt2*$^{+/-}$ *and Sirt2*$^{-/-}$ *and graphic representation of the free-running period in constant darkness*

Mice of the same age (8 weeks) were used. In the initial experiment numbers of animal were n=2 for *Sirt2*$^{+/+}$ and *Sirt2*$^{+/-}$ and n=1 for *Sirt2*$^{-/-}$. (We are currently expanding the *Sirt2*$^{-/-}$ cohort). Shading indicates darkness and the black vertical lines represent animal activity.

SIRT2 deacetylation activity is circadian

Since it has been shown (Nakahata et al., 2008 Asher et al., 2008) that SIRT1 activity oscillates in a circadian manner, we investigated whether SIRT2 activity may be under control of the circadian clock. We examined the expression of SIRT2 in wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse liver from mice killed at 4 hour intervals around the clock. Western blot analysis shows that the SIRT2 levels remained constant (Figure 8A). While we did not observe SIRT2 protein oscillations, we did observe oscillation of SIRT2 deacetylation activity. Using the same protein liver extracts obtained from WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ KO mice we examined SIRT2 deacetylation activity on α-tubulin as this was shown to be a SIRT2 substrate (North et al., 2003). Using an anti-acetylated-tubulin specific antibody we showed that the acetylation levels of tubulin oscillate, suggesting that SIRT2, although constant in level, exhibits a circadian deacetylation activity that peaks around ZT8-12 (Figure 8B).

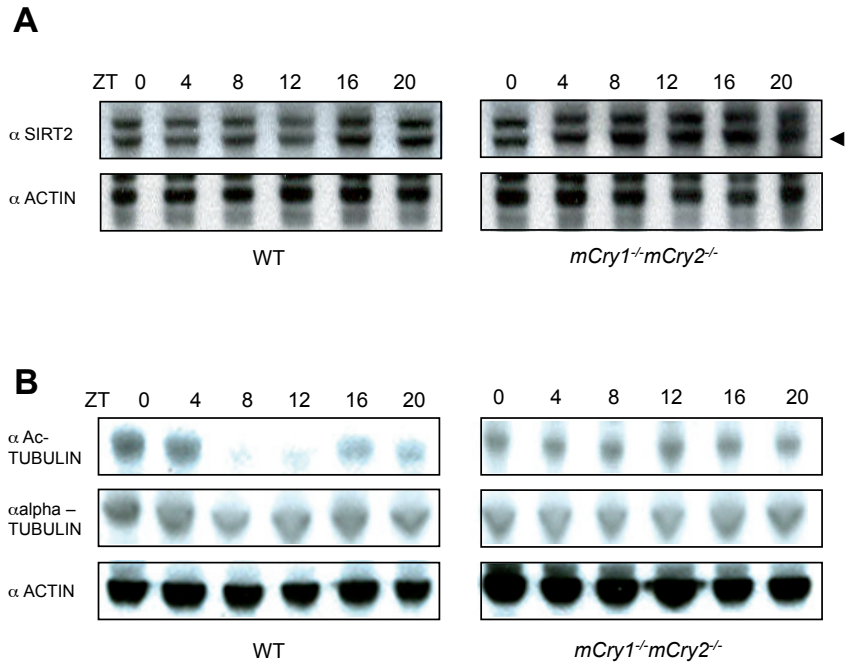

Figure 8. SIRT2 protein level is constant while SIRT2 deacetylation activity is circadian in the liver

A Endogenous SIRT2 and PER2 expression in the liver obtained from wild-type vs. *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animals, sacrificed at 4h intervals around the clock was determined by western blot.

B SIRT2 deacetylase activity was identified by detecting acetylated-tubulin (Ac-Tubulin) compared with non-acetylated tubulin levels in the liver obtained from wild-type vs. *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animals, sacrificed at 4h intervals around the clock was determined by western blot.

Discussion

In the present study we identified SIRT2 as a novel binding partner of the mammalian cryptochromes. We used our purified mCRY extended C-terminal domains fused to the MBP to perform pull down assays followed by mass spectrometry (MS) analysis. MS analysis revealed SIRT2, an NAD^+-dependent deacetylase, as a potential mCRY1extCT and perhaps mCRY2extCT binding partner. To verify this finding we performed a co-immunoprecipitation study. For that aim we used full-length mCRY proteins instead of mCRYextCT domains only. We confirmed that SIRT2 forms a complex with both mCRY1 and mCRY2.

The primary function of mammalian CRY proteins is to inhibit CLOCK/BMAL1 transcription (Kume et al., 1999; Griffin et al., 1999). In order to generate the circadian rhythms degradation of CRY repressors is required. Therefore CRY protein undergoes posttranslational modification involving phosphorylation and ubiquitylation: GSK-3β dependent CRY2 but not CRY1 phosphorylation (Harada et al., 2005), CRY phosphorylation by CKIε (Eide et al., 2002) and CRY ubiquitylation by Fbxl3 and Fbxl21 (Busino et al., 2007; Dardente et al., 2008). Our study for the first time demonstrates that CRY1 and CRY2 are acetylated proteins. Furthermore, we show that this acetylation is under control of SIRT2 identifying CRY1 and CRY2 as new SIRT2 substrates. Since protein ubiquitination and acetylation are both found on lysine residues, it is conceivable that the same lysine residue can be either acetylated or ubiquitinated. A direct competition between lysine acetylation and ubiquitination has been proposed as a major regulatory mechanism preventing protein ubiquitination and degradation via the ubiquitin/proteasome system (Sadoul et al., 2008). The same lysine that is a subject for both acetylation and ubiquitination was proposed in the case of e.g. p53 (Ito et al., 2002), SMAD7 (Gronroos et al., 2002), FOXO4 (Fukuoka et al., 2003), RUNX3 (Jin et al., 2003), p73 (Bernassola et al., 2004), NF-E4 (Zhao et al., 2004). Interestingly, recently it was shown that another member of sirtuin NAD^+-dependent deacetylase, SIRT1 promotes the deacetylation and degradation of a core clock protein PER2 (Asher et al., 2008). Taking into account all these data clearly leads to the proposition that SIRT2 via its deacetylation activity might influence CRY stability which in turn would add an additional level to regulation timing of the mammalian circadian clock. In recently obtained preliminary data, we see slightly more CRY1 proteins in livers obtained form *Sirt2*$^{-/-}$ animals at ZT=10, 14 and 22 (Supplementary Figure S2). At this stage we could test only 1 animal per time point thus to draw a solid conclusion we need to test more animals. Further work with more animals and (biochemical) assays using cells will be needed to test whether SIRT2 affects the protein stability of CRY1 and CRY2. Additionally we like to establish turnover of CRY1 and CRY2 in immortalised SIRT2 MEFs. By showing Supplementary Figure S1 we would like to provide evidence that we are in position of very good tools, namely antibodies, to proceed with further experiments.

We demonstrate that SIRT2 has an affect on CRY1 localisation and CRY1 inhibition activity. In contrast SIRT2 regulatory effects on CRY2 were not observed, even though this protein is also deacetylated by SIRT2. This observation suggests that SIRT2 regulates CRY proteins in a different way which could possibly lead to different contribution of CRY proteins in the circadian clock machinery. Notably, *mCry1* and *mCry2* knockout mice display accelerated and delayed free-running periodicity of locomotor activity, respectively (Van der Horst et al., 1999).

SIRT2 is a new circadian rhythms regulator as we have shown that knockdown of SIRT2 by siRNA or shRNA causes a shorter period in cultured fibroblasts whereas SIRT2 overexpression lengthens the period. Our preliminary data using *Sirt2*$^{-/-}$ mice show that SIRT2 contributes to circadian regulation *in vivo* as *Sirt2*$^{-/-}$ mice exhibit a shorter rhythm in constant darkness.

Sirtuins, NAD^+-dependent deacetylases, can dynamically sense cellular energy metabolism (Bordone et al., 2005). Therefore it is reasonable to speculate that the CRY/SIRT2 interactions described in this study represent one of the events linking the biological clock with metabolism. Studies of the relationship between circadian rhythms and metabolic systems are now under investigation (Eckel-Mahan and Sassone-Corsi, 2009). The first evidence was provided from experiments showing that the DNA-binding activity of the BMAL1/CLOCK heterocomplex is strongly influenced by the ratio of reduced and oxidized NAD cofactors, which are read-outs of the cellular metabolic state (Rutter J, 2001). Moreover a transcriptome profiling study revealed that many genes involved in metabolism are under circadian control (Panda et al., 2002, Ueda et al., 2002, Walker and Hogenesch, 2005, McCarthy et al., 2007). Recently a link between metabolism and circadian oscillations via sirtuins was made as it was shown that SIRT1 associates and deacetylates BMAL1 (Nakahata et al., 2008) as well as PER2 (Asher et al., 2008). Here we show that another member of the mammalian sirtuins family, SIRT2, binds to core clock proteins CRY. We also have found that SIRT2 NAD^+-dependent deacetylation activity may be under circadian regulation, whereas the SIRT2 protein level remains constant as was shown for SIRT1 (Nakahata et al., 2008). Data from wild-type MEFs showing that levels of cellular NAD^+ oscillate, which is absent in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ MEFs (Nakahara Y., 2009), provide a probable mechanism for the oscillation of the SIRT2 deacytelase activity. This suggests that NAD^+ is a key regulator of metabolic rhythms.

Metabolic changes are induced by a caloric restricted diet; the most potent anti-aging effect as it slows aging in a variety of species (Weindruch et al., 1988). Sirtuins are found to respond to nutrient deprivation and are known to regulate metabolic functions in lower organisms: yeast (Lin et al., 2000; Lamming et al., 2005), *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001) and *Drosophila melanogaster* (Rogina and Helfand, 2004). In addition, we and others have shown that sirtuins expression is also responsive to caloric restriction in mammals (Cohen et al., 2004; Shi et al., 2005; Wang et al., 2007). We have shown that in mice caloric restriction activates

expression of SIRT2. In a response to this SIRT2 deacetylates FOXO transcription factors: FOXO3a (Wang et al., 2007) and FOXO1 (Wang and Tong, 2009). FOXO are key components of the insulin/IGF signaling cascade; a crucial pathway that controls organism growth and metabolism. In *C. elegans* a mutation in the insulin receptor-like gene *daf-2* prolongs life span. This requires the activity of a second gene, *daf-16*, the FOXO ortholog (Kenyon et al., 1993). We have previously shown that caloric restriction regulates the function of SIRT2 and we propose that it is likely that SIRT2 has a role in regulation of aging in higher organism (Wang and Tong, 2009). In the present study we show, for the first time, that SIRT2 regulates the mammalian circadian clock as SIRT2 deacetylates cryptochromes. Consequently, it is likely that SIRT2 is an additional link between the mammalian circadian clock, metabolism and aging. Moreover, recently it was shown that in *Drosophila* FOXO regulates the sensitivity of the circadian clock to the oxidative stress (Zheng et al., 2007). Therefore studying FOXOs regulatory effect on the mammalian circadian clock will further help in understanding the mechanistic connection between the mammalian circadian clock, metabolism and aging.

In contrast to caloric restriction, DNA damage which accumulates with age, mutations caused by errors during DNA repair and environmental stimulus may lead to premature aging (Lombard et al., 2005; Saunders and Verdin, 2007). Our lab has shown that DNA-damaging agents can reset the phase of the mammalian circadian clock (Oklejewicz et al., 2008). Moreover the circadian genes *per1* and *tim* have been shown to play an important role in DNA-damage control (Gery et al., 2006; Unsal-Kac et al., 2005). Recently it has been shown that sirtuins are critical regulators at the crossroad between cancer and aging (Saunders and Verdin, 2007). They regulate the cellular responses to stress and ensure that damaged DNA is not propagated and that mutations do not accumulate. It was shown that SIRT2 is down-regulated in gliomas (Hiratsuka et al., 2003) and human brain tumors (Voelter-Mahlknecht, et al., 2005) suggesting a role of SIRT2 as a tumor suppressor, supporting its potential role as an inhibitor of cellular proliferation. It is known that certain types of cancer are strongly associated with obesity, a disease known to reduce life expectancy. Increasing evidence indicates that PPARγ, peroxisome proliferator-activated receptor γ, plays a role in cancer and obesity. PPARγ, is an important regulator of adipocyte development (Tontonoz et al., 1994). That link between cancer, obesity and the mammalian circadian clock via PPARγ has been made as it was shown that the level of PPARγ oscillates in a circadian manner (Fontaine, et al., 2003). This oscillation is altered in mice exposed to high-fat diet along with other clock transcription factors and expressions of the core clock genes (Kohsaka et al., 2007). Additionally PPARγ coactivator-1α (PGC-1α) was found to be deacetylated by SIRT1 in an NAD^+-dependent manner (Rodgers et al., 2005). Both PGC-1α and SIRT1 regulate metabolic state and circadian rhythm as PGC-1α along with RORα activates the *Bmal1* promoter (Liu et al., 2007). We have previously shown that SIRT2 represses transcription activity of PPARγ as SIRT2 deacetylates FOXO1

and promotes FOXO1's binding to PPARγ, in consequence SIRT2 inhibits adipocyte differentiation. Therefore the identification of SIRT2 as a new additional element regulating circadian rhythms via CRY association and deacetylation provides a new platform to study the connection between circadian metabolism, aging and cancer.

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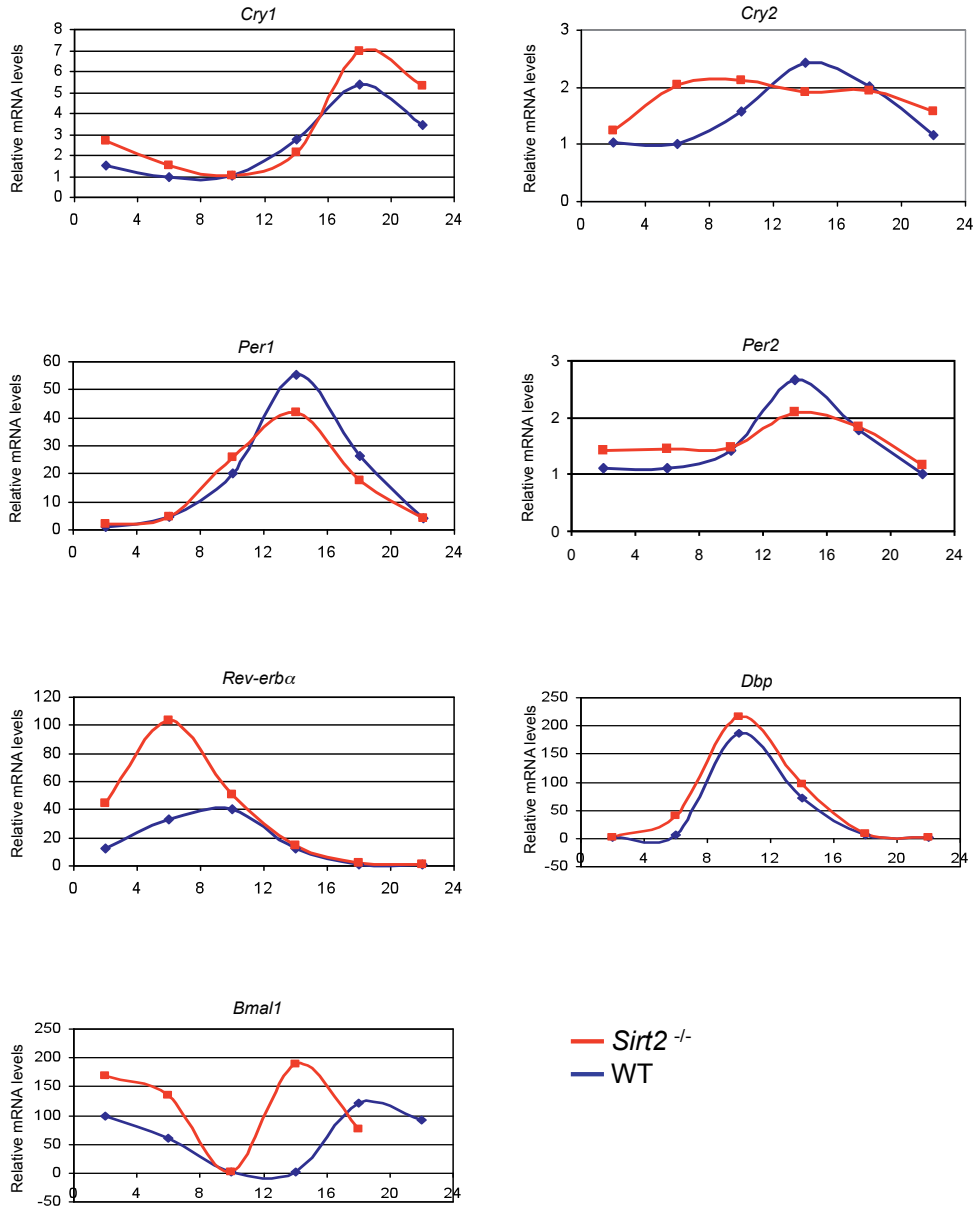
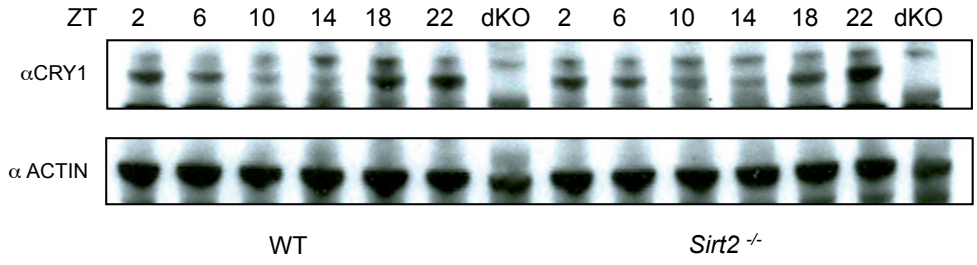


Supplementary Figure S1. SIRT2 influences expression of endogenous core clock genes
RNA was obtained from livers from wt and *Sirt2*$^{-/-}$ mice killed every 4h round the clock (n=1 per time point). The expression for all genes was normalized by comparing their expression with the expression of the control gene beta-2-microglobulin. For each the gene, the time point with the lowest expression was set to 1 and the other time points are relative to that.



Supplementary Figure S2. CRY1 protein level in the liver of SIRT2$^{-/-}$ animals
The CRY1 protein level in livers obtained from wild-type vs *Sirt2*$^{-/-}$ animals, sacrificed at 4h intervals around the clock was determined by western blot. Cry dKO livers are shown as negative controls.

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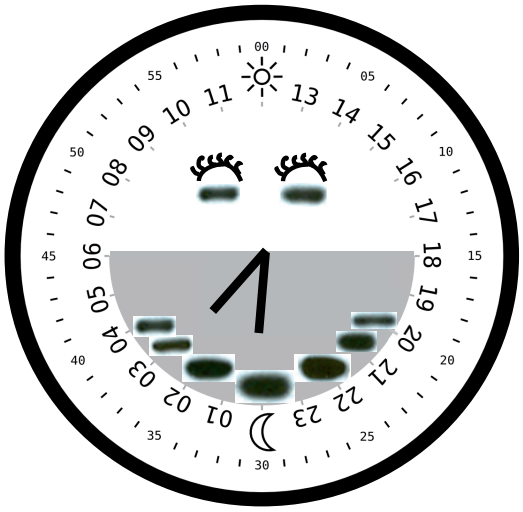
Appendix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List of abbreviations

(6-4)PP	pyrimidine (6-4) pyrimidine photoproducts
3'UTR	three prime untranslated region in mRNA
8-HDF	8-hydroxy-7,8-didemethyl -5-deazaflavin
FO	8-hydroxy-7,8-didemethyl--5-deazariboflavin
*At*CRY	*Arabidopsis thaliana* CRYptochrome
BMAL1	aryl hydrocarbon receptor nuclear translocator-like, also known as ARNTL, or MOP3,
BER	base excision repair
bHLH-PAS	basic-helix–loop-helix PER-ARNT-SIM transcription factor
CC	coiled-coil domain
CCG	circadian controlled genes
*At*CCT1	*Arabidopsis thaliana* CRY1 C-terminal domain
CKIe	casein kinase Iε
CLOCK	circadian locomotor output cycles kaput
COP1	constitutive photomorphogene 1
CPD	cyclobutane pyrimidine dimers
CRY	CRYryptochrome
Cry-DASH	cryptochrome - *Drosophila, Arabidopsis, Synechocystis,* human
CT	C-terminal tail
DBT	double time
dCRY	*Drosophila* CRYptochrome
DD	dark dark
DNA	deoxiribonucleic acid
DpCRY	*Danaus plexippus* CRYptochrome
EGFP	enhanced green fluorescent protein
ES	embryonic stem cells
extCT	extended C-terminal domain
FAD	flavin adenine dinucleotide
Fbxl21	F-box and leucine-rich repeat protein 21
Fbxl3	F-box and leucine-rich repeat protein 3
FOXO	Forkhead box O
FRET	fluorescent energy transfer
GSK-3β	glycogen synthase kinase 3β
H3K56	histone H3 at lysine 56
H4K16	histone H4 at lysine 16
HA	hemagglutinin
hCRY	human CRYptochrome

HY5	long hypocotyl 5
IN	input
IP	immunoprecipitaion
LD	light dark
MBP	maltose binding protein
mCRY	mammalian CRYptochrome
MDF	mouse dermal fibroblast
MEF	mouse embryonic fibroblast
mRNA	messenger ribonucleic acid
MTHF	5,10-methenyl-tetrahydrofolate
NAD^+	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NER	nucleotide excision repair
NES	nuclear export sequence
NLS	nuclear localisation sequence
ORF	open reading frame
PER	period
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
PHL	photolyases
PL	photolyases
PPARγ	peroxisome proliferator-activated receptor γ
PRC	phase response curve
*Pt*CPD PhL	*Potorous tridactylus* CPD-photolyase
*Pt*CPF	*Phaeodactylum tricornutum* photolyases/cryptochrome family
qPCR	quantitative polymerase chain reaction
REV-ERBα	reverse of ErbA alpha
RHT	retinohypothalamic tract
RORα	RAR-related orphan receptor-α
SCN	suprachiasmatic nucleus
SDS-PAGE	sodium dodecyl sulfate - poly acrylamide gel electroforeses
SIRT	sirtuin; silent mating type information regulation 2 homolog of *S. cerevisiae*
TIM	timeless
UV	ultraviolet light
WB	western blot
YFP	yellow fluorescent protein
ZT	*Zeitgeber* time

Summary

All biological clocks are adaptations to life on a rotating planet.
Colin Pittendrigh, undated

We live in a clockwork universe, therefore most living organisms have adapted by evolving their own internal clockwork, known as the biological clock, tuned to a 24-hour day/night cycle to adapt their behaviour, physiology and metabolism. The core oscillator of the mammalian circadian clock is composed of ingeniously designed auto-regulatory negative and positive transcription/translation feedback loops. In those loops CLOCK and BMAL1 transcriptionally activate the *Cryptochrome* (*mCry1* and *mCry2*) and *Period* (*mPer1*, *mPer2* and *mPer3*) genes. The protein products of these genes negatively regulate their own expression by inhibiting CLOCK/BMAL1, generating rhythms in gene expression with approximate 24-hour periodicity. Interestingly, whereas mammalian CRY proteins act as light-independent transcriptional inhibitors, plant and *Drosophila* CRY have a photoreceptor function.

Cryptochromes belong to the photolyase/cryptochrome flavoprotein family, members of which are thought to have the ability to capture light energy with the aid of one or two chromophores. Photolyases use this energy to repair ultraviolet light induced DNA lesions [(6-4)PP or CPD], whereas cryptochromes have lost the ability to repair DNA and instead are involved in another light-interactive process: (blue) light perception. Cryptochromes and photolyases share a structurally homologous core domain with 20-25% aa sequence identity with microbial photolyases and 40-60% aa sequence identity with *Arabidopsis thaliana* 6-4 photolyase. In addition to this core domain, cryptochromes have a unique C-terminal extension, which is not conserved amongst species. The function of the C-terminal domain of *Arabidopsis thaliana* and *Drosophila melanogaster* cryptochromes is to mediate phototransduction. In plants, intramolecular interactions between the C-terminal domain and the core domain have been shown to induce a stable tertiary structure in the C-terminal domain. The intriguing question whether in evolution there was a common ancestor with both functions (repressor and DNA repair) and whether there is a naturally occurring photolyase that can (partially) function in the circadian clock remains to be answered.

Recently, we have performed a functional analysis of the C-terminal domain of mammalian CRY1. We have shown that the C-terminal extension of mCRY1 contains a nuclear localization signal and a putative coiled-coil domain, both involved in regulating the subcellular localization (nuclear vs. cytoplasmic) of the shuttling mCRY1/mPER2 complex. Furthermore the C-terminus is necessary, but not sufficient for inhibition of CLOCK/BMAL1 mediated transcription (the prime function of mCRY proteins in the core oscillator). Interestingly, when the C-terminus of mCRY1 is fused to

a plant photolyase, the fusion protein gains this key clock function. Thus the C-terminal extension of cryptochrome seems to be very important for the clock function of mCRY proteins. Yet, the C-terminal domain of cryptochromes has not been crystallized and structural information is lacking. Moreover, our attempts and those of others to purify mammalian cryptochromes at a large scale have been unsuccessful thus far.

Phylogenetic analysis of the amino acid sequence of the core domain of all members of the photolyase/cryptochrome family indicates a clear relationship, yet they are functionally divergent. **Chapter 2** is focusing on two novel photolyase (phr) genes identified in plusiine-infecting group II nucleopolyhedroviruses (NPVs), *Chrysodeixis chalcites* (*ChCh*NPV), called respectively *Cc-phr1* and *Cc-phr2*. Expression of *Cc*-phr2 but not *Cc*-phr1 confers a photolyase deficient *E. coli* strain with photoreactivating activity. We overexpressed, purified and analysed *Cc*-phr2 photolyase. Spectral measurements indicated the presence of the flavin chromophore (FAD) in *Cc*-phr2 photolyase and the reconstitution study with FO (functions as the antenna chromophore) suggests that *Cc*-phr2 is an 8-HDF type photolyase. In this study we demonstrate for the first time that a functional photolyase is encoded by an insect virus.

To learn more about the functional diversity between cryptochromes and photolyases and whether in evolution there was a common ancestor of the photolyase/cryptochrome family with both functions, we investigated the effect of two different photolyases on the circadian oscillator, both *in vitro* and *in vivo*. In **Chapter 3**, using earlier generated and described transgenic mice expressing the *Arabidopsis thaliana* (6-4)PP-photolyase and/or the *Potorous tridactylus* CPD-photolyase, we demonstrate that, in contrast to (6-4)PP-photolyase, the CPD-photolyase, despite being a more distant relative of mCRY1, can function in the molecular oscillator. Transgenic mice, which carry this protein, show an altered free-running period of locomotor activity, which is approximately 20 minutes shorter than that of wild type mice. Furthermore, the CPD-photolyase affects the amplitude of circadian oscillation in cultured cells and is capable of inhibiting CLOCK/BMAL1 driven transcription. Our unexpected finding lets us postulate that *Potorous tridactylus* CPD photolyase might represent a missing link in the evolution of the cryptochrome/photolyase family.

To further investigate the function of mammalian cryptochromes using biochemical analyses, we tested whether mCRY1 and mCRY2 homo- or heterodimerize. In **Chapter 4** show that mCRY1 and mCRY2 do not homo- or heterodimerize, suggesting that mammalian cryptochromes do not require homo- or heterodimerization for their functioning.

A novel knock-in mouse model which expresses a mutant version of *mCry1* lacking the Coiled-Coil domain, *Cry1ΔCC*, helped us to gain more knowledge about the functionality of mammalian CRY1 protein *in vivo*. The circadian behavioural analyses of those mice, presented in **Chapter 5**, suggest that although the Coiled-Coil domain of CRY1 is required to generate oscillations with a correct period and maintain

rhythmicity in an *mCry2-deficient* background (*mCry1*$^{\Delta CC/\Delta CC}$*mCry2*$^{-/-}$ mice), it is not required to maintain oscillations in peripheral tissues synchronized *ex vivo*.

Finally, in the study presented in **Chapter 6** we used purified extCRY1CT and extCRY2CT domains (both fused to the MBP protein for efficient purification) to probe mouse tissue (kidney) lysates coupled with MALDI-TOF mass spectrometry to identify proteins interacting with the CTs of mCRY1 and mCRY2. We found the NAD^+-dependent deacetylase SIRT2 as a novel cryptochrome partner. Furthermore, we showed that mCRY proteins are acetylated and that SIRT2 reduced CRY-acetylation. Importantly, we demonstrate that SIRT2 modulates the period of the mammalian circadian clock *in vitro*. Coupled with our data suggesting that SIRT2 activity oscillates, these findings provide a new platform to study the connections between the circadian clock and metabolism and possibly other SIRT2 dependent processes.

In conclusion, the results presented in this thesis have increased our insight into the molecular mechanism underlying cryptochrome function and our insight in the evolutionary link between cryptochromes and photolyases.

Samenvatting

Alle biologische klokken zijn adaptaties aan het leven op een roterenden Aarde.
Colin Pittendrigh, ongedateerd

We leven in een wereld die dagelijks verandert. Om hiermee om te gaan hebben veel organismen een eigen interne klok ontwikkeld, de biologische of circadiane klok, waarmee ze hun gedrag, metabolisme en allerlei fysiologische processen kunnen aanpassen aan de dagelijkse dag/nachtcyclus. In zoogdieren wordt de klok aangestuurd door een ingenieus moleculair mechanisme bestaande uit zelfregulerende negatieve en positieve transcriptie/translatie terugkoppelingscycli waarin de CLOCK en BMAL1 eiwitten de genexpressie verhogen van de *Cryptochroom* (*mCry1* en *mCry2*) en *Period* (*mPer1*, *mPer2* en *mPer3*) genen. De eiwitproducten van deze genen verlagen hun eigen expressie door CLOCK/BMAL1 te inhiberen waardoor ritmes in genexpressie onstaan met een periodiciteit van ongeveer 24 uur. In tegenstelling tot planten (*Arabidopsis thaliana*) en fruitvliegjes (*Drosophila melanogaster*), waar CRY als een fotoreceptor functioneert, functioneert CRY in zoogdieren als een (negatieve) transcriptionele regulator, onafhankelijk van licht.

Cryptochromen behoren tot de fotolyase/cryptochroom flavo-eiwit familie, waarvan de leden worden gekenmerkt door hun capaciteit om lichtenergie op te vangen met behulp van een of twee chromoforen. Fotolyases gebruiken deze energie om door UV geïnduceerde DNA-schade te herstellen [(6-4)PP of CPD], terwijl cryptochromen deze functie verloren hebben en in plaats daarvan betrokken zijn bij (blauw) lichtwaarneming. Cryptochromen en fotolyases delen een structureel vergelijkbare kern waarin 20-25% van de aminozuren hetzelfde zijn als in microbiele fotolyase en 40-60% als in plant 6-4 fotolyase. Naast dit fotolyase domein hebben cryptochromen een speciale C-terminale (CT) extensie die uniek is in de verschillende organismen. In planten en fruitvliegjes zorgt deze CT extensie voor lichttransductie. In planten veroorzaken intramoleculaire interacties tussen het CT domein en het kerndomein een verandering in de structuur van het CT domein. De intrigerende vragen of er ergens in de evolutie een gemeenchappelijke voorloper was die beide functies had (lichtwaarneming en DNA-herstel) en of er een natuurlijke fotolyase is die in de klok kan functioneren, blijven vooralsnog onbeantwoord.

Recentelijk hebben we een functionele analyse van het CT-domein van zoogdier CRY1 uitgevoerd. Hier kwam uit dat het CT-domein twee sequenties bevat die samen de localisatie van het mCRY1/mPER2 complex binnen een cel (cytoplasma of kern) reguleren. Verder werd gevonden dat het CT-domein nodig is voor inhibitie van door CLOCK/BMAL1 gestimuleerde transcriptie (de voornaamste functie van de mCRY eiwitten). Het CT-domein alleen volstaat echter niet. Wanneer het CT-domein van mCRY1 gefuseerd wordt aan plant fotolyase, dan krijgt dit nieuwe

fusie eiwit het vermogen om als klokeiwit te functioneren. Derhalve kan geconcludeerd worden dat het CT-domein van zoogdier cryptochromen zeer belangrijk is voor het functioneren binnen de klok van deze eiwitten. Desondanks is het CT-domein van cryptochromen nog niet gekristalliseerd en ontbreekt structurele informatie. Onze pogingen en die van anderen om zoogdier cryptochromen op grote schaal te zuiveren voor structurele analyse zijn tot nu toe niet geslaagd.

Vergelijking van de aminozuursequentie van alle fotolyase/cryptochroom familieleden wijzen op een duidelijke relatie, niettemin zijn er sterkte functionele verschillen. **Hoofdstuk 2** richt zich op twee nieuwe fotolyase genen die geïdentificeerd zijn in de plusiine-infecterende groep II nucleopolyhedrovirussen (NPV), *Chrysodeixis chalcites* (*ChCh*NPV), genoemd *Cc-phr1* and *Cc-phr2*. We hebben *Cc*-phr2 tot overexpressie gebracht, opgezuiverd en geanalyseerd. Expressie van *Cc*-phr2, maar niet *Cc*-phr1, verleent fotoreactivatie activiteit aan een fotolyase-negatieve *E. coli* soort. Spectrale metingen wijzen op de aanwezigheid van de flavine chromofoor (FAD) in *Cc*-phr2 fotolyase en reconstitutiestudies met FO (functioneert als de antennechromofoor) suggesteren dat *Cc*-phr2 een 9-HDF type fotolyase is. Hiermee tonen we voor het eerst aan dat een virus een functionele fotolyase heeft.

Om meer te leren over de functionele diversiteit tussen cryptochromen en fotolyases en om te bepalen of er een gemeenschappelijke voorloper was van de fotolyase/cryptochroom familie met de functie van beide, hebben we het effect bestudeerd op de klok van twee verschillende fotolyases, zowel *in vitro* als *in vivo*. In **hoofdstuk 3**, gebruik makend van eerder gegenereerde en bestudeerde transgene muizen die *Arabidopsis thaliana* (6-4)PP-fotolyase en/of de *Potorous tridactylus* CPD-fotolyase tot expressie brengen, laten we zien dat, in tegenstelling tot de *Arabidopsis thaliana* (6-4)PP-fotolyase, de CPD-fotolyase, ondanks dat deze verder van mCRY1 staat, kan functioneren in de klok. Transgene muizen met dit gen hebben een gewijzigde periode van activiteit die 20 minuten korter is dan gewone muizen. Verder kan de CPD-fotolyase de amplitude van circadiane oscillaties beïnvloeden en CLOCK/BMAL1 activiteit verminderen in gekweekte cellen. Gebaseerd op deze onverwachte resultaten kunnen we postuleren dat *Potorous tridactylus* CPD wellicht een ontbrekende schakel is in de evolutie van de cryptochroom/fotolyase familie.

Om het functioneren van zoogdier cryptochromen verder te onderzoeken met behulp van biochemische technieken, hebben we getest of mCRY1 en mCry2 homo- of heterodimeriseren. In **hoofstuk 4** laten we zien dat mCRY1 en mCry2 niet homo- of heterodimeriseren, dit suggereert dat het functioneren van mCRY1 en mCRY2 niet afhankelijk is van homo- en heterodimerisatie.

Een nieuw muismodel waarin het originele *mCry1* gen vervangen is door een mutante versie van *mCry1* zonder het Coiled-Coil domein, *mCry1ΔCC*, heeft ons meer inzicht verschaft in het functioneren van het mCRY1-eiwit *in vivo*. De analyse van het circadiane gedrag van deze muizen, gepresenteerd in **hoofdstuk 5**, suggereert dat

hoewel het Coiled-Coil domein van mCRY1 nodig is om oscillaties te genereren met een correcte periode en om oscillaties te behouden in een *mCry2-deficiënte* omgeving (*mCry1*$^{\Delta CC/\Delta CC}$*mCry2*$^{-/-}$ muizen), is het niet noodzakelijk voor het behouden van oscillaties in perifere organen *ex vivo*.

Ten slotte, in het onderzoek gepresenteerd in **hoofstuk 6** hebben we gezuiverde extCRY1CT en extCRY2CT domeinen (beide gekoppeld aan het MBP-eiwit for efficiënte zuivering) gebruikt om in extracten van muizenorganen (nier) te onderzoeken welke eiwitten aan de CT-domeinen van mCRY1 en mCRY2 binden. Deze eiwitten werden geïdentificeerd met behulp van MALDI-TOF massaspectrometrie. We hebben de NAD^+-afhankelijke deacetylase SIRT2 als nieuwe cryptochroom gevonden. Verder hebben we laten zien dat de mCRY eiwitten geacetyleerd zijn en dat deze acetylatie verminderd wordt door SIRT2. SIRT2 moduleert de periode van circadiane oscillaties in vitro. Gekoppeld aan onze bevindingen die suggereren dat SIRT2-activiteit oscilleert, vormen deze observaties een nieuw platform om de connecties tussen de circadiane klok en metabolisme te onderzoeken en wellicht andere SIRT2-afhankelijke processen.

Concluderend kunnen we stellen dat de in dit proefschrift gepresenteerde resultaten ons inzicht hebben bevorderd in het moleculaire mechanisme achter de functie van cryptochromen en in de evolutionaire link tussen cryptochromen en fotolyases.

Translated by PhD E. Destici

Podsumowanie

Wszystkie biologiczne zegary są ewolucyjnym wyrazem przystosowania się do życia na obracającej się Ziemi.
Colin Pittendrigh, niedatowane

Wszystkie organizmy zamieszkujące naszą planetę Ziemię zsynchronizowały swój rozwój, fizjologię i metabolizm do 24-ro godzinnego cyklu dnia i nocy, rozwijając swój własny 24-ro godzinny zegar, zwany zegarem biologicznym. U ssaków, centralny zegar biologiczny oparty jest na zasadzie autonomicznego transkrypcyjnie/translacyjnego negatywnego i pozytywnego sprzężenia zwrotnego. Podstawowymi elementami regulujacymi owe sprzężenia są czynniki transkrypcyjne CLOCK i BMAL1, które aktywują transkrypcję genów zwanych: *Cryptochrome* (*mCry1* i *mCry2*) i *Period* (*mPer1, mPer2 i mPer3*). Produkty tych genów (białka mCRY i mPER), negatywnie regulują swoją własną ekspresję poprzez represje. Owa represja, uniemożliwia czynnikom transkrypcyjnym CLOCK/BMAL1 inicjację transcrypcji. W ten sposób, ekspresja genów zegara biologicznego przybiera charakterystyczną 24-ro godzinną oscylację. Należy zaznaczyć, że białko zwane kryptochromem (mCRY) u ssaków funkcjonuje niezależnie od światła jako białko represorowe podczas gdy u roślin (*Arabidopsis thaliana*) i muszki owocowej (*Drosophila melanogaster*) funkcjonuje dodatkowo jako fotoreceptor.

Kryptochromy należą do rodzinny białek zwanych fotoliaza/kryptochrom (PhL/CRY), w skład której wchodzą białka posiadające zdolność do absorpcji enegri świetlnej z udziałem jednego lub dwóch flowoproteinowych chromoforów. Fotoliaza pod wpływem światła widzialnego usuwa indukowane promieniami ultrafioletowymi uszkodzenia DNA [dimery pirymidynowe: (6-4)PP lub CPD]. Podczas gdy kryptochrom utracił zdolność do naprawy uszkodzeń DNA, białko to jest receptorem światła niebieskiego, składowej światła widzialnego. Kryptochrom i fotoliaza posiadają wspólną, strukturalnie homologiczną, centralną domenę. Owa domena, jest homologiczna w 20-25% do bakteryjnej fotoliazy i w 40-60% do *Arabidopsis thaliana* 6-4 fotoliazy. Poza centralną domeną, dodatkowo kryptochromy posiadają wyjątkowy i charakterystyczny C-końcowy fragment, który nie wykazuje żadnego podobieństwa do znanych dotąd polipeptydów. U *Arabidopsis thaliana* i *Drosophila melanogaster* C-końcowy fragment kryptochromu zapewnia transdukcje sygnału świetlnego. Niedawno zaprezentowano, że u roślin intramolekularne oddziaływanie pomiędzy C-końcowym fragmentem a centralną domeną kryptochromu zapewnia stałą trzeciorzędową structurę C-końcowego fragmentu. Intrygujące pytanie: czy podczas ewolucji było białko wczesne posiadające obie funkcje (białko represorowe i DNA naprawcze) jak rownież czy istnieje fotoliaza, która spełnia (cześciowo) funckję represorawą w zegarze biologicznym, pozostaje jak dotąd bez odpowiedzi.

W naszym laboratorium, przeprowadziliśmy szczegółową analizę funkcjonalną C-końcowego fragmentu mysiego kryptochromu. Wykazaliśmy, że C-końcowy fragment kryptochromu (mCRY) posiada sygnał lokalizacji jądrowej (NLS) i Coiled-coil (CC) domenę; obie domeny są odpowiedzialne za wewnątrzkomórkową lokalizację (jądro vs cytoplazma) mCRY1/mPER2 compleksu. Co więcej, C-końcowy fragment kryptochromu jest niezbędny ale nie wystarczający do represji czynników transktypcyjnych CLOCK/BMAL1 inicjujących transckrypcję (co jest główną, mechanistyczną funcją mysiego kryptochromu). W przeciwieństwie do oczekiwanego wyniku, podczas gdy C-końcowy fragment kryptochromu połączony jest do roślinnej fotoliazy, owa chimera białkowa odzyskuje na nowo kluczową funkcję kryptochromu. Tak więc, C-końcowy fragment kryptochromu jest bardzo ważną domoną odpowiedzialną za poprawne funkcjonowanie mCRY białka. Jak do tej pory, C-końcowy fragment kryptochromu nie został wykrystalizowany tak więc nie posiadamy strukturalnej informacji. Dodatkowo, pomimo starań wielu grup - w tym naszej, oczyszczenie mysiego kryptochromu na dużą skalę pozostaje bez sukcesu.

Na podstawie drzewa genealogicznego, analiza aminokwasowej sekwencji centralnej domeny obecnej u wszystkich białek należących do rodzinny PhL/CRY, wykazuje na wyraźny związek pomiędzy fotoliazą a kryptochromem, pomimo to białka te są funkcjonalnie bardzo zróżnicowane.

Rozdział 2 opisuje jeden z dwóch nowych genów fotoliazy (*phr*) zidentyfikowanych u *Chrysodeixis chalcites* bakulowirusów (specyficzne owadzie wirusy), nazwanych odpowiednio *Cc-phr1* and *Cc-phr2.* Ekspresja białka *Cc*-phr2 lecz nie *Cc*-phr1 u szczepu *E. coli* nie posiadającego fotoliazy, powoduje że bakterie nabywają fotoreaktywną aktywność. Dlatego też, do dalszych badań (ekspresja, oczyszczanie, analiza) użyliśmy *Cc*-phr2 fotoliazę. Spektralne pomiary *Cc*-phr2 fotoliazy, wykazały obecność flawoproteinowego chromoforu FAD a udana rekonstytucja z FO (chromofor funkcjonujący jako antena) sugeruje, ze *Cc*-phr2 fotoliaza należy do grupy 8-HDF photoliaz. W tym rozdziale, po raz pierwszy prezentujemy, że funkcyjna fotoliaza jest kodowana przez wirusa owadziego.

Aby poszerzyć naszą wiedzę na temat funkcjonalnego zróżnicowania pomiędzy kryptochromen i fotoliazą jak również odpowiedzieć na pytanie czy podczas ewolucji było obecne wczesne białko (przodek) w rodzinie PhL/CRY pełniące obie funkcje, badaliśmy w tym celu efekt dwóch różnych fotoliaz w kontekście zegara biologiczego zarówno *in vitro* jak *in vivo*. W **rozdziale 3** przeprowadziliśmy badania na wcześniej powstałych i opisanych transgenicznych myszach ekspresujących geny: *Arabidopsis thaliana* (6-4)PP-fotoliazy oraz *Potorous tridactylus* CPD-fotoliazy pod kontrolą beta aktyny lub keratynocyto-specyficznego K14 promotora. Wykazaliśmy, że CPD-fotoliaza [daleki przodek mysiego kryptochromu w porównaniu z (6-4)PP fotoliazą] pełni funkcję podobną do kryptochromu zegara biologicznego. Transgeniczne myszy z CPD-fotoliazą mają uszkodzony okres swego zegara biologicznego (“free-running period

of locomotor activity") tak że, jest on około 20 minut krótszy od charakterystycznej dla dzikiej myszy aktywności. Dodatkowo badania na hodowlach komórkowych wykazały, że CPD-fotoliaza ma efekt na amplitudę oscylacji zegara biologicznego poprzez represje czynników transkrypcyjnych CLOCK/BMAL1 inicjujących transkrypcję. Na podstawie naszych nieoczekiwanych wyników, postulujemy, ze *Potorous tridactylus* CPD fotoliaza być może jest przodkiem rodziny PhL/CRY.

W **rozdziale 4** opisujemy, jak za pomocą dostępnych nam biochemicznych metod wykazaliśmy, że homo- lub heterodimeryzacja nie jest wymagana do poprawnego funcjonowania kryptochromów (mCRY1 i mCRY2).

Nowo zgenerowany *mCRY1*$^{\Delta CC}$ mysi model, pomógł nam poszerzyć nasza wiedzę na temat funcji mysiego kryptochromu *in vivo*. Nasze analizy zeprezentowane w **rozdziale 5** sugerują, że CC domena kryptochromu jest wymagana do utrzymania odpowiedniej oscylacji zegara biologicznego jak rownież do utzymania oscylacji u myszy nie posiadających białka mCRY2. W przeciwieństwie do tych wyników, ta domena (CC) kryptochromu nie jest niezbędna do utrzymania oscylacji w peryferyjnych tkankach (skóra) zsynchronizowanych *ex vivo*.

W **rozdziale 6** używając oczyszczonych MBP-extCRY1CT i MBP-extCRY2CT zimmobilizowanych do nośnika białek, przeprowadziliśmy oczyszczanie na kolumnie. Za pomocą, MALDI-TOF spektrometrii masowej, używając mysich tkanek (nerki) zidentyfikowaliśmy nowe białka oddziałujące z C-końcowy fragmentem ktyptochromu 1 i 2. Znaleźliśmy, NAD^+-zależną deacetylazę SIRT2 jako nowego partnera oddziałującego z kryptochromem. Jako pierwsi pokazaliśmy, że kryptochromy są acetylowanymi białkami i że SIRT2 jest odpowiedzialny za jego deacetylację. Wreszcze wykazaliśmy, że SIRT2 moduluje oscylację mysiego zegara biologicznego *in vitro.* Nasze badania mogą stać się nową platformą do studiowania zwiazku pomiedzy zegarem biologicznym a metabolizmem.

Podsumowując, zawarte badania w prezentowanej pracy, poprzez przeprowadzenie wnikliwej analizy funkcjonalnej kryptochromu i badań ewolucyjnego zwiazku pomiedzy kryptochromen i fotoliazą, mają na celu poszerzenie wiedzy o molekularnym mechaniźmie zegara biologicznego.

Curriculum vitae

Name:	Monika Iwona Bajek-Vennix
Date of birth:	14.03.1977
Place of birth:	Stalowa Wola, Poland
Nationality:	Polish

1996-2001	Student of Maria Curie-Skłodowska University in Lublin, Poland, Faculty of Biology, Department of Biochemistry, Specialization – Molecular Biology
1997-2000	Member of Student's Biochemical Society
2000-2001	Master degree prepared at Laboratory of Oxidative DNA Damage Department of Molecular Biology, Institute of Biochemistry and Biophysics Polish Academy of Science – Warsaw, Poland Promoter: Prof.dr. B. Tudek / Prof.dr J.T. Kuśmierek
2002-2003	Researcher in the Department of Radiation Genetics and Chemical Mutagenesis, LUMC, Leiden, The Netherlands Promoter: Prof.dr. M. Zdzienicka / Prof.dr. L.H.F. Mullenders
2003- 2009	PhD Student at at the Department of Genetics, Chronobiology and Health Researcher Group, Erasmus MC, Rotterdam, The Netherlands Promoters: Prof.dr. G.T.J. van der Horst Prof.dr. J.H.J. Hoeijmakers
2009 (Feb-Mar)	Visiting Scientist at the lab of Prof.dr. Qiang Tong - Children's Nutrition Research Center, Baylor College of Medicine, Houston, USA

Awards

2000-2001	I was granted a fellowship for one year by the Polish Ministry of Education for excellent students

List of publications

Bajek M., Cieśla J.M. and Tudek B.
2002 Opposite base specificity in excision of pyrimidine ring-opened 1,N^6-ethenoadenine by thymine glycol-DNA-glycosylases. *DNA Repair* **3**:251-7.

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Bajek M.I., Destici E., Chaves I., Eker A.P., Wang F., Tamanini F., Demmers J., Tong Q. and Horst G.T.J.van der
SIRT2 modulates the speed of the mammalian circadian clock via Cryptochromes. *Manuscript in preparation.*

ERASMUS UNIVERSITEIT ROTTERDAM

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: **M.I. Bajek** Erasmus MC Department: Genetics, Chronobiology and Health Researcher Group Research School: MGC	PhD period: 2003-2009 Promoters: G.T.J. van der Horst J.H.J. Hoeijmakers

PhD training	**Year**
General academic skills	
- Laboratory animal science (Article 9)	2003
- Risk and quality management in laboratories	2003
- Biomedical English Writing and Communication	2005
Research skills	
- Confocal course, Rotterdam	2007
In-depth courses (e.g. Research school, Medical Training)	
- Molecular and Cell Biology	2004
- From Development to Disease	2004
- Reading and discussing Literature	2005
- Transgenesis and Gene targeting	2005
- In Vivo Imaging "From Molecule to Organism"	2006
Presentations	
- Centre for Timing Research Meeting, Rotterdam Presentation's titel: m SIRT2 a novel mCRY1 binding partner	2008
International conferences	
- MGC/Cancer UK PhD meeting, Lille, France	2004
- MGC/Cancer UK PhD meeting, Liège, Belgium	2005
- MGC/Cancer UK PhD meeting, Oxford, UK	2006
- Gordon Research Conference, Photosensory Receptors & Signal Transduction, Il Ciocco, Lucca, Italy Poster's title: " Structural analysis of mammalian Cryptochromes"	2006
- SRBR Meeting, Chronobiology, Sandestin, Destin, Florida Poster's title: "Functional analysis of mCRY1: in vitro vs. in vivo"	2008
- Gordon Research Conference, Chronobiology, Salve Regina University, Newport, RI, USA Poster's title: SIRT2 regulates mammalian circadian rhythms via cryptochromes	2009
Seminars and workshops	
- Autumn school, Circadian Clocks, Berlin	2003
Supervising practicals	
- HBO student supervision, Rotterdam	2009
PhD student supervision, Wageningen University	2009
Organisation skills	
- EPAR, The PhD candidate Association of the Erasmus University Rotterdam, board member	2005-2006

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Nic dwa raz się nie zdarza
i nie zdarzy. Z tej przyczyny
zrodziliśmy sie bez wprawy
i pomrzemy bez rutyny.

Wiesława Szymborska

Nothing can ever happen twice.
In consequence, the sorry fact is
that we arrive here improvised
and leave without the chance to practice.

Translated by Clare Cavanagh and Stanisław Barańczak

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Monika