Biochemical studies of the clock proteins BMAL1 and CRYs in *Xenopus*: Regulation of the CRYs subcellular localization and implications on their role in the modulation of the circadian loop

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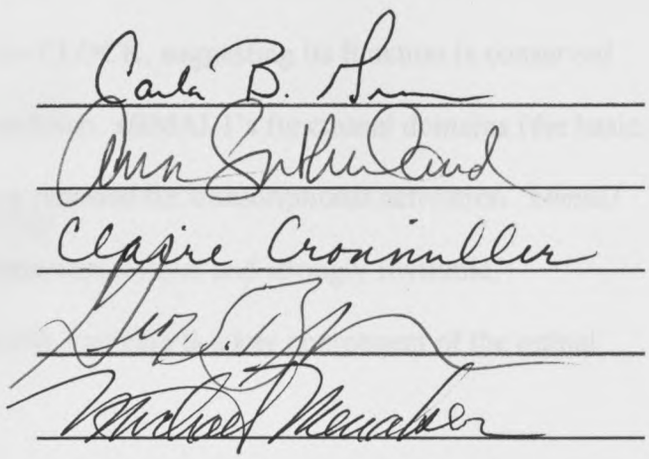

Abstract

The photoreceptor layer in the retina of *Xenopus laevis* harbors a circadian clock. This model system displays a wide array of rhythms, including melatonin release, electroretinograms rhythms and retinomotor movements, suggesting that the ocular clock is important for proper retinal function. Many molecular components known to drive the molecular circadian clock in other organisms have been identified in *Xenopus*, such as *xClock*, *xPers*, *xCry1* and *xCry2b*, and, as described in this dissertation, *xbmal1*, demonstrating phylogenetic conservation. The development of the transgenic technique in *Xenopus*, which leads to the fast generation of transgenic tadpoles, allows precise and reliable molecular perturbations, since it is possible to follow rhythms in eyecups obtained from adults or tadpoles. Because of these reasons, I have investigated the function of two key clock components, xBMAL1 and xCRY1 and xCRY2b, in the *Xenopus* retina. These components are part of the activation (BMAL1) and repression (CRYs) branch of the mammalian molecular circadian loop. In mammals, CLOCK and BMAL1 heterodimerize and activate transcription of clock genes and are then repressed by the CRYs; it is not known how the two CRYs contribute differently to repression, but this contribution is known not to be redundant. I have found that xBMAL1 is a transcription factor and that it binds to xCLOCK, suggesting its function is conserved between mammals and *Xenopus*. In addition, xBMAL1's functional domains (the basic, helix-loop-helix and PAS domains) are required for transcriptional activation. *xbmal1* mRNA is highly expressed in the photoreceptor layer and strongly rhythmic, strengthening the hypothesis that xBMAL1 protein is a key component of the retinal

clock. The xCRYs have previously been shown to repress xCLOCK/xBMAL1-mediated transcriptional activation in tissue culture. Here, I describe the characterization of nuclear localization signals present in the C-termini of the proteins. By generating several C-terminal mutants, I have shown that the NLSs are differentially regulated between xCRY1 and xCRY2b. xCRY2b contains a canonical NLS, which is regulated by phosphorylation, while xCRY1's NLS does not resemble any known sequence. These results offer a possible explanation of how these two proteins contribute differently to the molecular loop. *Xenopus* offers an excellent system to study the *in vivo* effects on the circadian system of xBMAL1 and xCRYs mutants.

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Chapter I

Introduction: the circadian clock and two of its molecular components, BMAL1 and CRY

Introduction

All biological processes adapt to their environment. In order to deal with the transition between night and day, organisms have developed circadian rhythms, which have become part of the biology of most, if not all, organisms on Earth. Circadian rhythms, from the Latin *circa diem* -almost one day- are biological daily oscillations. As the Earth rotates on its axis, the environment experiences dramatic changes in light intensity, temperature, weather patterns. Organisms have evolved to take advantage of these very changes, to use the sun as a source of energy, food, direction, or to use the cover of night for protection, predation and recovery. Specifically, evolution gave life a clock, a molecular timing mechanism set to the duration of one revolution of the Earth around its axis, 24 hours.

The necessity for an intrinsic clock is not immediately apparent, since changes in light intensity between the day and night could theoretically suffice as a regulatory timing mechanism. Nevertheless, the clock is not simply a clock, but it is part of the organism itself, regulating its biology as a whole, from brain function to metabolism, behavior, body temperature, hormone levels, cellular morphology, proteins and RNA levels and enzymatic activity. Anticipation of sunrise or sunset provides the organism with a necessary advantage, with a sense of actual time even in conditions of low sunlight, such as shade, overcast weather, or darkness in a cave or a burrow. If organismal physiology depended solely upon presence or absence of sunlight, it would fluctuate dangerously multiple times each day, wasting metabolic energy and leading to vulnerability. Since life forms from bacteria to mammals have a clock, and since day and night were always

present in Earth's life history, we can assume clocks evolved very early and most likely multiple times.

Evolution of Clocks

The primordial Earth was subjected to daily fluctuations of considerable amplitude, such as temperature, UV light intensity and radiations: anticipating these changes could protect the life forms. Clocks might have evolved to protect the organism from these changes, becoming intertwined with the organism's physiology (Pittendrigh, 1993; Gehring and Roshbash, 2003).

Examples of the advantages a clock gives to the organism are numerous: nocturnal predators are renowned for carrying out most of their hunting at dusk, when their prey is most vulnerable. Diurnal birds start their calls long before the sun has risen. Testing the fitness and advantage provided by circadian rhythms has been difficult, because natural behavior is not easily reproduced in a laboratory setting, forcing scientists to perform these experiments in the field, where the parameters are much more difficult to control (reviewed in Sharma, 2003). The strongest data supporting the adaptive value of circadian clocks come from *Arabidopsis thaliana*, *Drosophila melanogaster*, cyanobacteria, and mammals. Clock-disrupted cyanobacterial strains grown in competition with strains with a functional biological clock are out-competed: this advantage disappears in a non-rhythmic environment. In addition, cyanobacterial strains with different circadian periods compete most efficiently in an environment when the frequency of their internal biological oscillator and that of the environment are similar (Woelfle *et al.*, 2004). In *Arabidopsis*, loss of the Circadian Clock Associated 1 gene

(CCA1) causes lower viability under short-day conditions than the wild type counterparts (Green *et al.*, 2002). In addition, naturally occurring variations in the *Arabidopsis* circadian clock show that the period length is correlated with the day length at the latitude of origin, implying that a corrected regulated timing has important adaptive significance (Michael *et al.*, 2003). In *Drosophila*, males lacking a functional clock show a significant decline in the amount of sperm released from the testes to the seminal vesicles, resulting in approximately 40% fewer progeny than from wild type flies (Beaver *et al.*, 2002). The necessity of a circadian clock for survival was assessed in a study on chipmunks. SCN-lesioned chipmunks were released into their natural habitat and monitored for survival and reproduction. Although the sample size was small, the researches noticed higher mortality in the SCN-lesioned group versus the control group (DeCoursey and Krulas, 1998). These results demonstrate the adaptive advantage of circadian rhythms.

The importance of anticipating change is true not only for the organism as a whole, but also for individual tissues. For example, the *Xenopus laevis* retina contains an endogenous clock (Besharse and Iuvone, 1983). The *Xenopus* photoreceptor layer, the retinal cell layer responsible for vision, is comprised of rods and cones. Dark-mediated cone elongation and light-mediated rod disc shedding are the rhythmic cellular behaviors demonstrated in *Xenopus* (reviewed by Besharse, 1982; Besharse *et al.*, 1988; Cahill and Besharse, 1995). Cones elongate at night and contract during the day, while rods shed their outer segments shortly after dawn. These adaptive behaviors exhibit light-induced and circadian characteristics: they are acutely induced by light, and they persist in constant darkness (reviewed by Besharse *et al.*, 1988), demonstrating that they are clock-

controlled. A subset of photoreceptive cells, the rod cells, are active at night, while cone cells are active during the day. Retinas must adapt to the coming sunrise or sunset, to prepare the organism for day or night vision. Changes in tissue morphology happen in a matter of minutes at best, most times in a matter of hours. Anticipation, again, prepares the organism and promotes survival (reviewed in Anderson and Green, 2000). How did evolution design a biological clock? Which properties does a clock need to maintain accuracy and stability? How do you create a 24-hour rhythm or, more accurately, a *circadian rhythm*, a cycle of *approximately* 24 hours?

General Properties of the Clock

The characteristics of circadian rhythms can be defined in biophysical terms (reviewed in Menaker, 1969; Pittendrigh, 1981a,b). As already mentioned, all clocks are endogenous; they display a period of approximately 24 hours and a phase that defines the location in time of the peak and the trough (reviewed in Menaker, 1969). In the absence of light cues, a nocturnal mouse will still display locomotor activity during the "subjective" night and will do so with a period slightly shorter than 24 hours.

Endogenous period length unveiled under constant conditions, or *free running period*, fluctuates slightly between organisms, demonstrating its endogenous nature. Light can "reset" the clock and synchronize it to the current light cycle. Our physiology adjusts to short days and longer nights during the winter and to long days and shorter nights during the summer. Nevertheless, the intrinsic period remains constant and can be revealed again in constant conditions. Circadian biologists refer to the light/dark cycle as Zeitgeber Time (ZT time), where, in a 12 hour light-12 hour dark cycle, ZT0 is the onset

of the light period, ZT 12 refers to the transition between light and dark, and ZT24/0 to the transition from dark to light. Circadian time (CT time) is time within the subjective light cycle, with CT0 representing the subjective lights-on time. CT time is used as a point of reference when the organism is kept in constant conditions to reveal the endogenous clock's free running period. The clock is also temperature compensated, with the period remaining relatively constant at a range of temperatures (Sweeney and Hastings, 1960).

According to the general clock model, environmental light cycles reset a central clock, which in turn regulates the physiological output. Output can refer to events as diverse as locomotor activity, sleep and wake cycles, hormone levels and transcriptional regulation. The question of how the central clock regulates the output has been extensively studied in the mammalian model. Lesion studies have shown that the suprachiasmatic nucleus (SCN), a small structure located above the optic nerve chiasm, is the site of the central clock in mammals, since its ablation leads to loss of rhythmic locomotor activity (Reviewed in Klein et al., 1991, Ralph *et al.*, 1990; Reppert and Weaver, 1997). The proteins comprising the clock have been discovered in almost all of the tissues tested, so scientists hypothesized that the central clock drives the rhythmicity of the tissues and of the organisms as a whole.

The generation of transgenic rats carrying a rhythmic reporter gene led scientists to a better understanding of the relationship between the central clock in the SCN and the peripheral clocks in tissues (Yamazaki *et al.*, 2002). Because of its short half-life, the reporter of choice for these studies was Luciferase. Firefly Luciferase cleaves its substrate, luciferin, and emits light (dWet *et al.*, 1985). Unlike Green Fluorescent Protein

(GFP), luciferase is short lived: by cloning a rhythmic promoter from the mouse, *period1 or period 2 (mper1, mper2)*, upstream of the luciferase gene, it has recently been possible to visualize rhythmic gene activity in whole tissues and single cells. The advances brought by the *Period-Luciferase* transgenic rats and mice have led to a further understanding of how central and peripheral circadian oscillators are controlled (Yamazaki *et al.*, 2002; Yoo et al., 2004). Yamazaki *et al.* (2002) have shown that isolated tissues are indeed rhythmic. Rhythmicity in the tissues dampens after two to seven cycles, while it remains constant in the SCN for up to 32 days *in vitro*. Responding to advances or delays in the light cycle, the SCN shifts more quickly than the peripheral tissues or locomotor activity (Yamazaki et al., 2002). Even though these studies indicate that the SCN entrains peripheral tissues and is necessary for their rhythmicity, recent findings from the mouse model suggest that peripheral tissues might contain self-sustained, rather than dampened, circadian oscillators. Yoo *et al.* examined the persistence of circadian rhythms in peripheral tissues as compared to the SCN of mPER2:LUCIFERASE (mPER2:LUC) knockin mice. While the rats generated by the Yamazaki *et al.* group carry the *Luciferase* gene driven by the *mper1* promoter, the mice generated by the Yoo *et al.* group carry a fusion protein between mPER2 and LUCIFERASE. The researchers have found that both the SCN and peripheral tissues explants display robust and self-sustained oscillations lasting at least 20 days (Yoo *et al.*, 2004). Part of the discrepancy between these studies might be explained by the regulatory differences between a fusion protein reporter as opposed to an mRNA reporter. Possibly, the post-translational modifications in the mPER2:LUC animals might be needed for persistent rhythmicity.

Nevertheless, the question of how rhythmicity is lost in tissue explants (Yamazaki *et al.*, 2000) or in immortalized cells (Basalobre *et al.*, 1998) still remains. By imaging individual rat-1 fibroblasts expressing the *Luciferase* gene driven by the mouse *Bmal1* (*mBmal1*) promoter, Welsh *et al.* found that isolated fibroblasts display self-sustained oscillations that are independent of other cells and persist with undiminished amplitude. Because of these results, these researchers hypothesized that arrhythmicity in culture is due to lack of oscillator coupling. Since every cell displays an endogenous and slightly different period, the uncoupled cells eventually reach asynchrony, leading to damping of the population rhythm (Welsh *et al.*, 2004). This implies that in tissues individual cells do not lose their rhythmic quality, but possibly they fall out of synchrony, leading to loss of apparent rhythmicity. Clocks are cellular properties, so the level of organization quickly increases in complexity in multicellullar organisms. The SCN does not completely drive tissue rhythmicity, but possibly functions as a point of reference (Yamazaki et al., 2002, Yoo et al., 2004). It is thought that synchrony within a tissue or amongst different tissues is maintained via neurochemicals, gap junctions or hormones yet to be identified.

The Molecular Clock

As mentioned, clocks have been identified in all 5 kingdoms of life, from bacteria, to plants, to animals. The molecular clock has been examined mostly in a few model organisms spanning the phyla: cyanobacteria, *Neurospora crassa*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Danio rerio* and *mus musculus* (reviewed in Dunlap, 1999). The differences among the molecules regulating the clock and the similarities among the

mechanisms regulating it (for example, mRNA rhythmicity and post-translational modifications) demonstrate that the clock must have evolved more than once. Similar proteins assumed different roles, demonstrating how their roles changed to fulfill better the organism's niche. For example, cryptochrome is a photoreceptor in plants (reviewed in Lin et al., 2003; Cashmore at al., 1999) and flies (Emery et al., 1998; Stanewsky et al., 1998), while it is a transcriptional repressor in vertebrates such as mice (van der Horst et al., 1999; Kume *et al.*, 1999) and frogs (Zhu and Green, 2001). I will describe the vertebrate molecular clock and the similarities and differences with other organisms.

Rhythmicity at the genetic level is regulated by a transcriptional/translational feedback loop. Transcriptional activators regulate the expression of multiple genes, including their own repressors. As the protein level of the repressors rises, transcriptional activation is blocked. When the repressors' activities and levels fall again, transcription begins again, therefore leading to a cyclic system (reviewed in Fukada, 2003).

Multiple levels of regulation, including protein stability and phosphorylation, nuclear translocation and competitive interaction modulate the cycle and stretch it over a period of approximately 24 hours. This loop is not enclosed: transcriptional activation and repression expand to many genes regulating the organism's physiology. For example, oligonucleotide microarray screens in *Arabidopsis* have shown that the mRNA encoding enzymes required for synthesis of photosynthetic enzymes peak right before dawn; the genes required for biosynthesis or storage of sugar compounds accumulated during photosynthesis peak at the end of the day (Harmer *et al.*, 2000). This regulation is also tissue specific, demonstrating that the clock has adapted its output functions to each tissue. In *Drosophila* and in the mouse, screens carried out via oligonucleotide-based

arrays have demonstrated that many genes involved in transcriptional regulation of physiology are under circadian control. In addition, only a small portion of all cycling transcripts overlap between tissues (Ceriani *et al.*, 2002; Panda *et al.*, 2002). In the *Xenopus laevis* retina, melatonin is only expressed during the night (Cahill and Besharse, 1990). Which molecules are responsible for this rhythmic regulation of the organism's physiology?

Most of the clock components have been identified by genetic and homology screens. *Clock* was the first vertebrate circadian gene identified through an N-ethyl-N-nitrosourea (ENU) mutagenesis screen in mice, and it was then cloned by the same group (Vitaterna et al, 1994; King *et al.*, 1997; Antoch *et al.*, 1997). The *Clock* mutation is semidominant, leading to a range of phenotypes, from lengthening of period to loss of rhythmicity, an indication that this is a core clock component (Vitaterna *et al*, 1994). The other identified clock components include *Bmal1*, CLOCK's binding partner (Gekakis *et al.*, 1998), three *Period* gene paralogs, *mPer1*, *mPer2* and *mPer3*, (Takumi *et al.*, 1984; Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; Takumi *et al.*, 1998) and two cryptochromes, *mCry1* and *mCry2* (Hsu *et al.*, 1996; Todo *et al.*, 1996; van der Spek *et al.*, 1996). In the late 1990s, studies focused on the clock genes' regulation and possible light induction: today, the focus has shifted to a more biochemical approach to understand the kinetics of their interactions.

In mouse, mCLOCK (Vitaterna et al, 1994; Antoch *et al.*, 1997; King *et al.*, 1997) and BMAL1 (mBMAL1) (Hogenesh *et al.*, 1998; Gekakis et al, 1998) are the two transcriptional activators comprising the positive arm of the feedback loop. This is also the case in *Drosophila melanogaster* (reviewed in Dunlap, 1999 and Young, 1998). Both

contain basic helix-loop-helix (bHLH) and PAS (PER-ARNT-SIM) domains, which have both been shown to function as protein-protein interaction domains (Vitaterna *et al.*, 1994; Gekakis *et al.*, 1998). By comparing them to other known bHLH-PAS proteins, Vitaterna *et al.* and Gekakis *et al.* hypothesized mCLOCK and mBMAL1 bind DNA via the basic domain, and bind to each other, to their repressors and regulators possibly via the protein-protein binding domains, the HLH and the PAS domains. As a heterodimer, CLOCK and BMAL1 recognize a short enhancer element – CACGTG – called the E-box and activate transcription of genes containing the E-box in their promoters (Etchegaray *et al.*, 2003; Hogenesh *et al.*, 1998; Gekakis *et al.*, 1998; King *et al.*, 1997). Some of these genes are the three *period* genes (*per1, per2* and *per3*) and the two *cryptochrome* genes (*cry1* and *cry2)* (Gekakis *et al.*, 1998; Hogenesh *et al.*, 1998; Jin *et al.*, 1999; Kume *et al.*, 1999). As *per* and *cry* transcription increases, their mRNA levels rise, and the proteins begin to accumulate in the cytoplasm. Upon reaching critical levels, the proteins enter the nucleus and function as transcriptional repressors by binding to the CLOCK/BMAL1 heterodimer and blocking the transcriptional activation of many genes, including their own (Griffin *et al.*, 1999; Kume *et al.*, 1999; Lee *et al.*, 2001). Repression is relieved when CRY and PER protein levels fall, allowing CLOCK and BMAL1 to re-initiate transcription and to restart the loop.

Recent evidence demonstrates that auxiliary and interconnected loops function to strengthen and maintain a high-amplitude oscillation. BMAL1 down-regulates its own transcript, which is also upregulated by CRY1, CRY2 and PER2 (Yu *et al.*, 2002). Both *Clock* and *Bmal1* mRNAs are suppressed by REV-ERBa, a clock-controlled gene and nuclear orphan receptor (Peitner *et al.*, 2002; Ueda *et al.*, 2002). In addition, the genes

activated by CLOCK and BMAL1 might also be downregulated by other transcription factors, such as DEC1 and DEC2 (Honma *et al.*, 2002). The model is likely to increase in complexity, as more clock controlled genes, kinases, phosphatases and, in general, regulators of the loop are cloned (Figure 1).

Maintenance of the 24-hour period

Many of the clock components or the clock-controlled genes (CCG) were discovered because of their high-amplitude mRNA rhythmicity. Therefore, it was thought that timing of mRNA transcription and translation was one of the most important steps in the generation of a 24-hour period. Further understanding of the biochemistry underlying the protein-protein interactions regulating the clock has unveiled many levels of regulation, from timing of transcription, to mRNA stability, to translational and protein stability and subcellular localization control.

Several observations showed that simple presence or absence of mRNA and protein is not the only regulatory step in the maintenance of a 24-hour period. For example, *Clock* transcript is not rhythmic in the SCN or the liver (Lee *et al.*, 2001), and Bmal1's rhythmicity is strong in the SCN, but less prominent and of reversed phase in the piriform and parietal cortex (Honma *et al.*, 1998). In addition, evidence from the liver demonstrates that the CLOCK/BMAL1 heterodimer is bound to the E-box in the liver throughout the day, supporting the hypothesis that presence or absence of the transcription factors is not the major regulatory step of the loop . On the other hand, nuclear entry of the repressors, CRYs and PERs, is strongly rhythmic (Lee *et al.*, 2001). These experiments indicate that many key steps must fall into place in order to achieve a

24-hour rhythm. Not only do the protein levels have to be rhythmic, but the proteins themselves have to dimerize and enter the nucleus in a rhythmic manner. The exact period, 24 hours instead of 20 or 30, seems to be maintained via several delays such as phosphorylation, one of the best understood.

The discovery that the length of the period is partly regulated by kinases came from the identification of the genetic defect in the Syrian hamster *tau*. The *tau* hamster, identified in 1988, was the first vertebrate circadian mutant: it displayed a short period, 2 hours shorter in the heterozygotes and 4 hours shorter in the homozygotes (Ralph and Menaker, 1988). Novel forward genetic techniques (Lander, 1993) allowed Lowrey *et al.* to localize the hamster *tau* mutation to the catalytic domain of casein kinase Iε. Wild type CKIε binds and phosphorylates PER1, while the *tau* mutation displays an arginine-to-cysteine substitution in the catalytic pocket presumably involved in substrate recognition, decreasing PER1's phosphorylation (Lowrey *et al.*, 2000). CKIε appears to play a similar role in mice, where CKIε has been shown to bind and phosphorylate mPER1 (Vielhaber *et al.*, 2000), and humans (Keesler *et al.*, 2000; Takano *et al.*, 2004), indicating that CKIε plays a major role in the circadian loop. In humans, a mutation in the putative CKIε phosphorylation site on hPER2 was linked to a shortened sleep-wake cycle (familial advanced sleep phase syndrome, FASPS) (Toh *et al.*, 2001). Furthermore, CKIε appears to play a protective role against certain sleeping disorders, such as delayed sleep phase syndrome (DSPS) and non-24 hour sleep wake syndrome (N-24). Takano *et al.* (2004) analyzed all of the coding exons of human CKIε. One of the variants they discovered, S408N, eliminates one of the auto-phosphorylation sites on the CKIε's C-terminus. *In vitro* kinase assays demonstrate that this CKIε mutant is 1.8 fold more

active than the wild type CKIε. The S408N allele is significantly less common in DSPS and N-24 patients, indicating that it plays a protective role against the development of these sleep disorders (Takano *et al.*, 2004).

The role of CKIε also seems to be conserved in *Drosophila*. The *Drosophila* CKIε homolog is *doubletime* (*dbt*) (Kloss *et al.*, 1998; Price *et al.*, 1998). Price *et al.* (1998) identified *doubletime* mutant alleles displaying shorter (*dbtS*) or longer (*dbtL*) periods. The mutants exhibit altered behavioral rhythms and molecular oscillations. In another mutant, *dbtP*, PER remains hypophosphorylated and accumulates in the cytoplasm. From these results, the researchers inferred that the normal role of DBT is to reduce PER stability and monomeric levels, therefore modulating the period (Price *et al.*, 1998). Cumulatively, these data demonstrate that the period of the clock can be modulated by the destabilization of PER via CKIε.

As previously mentioned, nuclear entry of the repressor proteins is strongly rhythmic, indicating that this step may also be important for accurate rhythmicity (Lee *et al.*, 2001). Nuclear entry of PER has been shown to be regulated by heterodimerization and phosphorylation. In transiently transfected cells, mPER2 can heterodimerize with mPER1, retaining it in the cytoplasm, but it is not clear if this is physiologically significant *in vivo*. In addition, phosphorylation of PER1 by CKIε causes masking of PER2's nuclear localization signal, also leading to PER1 cytoplasmic retention (Vielhaber *et al.*, 2000). In liver, all the PER proteins bind to the CRY proteins (Lee *et al.*, 2001). Here, I will show possible evidence that the CRY proteins are subjected to similar regulation of their subcellular regulation, therefore contributing to the period's stability.

Cryptochrome

Cryptochrome (*cry*), named after the hidden nature of its bound chromophore, was first identified in *Arabidopsis thaliana* (Ahmad and Cashmore, 1993). Since CRY has assumed diverse roles amongst the organisms where it has been identified (reviewed in Cashmore *et* al., 1999; Green, 2004), its study is important not only for the understanding of clocks, but also to investigate how organisms adapt to their environment.

All cryptochromes identified to this day are pterin/flavin-binding proteins, highly homologous and thought to have evolved from photolyase proteins. Photolyase is a DNA repair enzyme. UV light can cause pyrimidine dimers in the DNA strand; photolyase can revert the dimer via photoinduced electron transfer between the protein and the substrate (Sancar and Sancar, 1988; Feng Li *et al.*, 1991). Therefore, in photolyase the flavin group is required for the UV-induced redox reaction.

The strong sequence homology between the cryptochrome and photolyase family (over 85% in *Xenopus;* Zhu, Conte and Green, 2003) also supports the hypothesis that clocks have evolved as a protection mechanism from harmful UV radiation during the daytime and that the ability to predict the change between day and night developed later in evolution (Pittendrigh, 1993; Gehring and Rosbash, 2003)

The diverse functions of cryptochrome have made it a difficult, yet interesting protein to study. In plants, cryptochromes are blue light photoreceptors, and they entrain the clock (Somers *et al.*, 1998; Cashmore *et al.*, 1999). In *Drosophila*, cryptochrome also functions as a photoreceptor in the lateral neurons: in the presence of light, dCRY binds

to and causes the degradation of dTIM, which in turn leads to the degradation of dPER and therefore to a phase shift (Stanewsky et al, 1998; Ceriani *et al.*, 1999; Busza *et al.*, 2004; reviewed in Ashmore and Seghal, 2003).

In vertebrates - where two *cryptochrome* genes have been cloned from mice and humans (Adams *et al.*, 1995; Hsu *et al.*, 1996; Todo *et al.*, 1996; van der Spek *et al.*, 1996) – CRY has been shown to function as the repressors of CLOCK/BMAL1-mediated transcriptional activation (van der Horst *et al.*, 1999; Kume *et al*, 1999; Griffin *et al.*, 1999; Shearman *et al.*, 2000). Mice lacking both *cryptochromes* are behaviorally arrhythmic, demonstrating that the proteins are required for proper circadian function. Interestingly, single *cry* knockout mice have different and opposing phenotypes: cry1-/- have a period faster than 24 hours, while cry2-/- have a period slower than 24 hours, indicating that the two molecules possibly work in concert in wild type animals to maintain the period's regularity (van der Horst *et al.*, 1999).

Recently, much attention has been focused on the C-terminus of CRY, because while the core domain is extremely well conserved amongst CRYs in different organisms and between CRY and PHOTOLYASE, the C-termini are highly variable (Zhu, Conte *et al.*, 2003). The core region of the protein is very well conserved amongst organisms and between CRY and PHOTOLYASE, and it contains binding sites for pterin methenyltetrahydrofolate (MTHF) and flavin (FAD). In fact, it is thought that the light response in PHOTOLYASE, plant CRY and *Drosophila* CRY is mediated via the bound flavin and a few key residues that are part of the electron transport chain; the binding domain and the key residues are conserved (Payne *et al.*, 1987; Lin *et al.*, 1995; Park *et al.*, 1995; Stanewsky *et al.*, 1998; Emery *et al.*, 1999.) In *Drosophila*, mutation of the

flavin-binding domain leads to loss of photoreception and inability to phase-shift (Stanewsky *et al.*, 1998). In *Xenopus*, the same mutation causes loss of repressive function (Zhu and Green, 2001b). Surprisingly, mutating the FAD binding domain of mCRY does not inhibit its repressive function (Froy *et al.*, 2002), leading to the possibility that these conserved co-factors might not act via the same mechanism even amongst vertebrates. Nevertheless, the formation of the mCLOCK/mBMAL1 heterodimer seems to be controlled by the redox state of the cell (Rutter *et al.*, 2001), leading to the assumption that CRY's mechanism of repressive action might involve electron transfer to the CLOCK/BMAL1 heterodimer (Rutter *et al.*, 2002).

Cryptochrome in the *Xenopus laevis* retina

The retina of *Xenopus laevis* exhibits circadian control in many aspects of its physiology, biochemistry and gene regulation. Melatonin release is highly rhythmic (Cahill and Besharse, 1990), partially due to clock-regulated synthetic enzymes, some of which have been demonstrated to be transcriptionally controlled by the clock (reviewed by Green, 1998a,b). Besharse and Iuvone demonstrated the existence of a circadian clock located within the eye of *Xenopus laevis*. They showed that the activity of serotonin N-acetyltransferase (NAT), one of the enzymes in the melatonin synthetic pathway, is regulated by a circadian clock present in the eye (Besharse and Iuvone, 1983). The retinal NAT activity can be entrained to an opposite light-dark cycle *in vitro*, demonstrating that the endogenous retinal circadian clock, responding to a change in the Zeitgeber, is directing a phase shift in the enzyme's activity (Besharse and Iuvone, 1983).

These studies were carried out using a *Xenopus* eyecup preparation, obtained by surgical removal of the cornea, the iris and the lens. The resulting eyecup includes the retina, pigment epithelium, choroid and sclera, and can be cultured *in vitro* (Besharse *et al.*, 1980). *Xenopus* eyecups can be exploited not only for pharmacological studies, but also for *in vitro* circadian analyses. The ocular clock in these preparations persists in constant darkness and can be phase shifted (Besharse and Iuvone, 1983; Green and Besharse, 1994). Melatonin synthesis in particular can be monitored over time from a single eyecup; this, in combination with the development of the transgenic technique, allows researchers to perturb the circadian system and monitor the phenotype in an *ex vivo* system (reviewed in Green, 2003; Cahill and Besharse, 1990; Hayasaka *et al.*, 2002).

The development of the perfusion system to culture retinas (Takahashi *et al.*, 1980; Cahill and Besharse, 1990) and of the transgenic technique (Kroll and Amaya, 1996) highly improved the efficiency of circadian studies in *Xenopus*. In the perfusion system, each eyecup is maintained in a superfusion chamber, where culture medium supplied with 5-hydroxy-tryptophan (HTrp) and gas (5% CO_2/95% O_2) is delivered continuously (Cahill and Besharse, 1990; Cahill and Besharse, 1991). The system can be regulated for light cycles and temperature, being therefore perfectly fit for circadian studies of rhythmic melatonin output. Melatonin levels are then quantified with the radio-immuno assay previously described by Rollag and Niswender (1976). This system allows the use of melatonin release as a convenient output to study retinal circadian clock mechanisms and the effect on it by genetic perturbations (Hayasaka *et al.*, 2002).

In addition, these studies can be performed at the tadpole stage, allowing a quick turnover between generation of mutant animals and experimental setup (Green *et al.*,

1999). The development of the transgenic technique in 1996 by Kroll and Amaya allowed molecular manipulations in *Xenopus* never before possible (Kroll and Amaya, 1996). This approach exploits the numerous, large and easily injectable *Xenopus* eggs. Sperm nuclei are isolated from a male frog (Murray, 1991) and then incubated with egg interphase extract, which induces decondensation of the sperm DNA and swelling of the sperm nuclei. To insert the transgene into the sperm nuclei, the plasmid carrying the transgene is first linearized, and then incubated with sperm nuclei, accompanied by the enzyme used to linearize the plasmid. The sperm DNA is cut at non-specific sites and the plasmid is inserted into the genomic DNA by restriction-enzyme-mediated integration (REMI). The specific activity of the enzyme is kept low to prevent excessive damage to the sperm DNA. The sperm nuclei, now carrying the transgene, are then injected at an estimated concentration of 1-2 nuclei/injection volume into healthy eggs, which have been made more readily injectable by removing the jelly coat. The transgene is therefore inserted into the genome, and it acquires the appropriate temporal and spatial expression in the developing tadpole (Kroll and Amaya, 1996, Knox *et al.*, 1998). Confirmation of expected expression can be monitored using GFP as a reporter gene (Kroll and Amaya, 1996; Knox *et al.*, 1998). Toxic effects of GFP expression in the retina are unlikely, since electroretinograms are not disrupted by GFP overexpression in the photoreceptor layer (Engbretson *et al.*, 1999).

The transgenic technique carries some drawbacks. Occasionally, the REMI damages the genomic DNA, leading to a small percentage of mutant embryos. It is not possible to control reliably how many copies of the transgene will be integrated, sometimes leading to transgenic embryos containing concatemers of the transgene with

possible variable expression. The unfertilized eggs are sometimes damaged by the injection of sperm, since the needles necessitate a large diameter to accommodate the swollen nuclei. Many of these problems have recently been addressed: Sparrow *et al.* (2000) developed a simplified transgenic technique in which sperm DNA decondensation and REMI is not necessary. This technique resulted in a high percentage of transgenic embryos, improving the efficiency of the transgenic procedure, without any loss in efficacy (Sparrow *et al.*, 2000).

In primary transgenic animals generated using the methods described above, the level of expression in transgenic tadpoles can often vary. Extremely high expression can lead to deleterious effects, while low levels of expression can lead to uninterpretable results. The only possible method to control for this variability is the generation of lines of frogs expressing the transgene. This was achieved by Marsh-Armstrong *et al.* (1999): in this study, the researchers generated F0 founder transgenic tadpoles by the REMI method (Kroll and Amaya, 1996), and raised the tadpoles to sexual maturity. Progeny can be produced either by in vitro fertilization, where sperm is simply added to unfertilized eggs, or through natural mating. In this study, transmission of the transgene to F1 progeny was successful in every case. To confirm that the transmission or the level of expression in the F1s was not gene- or promoter-specific, the researchers tested a range of promoters and reporters, rearing transgenic F1s with all the constructs tested. Successful transmission to F2s was also achieved. The advantage of rearing transgenic F1s is the ability to control expression level of the transgene, and to have access to a large, easily obtainable supply of genetically identical transgenic tadpoles.

Therefore, the transgenic technique opens new possibilities for *Xenopus laevis* as a circadian model system, allowing genetic manipulations which can be monitored in a matter of weeks. In particular, the retina is an excellent system to explore cellular interactions in the circadian system. The photoreceptor layer-the cell layer responsible for vision- of *Xenopus* contains comparable amounts of rod cells (the night vision cells) and cone cells (the day vision cells) (reviewed in Anderson and Green, 2000; Rohlich and Szel, 2000). Study of rod specific expression has been expanded through the use of the *Xenopus* rod-arrestin upstream sequence (Mani *et al.*, 1999). Our laboratory has used the mouse interphotoreceptor retinoid-binding protein (IRBP) promoter (Bobola *et al.*, 1995) to obtain specific expression in the whole photoreceptor layer and to study the effect of circadian perturbation in only one cell type in the photoreceptor layer (Hayasaka *et al.*, 2003). Rhythmic, photoreceptor-specific promoters already exist in *Xenopus* (Liu and Green, 1999; Steenhard and Besharse, 1999), allowing tissue-specific circadian studies. Because of all the reasons mentioned above, *Xenopus* is an excellent system to study circadian genes in general and *bmal1* and *cryptochrome* in particular (reviewed in Green, 2003). In addition, many aspects of retinal physiology are regulated not only by light and dark, but also by the ocular clock. In our laboratory, Zhu cloned three *cryptochrome* genes from *Xenopus* and found their deduced amino acid sequences well conserved between *Xenopus* and mouse (Zhu and Green, 2001a). All three *crys* (*xcry1, xcry2a* and *xcry2b*) are expressed in the photoreceptor layer and are rhythmic in the light/dark cycle (LD) and in constant darkness (DD).

Role of CRY in vertebrates

The role of CRY in vertebrates remains controversial. While some think it may function as a photoreceptor, its role as a transcriptional repressor is strongly supported by *in vivo* and in *vitro* data (van der Horst *et al.*, 1999; Kume *et al*, 1999; Griffin *et al.*, 1999). Its striking similarity to the photoreceptive PHOTOLYASE, *Arabidopsis* and dCRY has lead some scientists to search for a biochemical photoresponse, but to this date there is no strong evidence, biochemical or genetic, that it functions as a photoreceptor in vertebrates. Therefore, it has been hypothesized that the difference in function between vertebrate and invertebrate CRY is due either to minor differences in their core domains, to their highly diverse C-termini, or possibly both. Likely, the mammalian CRY evolved from the plant PHOTOLYASE, not from the plant CRY, since it is more similar to the plant PHOTOLYASE (Cashmore *et al.*, 1999).

The function of the C-terminal domain has recently been investigated in several organisms. In *Drosophila* and *Arabidopsis*, the C-terminus plays a regulatory role by inhibiting CRY's photoreceptive function, while in *Xenopus*, where three *crys* have been cloned (Zhu and Green, 2001), it regulates CRY's subcellular localization (reviewed in Green, 2004). In plants, the C-terminus functions as a regulator of the light signal: while in the dark the C-terminus is inactive, it is activated by photon absorption in the light. The active C-terminus binds to constitutive photomorphogenesis 1 (COP1) and inhibits it, therefore initiating the phototransduction pathway (Yang *et al.*, 2001). Recent evidence from *Drosophila* demonstrates that the C-terminus inhibits CRY's function in the dark: in fact, overexpression of CRY lacking the C-terminus causes phenotypes mimicking constant light exposure. Exposure to light possibly causes a conformational change,

which allows dCRY to interact and degrade dPER and dTIM (Busza *et al.*, 2004; Dissel *et al.*, 2004; Rosato *et al.*, 2001; Ceriani *et al.*, 1999).

The role of the carboxyl terminus in vertebrates has been investigated mostly in *Xenopus*, where it regulates the protein's subcellular localization. Truncation of xCRY at the C-terminus leads to loss of nuclear localization and, therefore, loss of repressive activity. The repressive activity is nevertheless intact, because if xCRY is driven into the nucleus by a heterologous NLS, repression is restored (Zhu *et al.*, 2003; see Chapter III). These observations lead to a model where xCRY's repressive function lies in the core domain and is lost if the flavin-binding domain is disrupted. The C-terminus functions as a nuclear localization signal and therefore regulates xCRY's subcellular localization, a critical regulatory step for the maintenance of a 24-hour period.

Key questions on the function of CRYs in vertebrates still remain. The circadian photoreceptive pathway is still unknown, but much progress has been made in identifying photoreceptive molecules (Provencio *et al.*, 2000; Hattar *et al.*, 2003; Lucas *et al.*, 2003; Panda *et al.*, 2003; Sollars *et al.*, 2003). Since CRY has been shown to be sufficient as a transcriptional inhibitor *in vitro*, what are the roles of the PERIOD proteins *in vivo*? Maintenance of a 24-hour period is critical for the clock's function. In mammals, the PER proteins complex with each other and with the CRYs, possibly regulating CRY's stability and subcellular localization. (Kume *et al.*, 1999; Field *et al.*, 2000; Lee *et al.*, 2001), possibly modulating period length. This complex also includes CKIε, which has been shown to be important in period length regulation (Ralph and Menaker, 1988; Lowrey *et al*, 2000). By which mechanism does CRY carry out repression in vertebrates? The flavin binding site seems to be required for repressive function in

xCRY (Zhu and Green, 2001b); since the redox potential might be involved in the activation of the CLOCK/BMAL1 heterodimer (Schibler *et al.*, 2001), is it possible that the bound co-factors play a role in this molecular switch? Possibly, CRY could absorb photons via the co-factors and transfer the electrons to the CLOCK/BMAL1 heterodimer via its electron transport pathway, changing its repressive ability. And lastly, what, if any, is the functional difference between mCRY1 and mCRY2?

Here I will report results from studies of the function of the C-terminus in *Xenopus laevis*. I have found that regulation of xCRY1 and xCRY2b's subcellular localization is dictated by the C-terminus, with important differences between the two molecules, which could possibly explain their diverse roles *in vivo*.

Figure 1-1: Model of the circadian loop

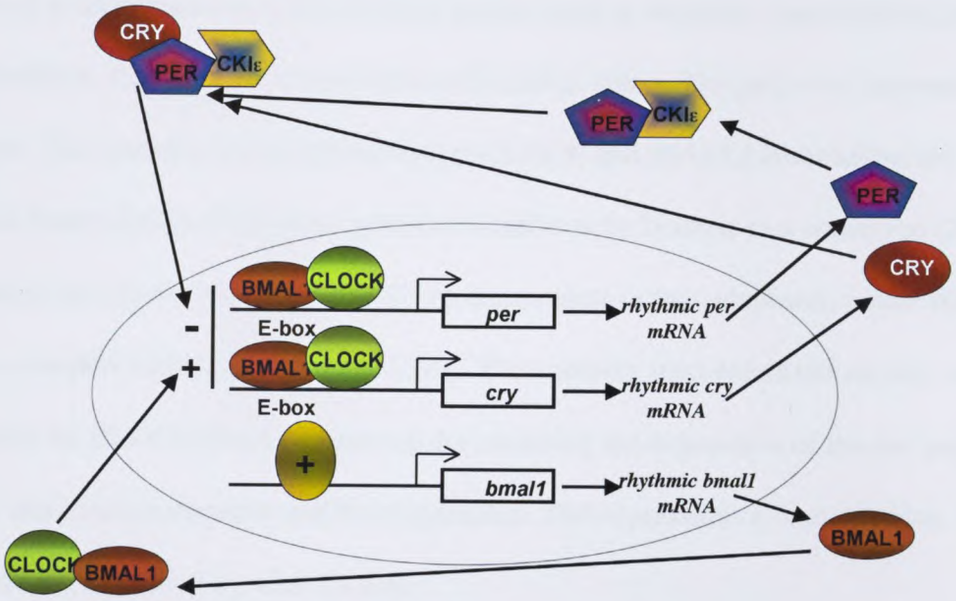


Figure 1-1: (Adapted from Thompson, C. and Sancar, A., 2002) **Simplified model of the circadian loop**. This figure illustrates some of the most important regulatory steps involved in the maintenance of a 24-hour period, such as rhythmic transcription, protein accumulation, degradation, dimerization and nuclear entry. The grey oval represents the nucleus. The circadian transcription factors CLOCK and BMAL1 heterodimerize and activate transcription of the clock genes *pers* and *crys* by binding to a conserved DNA sequence, the E-box. The PERs and CRYs accumulate in the cytoplasm, where they form a complex including the kinase CKIε. The complex then enters the nucleus and represses the CLOCK/BMAL1 heterodimer, reducing the expression of the *per* and *cry* genes; this leads to rhythmic mRNA expression. The expression of *bmal1* is also rhythmically regulated by other factors.

References

Adams, M.D., Kerlavage AR, Fleischmann RD, Fuldner RA, Bult CJ, Lee NH, Kirkness EF, Weinstock KG, Gocayne JD, White O, *et al.* (1995) Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. *Nature*, **377**: 3-174.

Ahmad, M., and Cashmore, A.R. (1993) HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature*, **366**: 162-166.

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

Anderson, F.E. and Green, C.B. (2000) A symphony of rhythms in the *Xenopus laevis* retina. *Microscop. Res. Tech.*, **50***:* 360-372.

Antoch, M.P., Song, E.J., Chang A.M., Vitaterna M.H., Zhao, Y., et al. (1997) Functional identification of the mouse Clock gene by transgenic BAC rescue. *Cell*, **89**: 655-667.

Ashmore, L.J. and Seghal, A. (2003) A fly's eye view of entrainment. *J. Biol. Rhythms*, **18**: 206-216.

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

Beaver, L.M., Gvakharia, B.O., Vollintine, T.S., Hege, D.M., Stanewsky, R., Giebultowicz, J.M. (2002) Loss of circadian clock function decreases

reproductive fitness in males of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.*, **99**: 2134-2139.

Besharse, J.C. (1982). The daily light-dark cycle and rhythmic metabolism in the photoreceptor-pigment epithelial complex. *Prog. Retinal. Res.*, **1**:81-124.

Besharse, J.C., and Iuvone, P.M. (1983) Circadian clock in *Xenopus* eye controlling retinal serotonin N-acetyltransferase. *Nature*, **305**:133-135.

Besharse, J.C., Iuvone, P.M., and Pierce, M.E. (1988) Regulation of rhythmic photoreceptor metabolism: A role for post-receptoral neurons. In *Progress in Retinal Research*, volume 7. Edited by N. Osborne and J. Chader, Pergamon Press, Oxford, England, pp. 21-61.

Besharse, J.C., Terrill, R.O., and Dunis, D.A. (1980) Light evoked disc shedding by rod photoreceptors in vitro: Relationship to medium bicarbonate concentration. *Invest. Ophthalmol. Vis. Sci.*, **19**:1512-1517.

Bobola, N., Hirsh, E., Albini, A., Altruda, F., Noonan, D., and Ravazzolo, R. (1995) A single cis-acting element in a short promoter segment of the gene encoding the interphotoreceptor retinoid-binding protein confers tissue-specific expression. *J. Biol. Chem.*, **270**:1289-1294.

Busza, A., Emery-Le, M., Rosbash, M. and Emery, P. (2004) Role of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science*, **304**: 1503-1506.

Cahill, G.M., and Besharse, J.C. (1990). Circadian regulation of melatonin in the retina of *Xenopus laevis*: Limitation by serotonin availability. *J. Neurochem.*, **54**:716-719.

Cahill, G.M., and Besharse, J.C. (1991). Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. *J. Neurosci.*, **11**: 2959-2971.

Cahill, G.M., and Besharse, J.C. (1995) Circadian rhythmicity in vertebrate retinas: Regulation by a photoreceptor oscillator. *Prog. Retin. Eye Res.*, **14**: 267-291.

Cashmore, A.R., Jarillo, J.A., Wu, Y., Liu, D (1999) Cryptochromes: blue-light receptors for plants and animals. *Science*, **284**: 760-765.

Ceriani, F.M., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J. and Kay, S.A. (1999) Light-dependent sequestration of TIMELESS by CRYTPOCHROME. *Science*, **285**: 553-556.

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

DeCoursey, P.J., Krulas, J.R. (1998) Behavior of SCN-lesioned chipmunks in natural habitat: a pilot study. *J. Biol. Rhythms*, **13**: 229-244.

Dissel, S., Codd, V., Fedic, R., Garner, K.J., Costa, R., Kyriacou, C.P., Rosato, E. (2004). A constitutively active cryptochrome in *Drosophila melanogaster*. *Nat. Neurosci.* 7: 834-840

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

DWet, J.R., Wood, K.V., Helinski, D.R., DeLucca, M. (1985) Cloning of firefly Luciferase cDNA and the expression of active Luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **82**: 7870-7873.

Emery, P., So, W.V., Kaneko, M., Hall, J.C., Rosbash, M. (1998) CRY, a Drosophila

clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*, **95**: 669-679.

Engbretson, G.A., Kefalov, V.J., Cameron, D.A., Cornwall, M.C., Knox, B.E., and Barlow, R.B. (1999) Responses and microspectrophotometry of *Xenopus* rods expressing GFP and mutant bovine rhodopsin. *Invest. Ophthalmol. Vis. Sci.*, **40**: S327

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

Feng Li, Y., Heelis, P.F., Sancar, A. (1991) Active site of DNA photolyase: Tryptophan-306 is the intrinsic hydrogen atom donor essential for flavin radical photoreduction and DNA repair in vitro. *Biochem.*, **30**: 6322-6329.

Field, M.D., Maywood, E.S., O'Brein, J.A., Weaver, D.R., Reppert, S.M., Hastings, M.H. (2000) Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanism. *Neuron*, **25**: 347-447.

Froy, O., Chang, D.C., Reppert, S.M. (2002) Redox potential: different roles in dCRY and mCRY functions. *Curr. Biol.*, **12**: 147-152.

Fukada, Y. (2003) Molecular oscillations behind the clockface. *J. Biochem.*, **134**: 773-775.

Gehring, W., and Rosbash M. (2003) The coevolution of blue-light photoreception and circadian rhythms. *J. Mol. Evol.*, **57**: S286-289.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P.,

Takahashi, J.S., and Weitz, C.J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science*, **280**:1564-1569.

Green, C.B. (1998a) How cells tell time. *Trends Cell Biol.*, **8**:224-230.

Green, C.B. (1998b) Time marches on. *Trends in Cell Biol.*, **8**:342-343.

Green, C.B. (2003) Molecular control of *Xenopus* retinal circadian rhythms. *J. Neuroendocrinol.*, **15**: 350-354.

Green, C.B., (2004) Cryptochromes: Tail-ored for distinct functions. *Curr. Biol.*, **14**: 847-849.

Green, C.B., and Besharse, J.C. (1994) Tryptophan hydroxylase expression is regulated by a circadian clock in *Xenopus laevis* retina. *J. Neurochem.*, **62**: 2420-2428.

Green, C.B., Liang, M.-Y., Steenhard, B.M., and Besharse, J.C. (1999) Ontogeny of circadian and light regulation of melatonin release in *Xenopus laevis* embryos. *Brain Res. Dev. Brain Res.*, **117**:109-116.

Green, R.M., Tingay, S., Wang, Z.Y., Tobin, E.M. (2002) Circadian rhythms confer a higher level of fitness to *Arabidopsis* plants. *Plant Physiol.*, **129**: 576-584.

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

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

Hattar, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G., Yau, K.-W. (2003) Melanopsin and

rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*, **424**: 76-81.

Hayasaka, N., LaRue, S.L., Green, C.B. (2002) In vivo disruption of *Xenopus* CLOCK in the retinal photoreceptor cells abolishes circadian melatonin rhythmicity without affecting its production levels. *J. Neurosci.*, **22**: 1600-1607.

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

Honma, S., Ikeda, M., Abe, H., Tanahashi, Y., Namihira, M., Honma, K., Nomura, M. (1998) Circadian oscillation of BMAL1, a partner of a mammalian gene Clock, in rat suprachiasmatic nucleus. *Biochem. Biophys. Res. Commun.*, **250**: 83-87.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., Honma, K. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature*, **419**: 841-844.

Hsu, D.S., Zhao, X., Zhao, S., Kazantsev, A., Wang, R.P., Todo, T., Wei, Y.F., Sancar, A. (1996) Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. *Biochemistry*, **35**: 13871-13877.

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

Keesler, G.A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C., Yao, Z. (2000) Phosphorylation and destabilization of human period 1 clock protein by human casein kinase I epsilon. *Neuroreport*, **11**: 951-955.

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

Klein, D.C., Moore, R.Y., and Reppert, S.M. (1991) *Suprachiasmatic Nucleus: the Mind's Clock* (New York: Oxford University Press)

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

Knox, B.E., Schlueter, C., Sanger, B.M., Green, C.B., Besharse, J.C. (1998) Transgene expression in *Xenopus* rods. *FEBS Lett.*, **423**: 117-121.

Kroll, K.L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*, **122**: 3173-3183.

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

Lander, E.S. (1993) Finding similarities and differences amongst genomes. *Nat. Genet.*, **4**: 5-6.

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

Lin, C., Ahmad, M., Gordon, D., Cashmore, A.R. (1995) Expression of an *Arabidopsis*

cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV A and green light. *Proc. Natl. Acad. Sci.* U.S.A., **92**: 8423-8427.

Lin, C., and Shalitin, D. (2003) Cryptochrome Structure and Signal Transduction. *Annu. Rev. Plant Biol.*, **54**: 469-496.

Liu, X., and Green, C.B. (1999) Analysis of protein-binding motifs within the proximal nocturnin promoter in *Xenopus laevis*. *Invest. Ophthalmol. Vis. Sci.*, **40**:S610.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., Takahashi, J.S. (2000) Positional synthetic cloning and functional characterization of the mammalian circadian mutation *tau*. *Science*, **288**: 483-491.

Lucas, R.J., Hattar, S., Tokao, M., Berson, D.M., Foster, R.G., Yeu, K.-W. (2003) Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science*, **299**: 245-247.

Mani, S.S., Besharse, J.C., and Knox, B.E. (1999) Immediate upstream sequence of arrestin directs rod-specific expression in *Xenopus*. *J. Biol. Chem.*, **274**:15590 15597.

Marsh-Armstrong, N., Huang, H., Berry, D.L., and Brown, D.D. (1999) Germ-line transmission of transgenes in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA*, **96**:14389-14393.

Menaker, M. (1969) Biological clocks. *Bioscience*, **19**: 681-689.

Michael, T.P., Salome, P.A., Yu, H.J., Spencer, T.R., Sharp, E.L., McPeek, M.A., Alonso, J.M., Ecker, J.R., McClung, C.R. (2003) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science*, **302**: 1049-1053.

Murray, A.W. (1991) Cell cycle extracts. *Methods Cell Biol.*, **36**:581-605.

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

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

Park, H.W., Kim, S.T., Sancar, A., Deisenhofer, J. (1995) Crystal structure of DNA photolyase from *Escherichia coli*. *Science*, **268**: 1866-1872.

Payne, G., Heelis, P.F., Rohrs, B.R., Sancar, A. (1987) The active form of *Escherichia coli* DNA photolyase contains a fully reduced flavin and not a flavin radical, both *in vivo* and *in vitro*. *Biochemistry*, **26**: 7121-7127.

Pittendrigh, C.S. (1981a) Circadian systems: General perspective. In *Handbook of Behavioral Neurobiology,* Vol. 4. Biological Rhythms. J. Aschoff, Ed. Plenum, New York, pp. 57-80.

Pittendrigh, C.S. (1981b) Circadian systems: Entrainment. In *Handbook of Behavioral Neurobiology*, Vol. 4. Biological Rhythms. J. Aschoff, Ed. Plenum, New York, pp. 95-124.

Pittendrigh, C.S. (1993). Temporal organization: reflections of a Darwinian clock watcher. *Annu. Rev. Physiol.*, **55**: 16-54.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls

circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell,* **110***:* 251-260.

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

Provencio, I., Rodriguez, I.R., Jiang, G., Hayes, W.P., Moreira, E.F., *et al.* (2000) A novel human opsin in the inner retina. *J. Neurosci.*, **20**: 600-605.

Ralph, M.R., Menaker, M. (1988) A mutation of the circadian system in golden hamster. *Science*, **241**: 1225-1227.

Ralph, M.R., Forster, R.G., Davis, F.C., Menaker, M. (1990) Transplanted suprachiasmatic nucleus determines circadian period. *Science*, **23**: 975-978.

Rollag, M.D., and Niswender, G.D. (1976) Radioimmunoassay of serum concentration of melatonin in sheep exposed to different lighting regimens. *Endocrinology*, **98**:482-48.

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., Costa, R., Kyriacou, C.P. (2001) Light-independent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxyl terminus of CRY. *Curr. Biol.* **11**: 909-917.

Reppert, S.M., and Weaver, D.R., (1997) Forward genetic approach strikes gold: cloning of a mammalian Clock gene. *Cell,* **89**: 487-490.

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

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

Sancar, A., Sancar, G.B. (1988) DNA repair enzymes. *Annu. Rev. Biochem.*, **57**: 29-67.

Schibler, U., Ripperger, J.A., Brown, S.A. (2001) Chronobiology – Reducing time. *Science*, **293**: 437-438.

Sharma, V.K. (2003) Adaptive significance of circadian clocks. *Chronobiology International*, **20**: 901-919.

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F., Reppert, S.M. (1997) Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron*, **19**: 1261-1269.

Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., van der Horst, G.T., Hastings, M.H., Reppert, S.M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science*, **12**: 943-944.

Sollars, P.J., Smeraski, C.A., Kaufman, J.D., Ogilvie, M.D., Provencio, I., Pickard, G.E. (2003) Melanopsin and non-melanopsin expressing retinal ganglion cells innervate the hypothalamic suprachiasmatic nucleus. *Vis. Neurosci.*, **20**: 601-610.

Somers, D.E., Delvin, P.E., Kay, S.A. (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science*, **282**: 1488-1490.

Sparrow, D.B., Latinkic, B., and Mohun, T.J. (2000) A simplified method of generating transgenic *Xenopus*. *Nucleic Acids Res.*, **28**:E12.

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

Steenhard, B.M., and Besharse, J.C. (1999) Analysis of the tryptophan hydroxylase gene promoter using a transgenic *Xenopus*. *Invest. Ophthalmol. Vis. Sci.*, **40**:S939.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. Lee, C.C. (1997) RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. ***Cell*, 90**: 1003-1011.

Sweeney, B. and Hastings, J.W. (1960) Effects of temperature upon diurnal rhythms. *Cold Spring Harbor Symposium*. **25**: 87-104.

Takahashi, J.S., Hamm, H.E., and Menaker, M. (1980) Circadian rhythms of melatonin release from individual superfused chicken pineal glands in vitro. *Proc. Natl. Acad. Sci. USA*, **77**:2319-2322.

Takano, A., Uchiyama, M., Kajiamura, N., Mishima K., Inoue, Y., Kamei, Y., Kitajima, T., Shibui, K., Katoh, M., Watanabe, T., *et al.* (2004) A missense variation in human casein kinase I epsilon gene that induces functional alteration and shows an inverse association with circadian rhythms sleep disorders. *Neuropsychopharmacology*, **29**: 1901-1909.

Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K., Maebayashi Y., Sakakida Y., Okumura K., Takashima N., Okamura H. (1998) A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. *Genes Cells*, **3**: 167-175.

Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara C., Maebayashi Y., Okumura K., Takekida S., Yamamoto S., *et al.* (1998) A light-independent oscillatory gene *mPer3* in mouse SCN and OVLT. *EMBO J.*, **17**: 4753-4759.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozkawa, R., Hirose M., Sakaki, Y. (1997) Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature*, **389**: 521-516.

Thompson, C. and Sancar, A. (2002) Photolyase/cryptochrome blue-light photoreceptors use photon energy to repair DNA and reset the circadian clock. *Oncogene*, **21**: 9043-9056

Todo, T., Ryo, H., Yamamoto, K., Toh, H., Inui, T., Ayaki H., Nomura T., Ikenaga M. (1996) Similarity among the *Drosophila* (6-4) photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. *Science*, **272**: 109-112.

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

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

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

van der Spek, P.J., Kobayashi, K., Bootsma, D., Takao, M., Eker, A.P., Yasui, A. (1996) Cloning, tissue expression, and mapping of a human photolyase homolog with similarity to plant blue-light photoreceptors. *Genomics*, **37**: 177-182.

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

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

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

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H. (2002) Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, **288**: 682-685.

Yang, H.Q., Tang, R.H., Cashmore, A.R. (2001) The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. *Plant Cell*, **13**: 2573-2587

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.O., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveal

persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci., U.S.A.*, **101**: 5339-5346.

Young, M.W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. *Annu. Rev. Biochem.*, **67**: 135-152.

Yu, W., Nomura, M., Ikeda, M. (2002) Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2 and PER2. *Biochem. Biophys. Res. Commun.* **290**: 933-941.

Zhu, H., and Green, C.B. (2001a) Three cryptochromes are rhythmically expressed in *Xenopus laevis* retinal photoreceptors. *Mol. Vis.*, **7**: 210-215.

Zhu, H., and Green, C.B. (2001b) A putative flavin electron transport pathway is differentially utilized in *Xenopus* CRY1 and CRY2. *Curr. Biol.*, **11**: 1945-1949.

Zhu, H., Conte, F.E. and Green, C.B. (2003) Nuclear localization and transcriptional repression are confined to separable domains in the circadian protein CRYPTOCHROME. *Curr. Biol.*, **13**: 1653-1658.

Chapter II

Functional Characterization of the Circadian Transcription Factor BMAL1 in the Retina of *Xenopus laevis*

Summary

Here, I describe the functional analysis of *Xenopus* BMAL1, a bHLH-PAS transcription factor and a critical component of the circadian molecular loop. During the positive arm of the loop, BMAL1 heterodimerizes with CLOCK and activates transcription of several clock-controlled genes, including its own repressors (the *per* and *cry* genes). Many of the regulatory mechanisms controlling *bmal1* mRNA and protein are still not understood. *Xenopus* is an excellent system to study *bmal1* because this organism contains a self-sustained oscillator in the retina, and because transgenic animals can be easily generated.

To study the role of *bmal1* in *Xenopus*, I have cloned *Xenopus bmal1* (*xbmal1*) by screening a retinal cDNA library. *In situ* hybridization and Northern blot analysis results show that *xbmal1* is strongly expressed in the photoreceptor layer of the *Xenopus* retina. *xbmal1* mRNA is strongly rhythmic in the light-dark cycle and in constant darkness, peaking at the transition between day and night. xBMAL1 protein is also rhythmic *in vivo*, with highest levels in the late night. Surprisingly, no mRNA rhythmicity was detected in the brain, heart or testis. Deletion of any of xBMAL1's functional domains (basic, HLH or PAS domains) causes loss of transcriptional activation. *In vitro*, xBMAL1 binds to xCLOCK. xBMAL1 is phosphorylated, and phosphorylation is not dependent upon the basic or HLH domains. These results suggest that xBMAL1 is a critical component of the *Xenopus* retinal clock. All of the conserved functional domains (basic, HLH and PAS domains) are necessary for transcriptional activation, and phosphorylation might also be important for interaction with xCLOCK and/or transcriptional activation.

Introduction

Brain and muscle Arnt-like protein 1 (BMAL1) belongs to the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) protein family. Members of the bHLH-PAS family of proteins are transcriptional regulators controlling a variety of developmental and physiological pathways, including neurogenesis, cell lineage, toxin metabolism, response to hypoxia, hormone receptor function and circadian rhythms (reviewed in Crews, 1998). Typically, these proteins activate transcription of target genes after heterodimerization (Rowlands and Gustafsson, 1997).

In bHLH-PAS proteins, the basic domain binds DNA by allowing specific contacts within the major groove of target regulatory elements, and the HLH domain promotes protein-protein dimerization (Kadesch, 1993; Murre *et al.*, 1994). The PAS domain typically comprises 250-300 amino acids, divided in a pair of highly degenerate 50 amino acid subdomains termed the A and B repeats (Jackson *et al.*, 1986; Hoffman *et al.*, 1991; Nambu *et al.*, 1991). In higher eukaryotes, the PAS domain functions as a small molecule-binding domain (Dolwick *et al.*, 1993; Coumailleau *et al.*, 1995) and as a dimerization domain with other PAS proteins (Huang *et al.*, 1993), with non-PAS proteins (Coumailleau *et al.*, 1995; Gekakis *et al.*, 1995) or with cellular chaperones such as the 90kDa heat shock protein (HSP90) (Dennis *et al.*, 1988; Perdew, 1988). For example, two bHLH-PAS proteins, the aryl hydrocarbon receptor (Ahr) (Burbach *et al.*, 1992; Ema *et al.*, 1992) and the aryl receptor nuclear translocator (Arnt) (Hoffman *et al.*, 1991), control the pathway for toxin metabolism. Normally, Ahr is a cytoplasmic protein, complexed with accessory proteins. Upon binding of aryl hydrocarbons to Ahr,

the accessory proteins are released, and Ahr dimerizes with Arnt. The Ahr::Arnt complex enters the nucleus, where it binds to genes involved in toxin metabolism that contain the xenobiotic response element (GCGTG) (Swanson and Bradfield, 1993; Harkinson, 1995; Whitlock *et al.*, 1996; Rowlands and Gustafsson, 1997).

Bmal1 was first identified via an iterative search of expressed sequence tags (EST) in the human database for PAS-like sequences (Ikeda and Nomura, 1997), but its circadian role was not discovered during this study. Gekakis *et al.* (1998) screened a mouse SCN cDNA library via yeast-two-hybrid to search for mCLOCK binding partners and identified BMAL1. Unlike other isolated clones, such as *Arnt2*, mouse *bmal1* (*mbmal1*) is expressed in a similar pattern to *mClock* and *mper1* in the SCN and the retina. Strengthening the hypothesis that BMAL1 could be the normal binding partner of CLOCK, the researchers found that CLOCK/BMAL1 heterodimers could activate transcription from E-box elements in the *mper1* promoter. In addition, dominant negative mCLOCK, lacking the activation domain (Vitaterna *et al.*, 1994; King *et al.*, 1997a; King *et al.*, 1997b), is still able to bind mBMAL1, but cannot activate transcription (Gekasis *et al.*, 1998). Therefore, in mammals CLOCK and BMAL1 heterodimerize and activate the transcription of three *per* genes and two *cry* genes containing E-box enhancer elements (CACGTG) in their regulatory sequences (Gekakis *et al.*, 1998; Hogenesh *et al.*, 1998; Jin *et al.*, 1999; Kume *et al.*, 1999). The generation of *bmal1*-knockout mice, which become arrhythmic under DD conditions, further demonstrated its role as an essential component of the circadian loop (Bunger *et al.*, 2000).

In vertebrates, the CRYs repress CLOCK/BMAL1-mediated transcriptional activation (van der Horst *et al.*, 1999; Kume *et al*, 1999; Griffin *et al.*, 1999; Shearman *et*

al., 2000), but the repressive mechanism is unknown. CLOCK/BMAL1 binding has been shown to be regulated by the redox state of nicotinamide adenine dinucleotide (NAD) cofactors. In fact, the reduced form of the redox cofactors, NAD(H) and NADP(H), strongly enhances DNA binding of the CLOCK/BMAL1 heterodimer (Rutter *et al.*, 2001). These observations raise the possibility that the switch regulating the CLOCK/BMAL1 transcriptional activation might indeed be due to the redox state of the heterodimer, and that the CRYs might affect this state by transferring electrons from the pterin/flavin cofactors through the electron transport chain and to the CLOCK/BMAL1 heterodimer (Rutter *et al.*, 2002).

Regulation of *bmal1* expression

In the rat SCN, *bmal1* expression is antiphasic to *per* (Oishi *et al.*, 1998). Therefore, *bmal1* and *per* transcription must be governed by different mechanisms. Preitner *et al.* (2002) identified the orphan nuclear receptor REV-ERBalpha as a repressor of cyclic *bmal1* transcription. Specifically, cis-acting regulatory elements within the *bmal1* promoter reveal that REV-ERBalpha controls the cyclic transcription of *bmal1*: REV-EBRalpha itself is activated by BMAL1 and CLOCK and repressed by PER1, PER2, CRY1 and CRY2 (Preitner *et al.*, 2002). BMAL1 also seems to have other binding partners to regulate transcription of circadian genes. The bHLH transcription factor Differentially Expressed in Chondrocytes 1 (DEC1) is expressed with a circadian profile in the SCN, where it seems to bind to BMAL1 and suppress CLOCK/BMAL1-activated promoters (Honma *et al.*, 2002). The interaction between BMAL1 and DEC1

and the suppressive activity are dependent upon the DEC1 bHLH domain (Sato *et al.*, 2004).

Bmal1 is expressed in the mouse and rat SCN with high amplitude rhythms peaking in the late subjective night from CT15 to CT18 (Abe *et al.*, 1998; Honma *et al.*, 1998; Oishi *et al.*, 1998; Oishi *et al.*, 2000). In the rat, dampened rhythmic *bmal1* expression was also observed in the piriform and parietal cortices (Honma *et al.*, 1998) and in peripheral tissues, such as brain (excluding the SCN), eyes, heart, kidney and lung (Oishi *et al.*, 1998).

Evidence from studies in mouse liver indicates that *mbmal1* mRNA and protein are rhythmic, with the mRNA peaking during the early night and the protein during the late night. Nevertheless, the same studies also show that the mCLOCK/mBMAL1 heterodimer is bound to DNA throughout the circadian cycle (Lee *et al.*, 2001), suggesting that *mbmal1* rhythmicity is not necessary. More information on the kinetics of the interactions between the clock proteins will be needed to resolve this issue.

Phosphorylation of BMAL1

Phosphorylation of mBMAL1 might be important for its subcellular localization. Lee *et al.* (2001) showed that during the night mCLOCK, mBMAL1, mPER1, mPER2, mCRY1, mCRY2 and CKIε are present in a large complex in the nucleus, which the authors hypothesize is the repressive complex. At CT15, mBMAL1 is phosphorylated, and, unlike mCLOCK, only the phosphorylated form is present in the nucleus, while the unphosphorylated mBMAL1 is present in the cytoplasm. This is also true at CT6, even though lower levels of mBMAL1 are present in the nucleus at that time (Lee *et al.*, 2001).

Phosphorylation of BMAL1 might also regulate its function. Sanada *et al.* (2001) found that chicken BMAL1 (cBMAL1) binds to mitogen activated protein kinase (MAPK) *in vitro*. Specifically, cBMAL1 associates preferentially with phosphorylated MAPK (P-MAPK) compared to the unphosphorylated form. In this study, the researchers showed that MAPK can phosphorylate cBMAL1 *in vitro*, and they also identified three putative MAPK phosphorylation sites on the C-terminus of cBMAL1 (S527, T534 and S599). Nevertheless, since mutation of these sites causes only partial loss of phosphorylation, they concluded that more sites must be present. These studies also suggested that MAPK phosphorylation of BMAL1 can inhibit CLOCK/BMAL1-mediated transcriptional activation by about 20-30% (Sanada *et al.*, 2001).

Specifically, phosphorylation of BMAL1 by CKIε might play a role in the regulation of its function. Eide *et al.* (2002) investigated the role of CKIε on the mCLOCK/mBMAL1-mediated transcriptional activation. They found that CKIε can phosphorylate mBMAL1 *in vitro*. They also found that co-expression of *mClock* and *mbmal1* with a dominant form of CKIε (K38A) in a Luciferase reporter assay in HEK293 cells causes a 40% decrease in Luciferase activity. The same reduction of Luciferase activity was recorded when endogenous CKIε was reduced in the cells by dsRNA-mediated interference (Eide *et al.*, 2002). BMAL1's phosphorylation seems to also depend on its interaction with CLOCK and, in turn, BMAL1 appears to regulate CLOCK's levels and subcellular localization. In 2003, Kondratov *et al.* discovered the appearance of a BMAL1-specific band of slower electrophoretic mobility only when *mClock* and *mbmal1* were co-expressed in HEK293 cells. This band, which the authors suggest may represent phosphorylated BMAL1, increases in abundance with increasing

CLOCK amount, suggesting that CLOCK is critical for inducing BMAL1 modification (Kondratov *et al.*, 2003).

The authors also found that co-expression of *xClock* with *xbmal1* results in a dramatic decrease in CLOCK protein amount, suggesting that CLOCK/BMAL1 heterodimerization induces CLOCK proteolytic degradation. These effects are not due to BMAL1 repressing the cytomegalovirus promoter (CMV) used to drive *Clock* expression in these assays or to the cell type. Therefore, highest amounts of phosphorylated BMAL1 correlates with lowest amounts of CLOCK and, at the same time, with highest transcriptional activation. BMAL1 also seems to influence CLOCK's subcellular localization: in mouse embryonic fibroblast (MEF) cells from *bmal1* knockout mice, CLOCK protein was detected only in the cytoplasm (Kondratov *et al.*, 2003).

Here I will describe the cloning and characterization of *Xenopus bmal1* (*xbmal1*). I will show that *xbmal1* is strongly rhythmic in the retina at the mRNA and protein level. Surprisingly, I was unable to detect mRNA rhythmicity in the peripheral tissues tested. *xbmal1* activates transcription when co-transfected with *xClock*, and this function is impaired, when the basic, HLH or PAS domains are deleted. In addition, xBMAL1 is phosphorylated in Cos-7 cells.

Methods

Cloning of *Xenopus bmal1* and generation of mutant constructs

The probes were generated by excision with NotI and NcoI of a PCR fragment of a *bmal1* cloned into the pGEM5 vector. The PCR fragment was a generous gift from Dr. C. Weitz (unpublished data). The PCR amplification was carried out with mouse *bmal1* degenerate primers to yield the following fragment:

GGGTTCCTGTTTGTGGTGGGGTGTGACCGAGGGAAAATCCTCTTTGTTTCAGA
ATCCGTTTTCAAGATCCTTAATTACAGCCAGAATGATCTGATTGGCCAGAGCC
TGTTTGACTACCTGCACCCCAAAGACATCGCCAAAGTCAAATCACT

The fragment was then random prime-labeled and used to screen a *Xenopus* retinal cDNA library (in λ-Hybrizap vector; Stratagene, La Jolla, CA) generated by pooling RNA isolated from retinas at four time points throughout the day. Screening was carried out as described in Green and Besharse, (1994), except the wash solution was changed to 0.1XSSPE, 0.1% SDS. The positive clones were plaque purified, excised using the ExAssist helper phage (Stratagene, La Jolla, CA) and sequenced.

Wild type *xbmal1* and *xClock* were subcloned into the pVAX1 expression vector (Invitrogen, Carlsbad, CA). The mutant constructs (xBMAL1Δb- deleted basic domain, xBMAL1ΔHLH- deleted helix-loop-helix domain, xBMAL1ΔPAS-A- deleted PAS-A domain, xBMAL1ΔPAS-B- deleted PAS-B domain) were generated by PCR-amplifying and ligating the regions outside of the deleted areas. All clones were expressed in the pCMV-Tag4B vector (Stratagene, La Jolla, CA) and confirmed by sequencing. For the co-immunoprecipitation experiments, *xClock* and *xbmal1* were cloned into expression

vectors: *xClock* was cloned into the pcDNA3.1 V5-His vector (Invitrogen, Carlsbad, CA), while *xbmal1* was cloned into the pCMV-Tag4B vector (Stratagene, La Jolla, CA).

Eyecup preparation and culture

Xenopus laevis (5-6.5) were purchased from Nasco (Fort Atkinson, WI) and were maintained in a 12-hour light/12 hour dark cycle, where they were entrained for at least two weeks before the experiment. Animal care and use was in accordance with federal and institutional guidelines. The eyecups were then dissected and cultured as previously described (Cahill and Besharse,1991) in a humidified atmosphere of 95% O_2/5% CO_2: the eyecups include the retina, the pigment epithelium, the choroid and the sclera.

The incubations were carried out in a rotary shaker (60 rpm), in a constant temperature incubator at 21+/-0.1 °C under the established light/dark conditions. All times are expressed as Zeitgeber Time (ZT) in hours: ZT0 is defined as the time of normal light onset (sunrise) and ZT12 is defined as time of normal dark onset (sunset).

RNA isolation and Northern blot analysis

Retinas were isolated and quickly frozen on dry ice from the culture conditions at the determined ZT times. After tissue homogenization, the RNA was isolated with Trizol Reagent (Life Technology, Gaithersburg, MD). Northern blot analysis was carried out in QuickHyb Hybridization solution (Stratagene, La Jolla, CA). Radioactive DNA probes were generated via the random-primed method by using the random-primed kit (Invitrogen, Carlsbad, CA). To avoid cross-hybridization between *xbmal1-a* and *xbmal1-b*, the 3'UTR regions of the clones were used. These regions exhibit the lowest

homology. The *xbmal1* DNA fragments were boiled for 5 minutes and then mixed with dATP, dGTP, dTTP and dCTP[α^{32}P]. Klenow fragment was added to the mix and the reaction was incubated at room temperature for 2 hours (see manufacturer for details). The probes were then purified from the unincorporated nucleotides with NucTrap columns (Stratagene, La Jolla, CA). I then determined the counts per million (cpm) by counting 1µl of probe on a scintillator. For each membrane, I used $2*10^7$ cpm. For normalization, filters were stripped by boiling twice for 10 minutes in 0.01X SPPE, 0.1% SDS and rehybridized with random primed probes made from *β-actin* clones (Cleveland et al., 1980).

In situ hybridization

After dissections, *Xenopus* eyes or tadpoles were fixed overnight at 4 °C in 4% paraformaldehyde (diluted in DEPC PBS). Cryoprotection was then achieved by incubating the tissues in 30% sucrose (diluted in DEPC PBS) for 3 hours or overnight at 4 °C. The tissues were then imbedded in Tissue Tek O.C.T. compound (Ted Pella, Redding, CA) and 14µm cryosections were obtained.

The digoxygenin (DIG) antisense and sense riboprobes were designed to the 3'UTR of both *xbmal1-a* and *xbmal1-b*. The probes were then hydrolyzed to an average length of 100-250 bases. After washing in DEPC PBS, the tissues were permeabilized in 0.3% TritonX-100 for 30 minutes and acetylated in acetylation buffer for 10 minutes. After a subsequent wash, the slides were pre-incubated with hybridization mix. They were then incubated with the DIG-labeled probes overnight at 65 °C in humidified

chambers. For each slide, 200ng/ml of probe was used. The *in situ* hybridization protocol was adapted from Schaeren-Wiemers and Gerfin-Moser, 1993).

Transient Transfection and Dual Luciferase Detection

Cos-7 cells were plated in 6-well plates at 60-70% confluency. The cells were incubated overnight in Dulbecco modified Eagle medium (DMEM) + 10% fetal bovine serum (FBS) in 5% CO_2 at 37° C. The cells were then transfected the following day with FuGENE (Roche, Basel, Switzerland) according to the manufacturer's recommendation. Transient transfections were assayed with the dual Luciferase (Luc) reporter system (Promega, Madison, WI). Firefly Luciferase is used as the reporter, and *Renilla* Luciferase is used as a transfection efficiency control. The *per* E-box-Luc reporter construct was a gift from Dr. Richard Day. The promoter contains three direct repeats of the *mper1* E-box (Gekakis *et al.*, 1998) and 87 base pairs of the rat prolactin basal promoter (Day, R., unpublished). The reporter constructs were cotransfected with expression plasmids containing the x*Clock,* x*bmal1* and *xbmal1* mutant cDNAs as indicated below each measurement. One microgram of total DNA was transfected for each measurement, and the total amount of DNA was held constant. Transcriptional activity was assessed by measuring luciferase activity from cell lysates and normalizing to R*enilla* luciferase (under the control of a basal promoter), which served as a control for transfection efficiency. Each value is an average of four replicas (+/- SEM) and each experiment was repeated at least three times with similar results.

Western blot detection

Cos-7 cells were seeded to 70% confluency the night before transfection into 10-cm dishes. The following morning the cells were transfected with the *xbmal1-Flag* and *xClock-V5* constructs. 3μg of total DNA was transfected with 8.6μl of FuGENE (Roche, Basel, Switzerland) for each plate. The plates were incubated overnight and lysed. The lysis buffer used was composed of 50mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton-X 100 (Sigma-Aldrich Inc., St. Louis, MO). Cell lysates (10μl each) were run on a 9% SDS-PAGE gel and then blotted onto PVDF membrane (BIO-RAD, Hercules, CA). The membranes were blocked with Blotto solution (0.1% Tween 20, 5% dry non-fat skim milk powder in PBS, pH 7.4) either for 2 hours at room temperature or overnight at 4 °C. The primary antibodies (mouse anti-Flag M2, Stratagene, La Jolla, CA; mouse anti-V5, Invitrogen, Carlsbad, CA; anti-BMAL1, Affinity Bioreagents Inc., Golden, CO) were diluted in Blotto (anti-Flag M2, 1:1000; anti-V5, 1:2000; anti-xBMAL1, 1:200). The secondary antibody (anti-mouse IgG-peroxidase- POD) was diluted 1:1000 in Blotto. The bound antibody was detected via light emission following oxidation of luminol by peroxidase. For this, the Chemiluminescence Western Blotting kit (Boehringer Mannheim) was used.

Co-immunoprecipitation

Cos-7 cells were seeded to 70% confluency the night before transfection into 10-cm dishes. The following morning the cells were transfected with the *xbmal1-Flag* and *xClock-V5* constructs. 3μg of total DNA was transfected with 8.6μl of FuGENE (Roche, Basel, Switzerland) for each plate. The plates were incubated overnight and lysed. The

lysis buffer used was composed of 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100 (Sigma-Aldrich Inc., St. Louis, MO. Product No. L 3412). The lysates were then run over beads linked with anti-Flag antibodies, and the samples were co-immunoprecipitated following the FLAG Tagged Protein Immunoprecipitation Kit (Sigma-Aldrich Inc., St. Louis, MO). Samples were eluted using the 3X Flag peptide (also used on the Western blot as a positive control, Product No. F4799). Samples were then detected via Western blot (see above) either with anti-Flag antibodies (for xBMAL1 detection) or anti-V5 antibodies (for xCLOCK detection).

Phosphorylation studies

Cell lysates were incubated with λ-phosphatase (New England Biolabs, Beverly, MA) following manufacturer's recommendation. For phosphatase inhibition, the samples were incubated with 100 mM sodium orthovanadate (Sigma-Aldrich Inc., St. Louis, MO). Cells were lysed as described in "Transient Transfection and Dual Luciferase detection".

Results

Cloning of *bmal1* from the retina of *Xenopus laevis*

I cloned the *Xenopus* ortholog of *bmal1* (*xbmal1*) from a *Xenopus* retinal cDNA library and obtained a 2.5kb clone and a 3kb clone (Figure 1). Sequencing shows that the 2.5kb clone (*xbmal1-b*) is missing the first few hundred base-pairs and the start-site. The 3kb clone (*xbmal1-a*), in addition to the 5' and 3' UTRs, contains a 1.9kb open reading frame. Sequencing of the complete *xbmal1-a* clone revealed that the deduced amino acid sequence (632 amino acids, Figure 2) is 86% identical and 91.5% similar to the mouse BMAL1 (mBMAL1), 82% identical and 90% similar to zebrafish BMAL1 and 56% identical and 71% similar to zebrafish BMAL2. Identity is highest across the basic helix-loop-helix and PAS domains (Figure 3). *xbmall1-a* has been submitted to GenBank (accession #). I will refer to *xbmal1-a* as *xbmal1* throughout this study.

xbmal1 is expressed in the photoreceptor layer

In situ hybridization analysis shows that *xbmal1-a* and *xbamal1-b* are expressed in the photoreceptor layer of the *Xenopus* retina where the mRNA seems to be localized to the inner segments of both rods and cones (Figure 4; *xbmal1-a* only shown). I observed no difference between *xbmal1-a* and *xbmal1-b* expression. The *Xenopus* photoreceptor layer is also the site of expression of other clock genes such as *Clock*, *Cryptochrome*, *Period 1* and *2* (Steenhard and Besharse, 2000). I noticed a diffuse staining in the inner nuclear layers of the retina, which is not present in the sense control. However, this staining appears to be excluded from the cell bodies and is of much lower

intensity than the photoreceptor layer staining. Further studies will be needed to determine the specificity of this staining.

xbmal1 mRNA and protein are strongly rhythmic in *Xenopus* retina

Adult *Xenopus laevis* eyecups were isolated during the middle of the light cycle. The isolated eyecups were then cultured as described previously (Cahill and Besharse, 1991), either in the same light cycle as the animals (LD) or in constant darkness (DD), and the collection was commenced at ZT0 of the next cycle. Retinas were frozen at defined time points (Figure 5). For the eyecups maintained in constant darkness, I isolated the same CT time points, but the eyecups and retinas were never exposed to light. Results showed that *xbmal1-a* and *xbmal1-b* mRNA was strongly rhythmic, peaking in amplitude at ZT12, or the transition between light and dark (Figure 5). Rhythmicity in DD conditions was dampened yet still strongly present. As in the *in situ* hybridization, no difference was noticed between *xbmal1-a* and *xbmal1-b* expression.

xBMAL1 protein was also rhythmic, peaking between ZT18 and ZT22 (Figure 6). Although the signal was faint, a xBMAL1-specific band could be seen at ZT 18 and ZT 22 and at no other ZT time during the circadian cycle. The peak of mRNA rhythm (ZT12) correlated with the peak of protein rhythm (ZT18-ZT22), expected to lag by a few hours.

Xbma11 mRNA is not rhythmic in peripheral tissues

To determine if *xbmal1-a* and *xbmal1-b* transcripts are also rhythmic in other tissues besides the retina, I carried out Northern blot analyses of mRNA samples isolated

from liver, testis, brain and heart. The tissues were isolated every 6 hours over one circadian cycle. Northern blot analyses were performed as described above and I included the retina as a rhythmic positive control. Results showed that the tissues studied, retina excluded, did not display strong rhythmicity (Figure 7, *xbmal1-a* only shown). As previously observed, no difference was noticed between *xbmal1-a* and *xbmal1-b* expression (data not shown).

Transcriptional activation of xBMAL1

Since I noticed no difference in mRNA expression between the two *xbmal1* clones, they are probably the result *Xenopus*'s tetraploidy. Therefore, the following studies on xBMAL1 protein focused on the *xbmal1-a* clone. Transfection analysis demonstrated that when *Xenopus* BMAL1 was co-expressed with xCLOCK, it strongly activated transcription of the Luciferase reporter gene driven by three E-boxes. Activation was only present when *xClock* and *xbmal1* were co-transfected suggesting that heterodimer formation was necessary for transcription (Figure 8).

Deletion of the basic, helix-loop-helix , PAS-A and PAS-B domains of xBMAL1 generates proteins defective in transcriptional activation

The biochemistry behind CLOCK/BMAL1 interaction is not well understood. It is known that the two proteins heterodimerize and bind to the E-box (Gekakis *et al.*, 1998), but it is not known which domains are responsible for the heterodimerization. Sequence analysis revealed that xBMAL1 contains an HLH and PAS domain comprised of two repeats, the PAS-A and PAS-B domains, which have both been shown to be

involved in forming hetero- and homodimers among bHLH/PAS transcription factors (Reyes *et al.*, 1992; Huang *et al.*, 1993; Matsushita, *et al.*, 1993; Lindebro, *et al.*, 1995). To determine the effect of deletion of the functional domains on BMAL1-mediated transcriptional activation, I deleted the HLH and the PAS domains. As a positive control, I also deleted the basic domain. In other bHLH-PAS proteins, deletion of the basic domain has been shown to generate a dominant negative protein (Sato *et al.*, 2004). In addition, a dominant negative BMAL1 was recently generated by mutating a conserved arginine (R91) in its basic domain, which is thought to mediate E-box recognition (Hosoda *et al.*, 2004). My results show that deletion of the basic domain also generated a dominant negative construct; co-transfection of the mutant missing the basic domain (*xbmal1Δb*) with *xClock* and wild type *xbmal1* reduced xCLOCK/xBMAL1-mediated transcriptional activation. The mutant lacking the basic domain was unable to activate transcription when co-transfected with xClock or by itself, demonstrating that the basic domain is necessary for transcriptional activation (Figure 9A). Individually deleting the HLH, PAS-A or PAS-B domains generated constructs unable to activate transcription, indicating that these domains are necessary for xCLOCK/xBMAL1-mediated transcriptional activation of E-box-containing promoters (Figure 9B). Western blot studies showed that the wild-type and mutant xBMAL1 constructs were expressed at comparable levels in these cells, indicating that the lack of repression was not due to lower protein levels (Figure 10).

xCLOCK co-immunoprecipitates with xBMAL1 *in vitro*

To test the hypothesis that xBMAL1 binds to xCLOCK, I co-immunoprecipitated the two proteins. Flag-tagged xBMAL1 was immunoprecipitated using highly specific bead-conjugated anti-Flag antibodies. Results show that wild type and mutant xBMAL1 (xBMAL1Δb and xBMAL1ΔHLH) bound to the bead-conjugated anti-Flag antibodies (Figure 11A, elution). Since the amount of protein detected after elution is higher compared to the amount of protein not bound to the beads (Figure 11A, flow through), the binding of xBMAL1 to bead-conjugated anti-Flag antibodies was very efficient. In addition, I carried out preliminary studies in which I co-immunoprecipitated xCLOCK bound to xBMAL1. Results show that when xCLOCK and xBMAL1 were overexpressed in Cos-7 cells they bound and co-immunoprecipitated, even though small levels of xCLOCK were detected in the eluted sample. This demonstrates that xBMAL1 can bind to xCLOCK *in vitro* (Figure 11B). Western blot of Cos-7 lysates transfected with *xClock* demonstrated that xCLOCK is a protein of about 100kDa, which is the same size as the protein co-immunoprecipitated with xBMAL1 (Figure 11B, right).

xBMAL1 is phosphorylated in Cos-7 cells

In mouse, BMAL1's phosphorylation seems to be important for its nuclear localization, transcriptional activation and heterodimer formation (Lee *et al.*, 2001; Sanada *et al.*, 2001; Eide *et al.*, 2002; Kondratov *et al.*, 2003). To investigate if xBMAL1 is phosphorylated, I treated wild type and mutant *xbmal1* cell lysates with phosphatase. Cos-7 cells were transfected with *xClock* and *xbmal1* wild type and mutant constructs (*xbmal1Δb* and *xbmal1ΔHLH*). I included *xClock* because there is evidence

that its presence might be necessary for BMAL1 phosphorylation (Kondratov *et al.*, 2003). The cells were then lysed, and the lysates were incubated with λ-phosphatase alone or with λ-phosphatase and sodium orthovanadate, a λ-phosphatase inhibitor.

My results show that wild type xBMAL1, xBMAL1Δb and xBMAL1ΔHLH were phosphorylated. Two xBMAL1 bands were present in both the wild type and mutant lanes, and all forms of the protein seemed to be phosphorylated. Even though previous evidence had suggested that BMAL1 might appear in two forms, one of which might be phosphorylated BMAL1 only when CLOCK is present (Kondratov *et al.*, 2003), this was not true for the studies described here. When *xbmal1* and *xbmal1ΔHLH* were transfected alone, two xBMAL1 bands were still present.

xBMAL1 induces xCLOCK proteolytic degradation

Kondratov *et al.* (2003) have found that co-expression of *mbmal1* with *mClock* causes a dramatic reduction in mCLOCK protein levels, possibly due to proteolytic degradation. They also found that this effect was not due to the cell type used, since they detected similar results in HEK293 and NIH3T3 cells. In addition, they suggested that reduced CLOCK amounts were not due to possible repression of the CMV promoter driving *mClock* by mBMAL1, although direct evidence to support this statement was not shown (Kondratov *et al.*, 2003). I have found a very similar effect of xBMAL1 on xCLOCK. xCLOCK levels were undetectable when *xClock* and *xbmal1* were co-expressed in Cos-7 cells. On the other hand, high levels of xCLOCK protein were detected when *xClock* was expressed alone (Figure 13).

Discussion

The study of *bmal1* in the retina is important to understand how the clock is regulated in *Xenopus* and in vertebrates in general. The biochemistry regulating the CLOCK/BMAL1 heterodimer is not well understood. The DNA binding activity of the CLOCK/BMAL1 is regulated by the redox state of NAD cofactors *in vitro* (Rutter *et al.*, 2001). In mouse liver, the promoter regions of *Per1/2* and *Cry1* exhibit rhythms in H3 histone acetylation and RNA polymerase II binding. The CRY proteins inhibit p300-induced transcriptional activation mediated by the CLOCK/BMAL1 heterodimer, and therefore H3 histone acetlyation (Etchegaray *et al.*, 2003).

Even though these studies shed some light on the mechanism of CLOCK/BMAL1-mediated transcriptional activation, many questions still remain. For example, it is not known which domain in BMAL1 is responsible for interaction with CLOCK. The xBMAL1 mutants I generated in this study, in combination with the transgenic technique in frogs, offer a tool to understand this interaction. *Xenopus* is an excellent system for the generation of transgenic frogs (Kroll and Amaya, 1996), and the phenotypes of the *bmal1* mutants generated in this study could be tested in transgenic tadpoles to better understand the biochemistry of BMAL1 and of its molecular interaction with CLOCK. Although the retina is not an ideal system to study the biochemistry of this protein because of small tissue size, as opposed to, for example, the liver, this downfall can be supplemented with tissue culture studies and the generation of transgenic animals.

In this study I have cloned two *xbmal1* clones: *xbmal1-a* and *xbmal1-b*. *xbmal1-a* is a 3 kb clone comprised of the 5'UTR, the coding region and the 3'UTR. The *xbmal1-b* clone is lacking the 5'UTR and a portion of the coding region (approximately the first 75 amino acids). Since *Xenopus laevis* is a tetraploid organism, and because I have not detected any difference in expression between *xbmal1-a* and *xbmal1-b* (as far as mRNA localization in retina and in tissues and rhythmicity in the retina and other tissues), the two clones are most likely the result of genome duplication: it is in fact unlikely that they display functional differences.

I have found that *xbmal1* is expressed in the photoreceptor layer, the retinal cells that have been demonstrated to contain a circadian clock (Cahill and Besharse, 1993). In the retina, *xbmal1* is strongly rhythmic in LD and DD, even though rhythmicity is dampened in DD. Dampening is possibly due to the photoreceptor cells losing synchrony. Recent evidence from rhythmic rat-1 fibroblasts and mouse embryonic fibroblasts demonstrates that single fibroblasts can oscillate robustly with undiminished amplitude and that dampening is most likely due to loss of synchrony amongst cells (Welsh *et al.*, 2004). Studies in the *Xenopus* retina show that dopamine mimics light in entraining and phase-shifting the retinal clock (Cahill and Besharse, 1989, 1990, 1991). Photoreceptor cells are joined via gap junctions, and they are also contacted by other retinal cell types, the horizontal cells and bipolar cells (Witkovsky, 2000). It is possible that photoreceptor cells necessitate either light input or neuronal connections with other cells to maintain long term synchrony in the dark, and that dissection of the retina might weaken these inputs. Surprisingly, no rhythmicity was detected in any of the non retinal tissues tested (brain, heart and testis), although the retinas isolated during the same study

were rhythmic. In mouse and rat most of the tissues display rhythmicity, which persist in culture for many days (Yamazaki *et al.*, 2002, Yoo et al., 2004). Failure to detect *xbmal1* rhythmicity in *Xenopus* tissues (retina excluded) could be due to a difference in the regulation of the promoter. For example, while rhythmic factors might control *xbmal1* rhythmicity in the retina, different non-rhythmic factors might control its expression in the tissues tested. This divergence between the tissues and the retina might lead to interesting models for how the clock is regulated.

BMAL1 is part of the bHLH-PAS family of proteins, known to regulate mechanisms including cell fate and division, protection from environmental contaminants, neurogenesis and circadian rhythms (Crews, 1998; Gu *et al.*, 2000). The mechanism by which the CLOCK/BMAL1 heterodimer is repressed is unknown, but one hypothesis is that repression is mediated via the PAS domain. The PAS domain functions in a variety of ways, which include protein-protein interaction and ligand binding. For example, the hypoxia-inducible factor alpha (HIF-1α), a ubiquitous bHLH-PAS transcription factor, plays a key role in the response to low oxygen. During normal oxygen conditions, defined as normoxia, HIF-1α binds to an ubiquitin ligase, and is subsequently degraded. During low-oxygen stress, HIF-1α's protein stability and transcriptional potency increases, and HIF-1α activates target genes encoding erythropoietin, vascular endothelia growth factors and enzymes involved in anaerobic energy production (Semenza, 2000). During low-oxygen conditions, hydroxylation of a conserved asparagine in the C-terminal transactivation domain (CAD) is abrogated, allowing the CAD to interact with p300 and activate transcription (Lando *et al.*, 2002). It is known that in liver the CLOCK/BMAL1 heterodimer is bound to DNA throughout the

circadian cycle (Lee *et al.*, 2001), indicating that simple heterodimerization and DNA binding are not sufficient for transcriptional activation. Although the switch activating the CLOCK/BMAL1 heterodimer is not known, it is known that it probably involves phosphorylation and binding of p300 to CLOCK (Etchegaray *et al.*, 2003).

It is likely that the CLOCK/BMAL1 heterodimer functions via a similar switch where, possibly, the PAS domain is used as a switch controlled by the redox state of its repressors, the CRYs (Rutter *et al.*, 2002). In fact, mutation of the flavin-binding domain of xCRY2b dramatically reduces its repressive activity (Zhu and Green, 2001). Here, I have shown that xBMAL1 binds to xCLOCK and that the heterodimer activates transcription of a Luciferase reporter gene driven by three CLOCK/BMAL1 binding sites. Deletion of either the HLH, PAS-A or PAS-B domains of xBMAL1 cause loss of transcriptional activation, indicating that all the domains are required. This suggests that both the HLH and PAS domains may be required for the interaction between CLOCK and BMAL1 and therefore for transcriptional activation. Another possibility is that only the HLH is required for heterodimer formation, and that the PAS domain is required for transcriptional activation.

Phosphorylation has been shown to be important for BMAL1 interaction with CLOCK and for transcriptional activation. In fact, co-expression of *mbmal1* with a dominant negative *mCKIε* reduces transcriptional activation by 40% (Eide *et al.*, 2002). In addition, co-expression of chicken *bmal1* with a constitutive active form of MAPK that phosphorylates BMAL1 *in vitro*, suppresses CLOCK/BMAL1-mediated transcriptional activation (Sanada *et al.*, 2001). Here I have shown that xBMAL1 is also phosphorylated and that phosphorylation is not dependent on the presence of the basic or

HLH domains. Phosphorylation of xBMAL1 might also control transcriptional activation and its subcellular localization, as appears to be the case in mouse (Kondratov *et al.*, 2003).

In these studies I observed that xBMAL1 induced the proteolytic degradation of xCLOCK. Although both proteins were driven by the CMV promoter, Kondratov *et al.* (2003), who also noticed a similar phenomenon, state that reduction of *Clock* expression is not due to BMAL1-mediate repression via the CMV promoter. Possibly, formation of the heterodimer could lead to the degradation of CLOCK, therefore introducing an additional level of regulation of the circadian cycle. These authors also showed that BMAL1 is only phosphorylated when co-expressed with *Clock*. They detected a 2-band pattern of BMAL1 similar to my results and hypothesized that the slower band represents phosphorylated BMAL1. In these studies they did not show direct phosphatase treatment (Kondratov *et al.*, 2003). In my studies, I observed the presence of the 2-band pattern when *xbmal1* was expressed alone and with *xClock*: in addition, treatment of the lysates with phosphatase did not cause the disappearance of either band, suggesting that the additional band was not due to phosphorylation, but instead to an alternate start-site.

These studies demonstrate that in the *Xenopus* retina *bmal1* is under the control of the clock. Many of the mechanisms controlling BMAL1 in mammals seem to be conserved in *Xenopus*, making this organism an excellent system to study the biochemistry regulating this circadian transcription factor. Generation of transgenic tadpoles expressing the *xbmal1* mutants I generated in this study will answer questions about the function of the different domains. In fact, no functional *in vivo* study exploring

loss of the BMAL1 HLH or PAS domains has yet been carried out, and it is not known how CLOCK and BMAL1 bind, or how BMAL1 contributes to transcriptional activation.

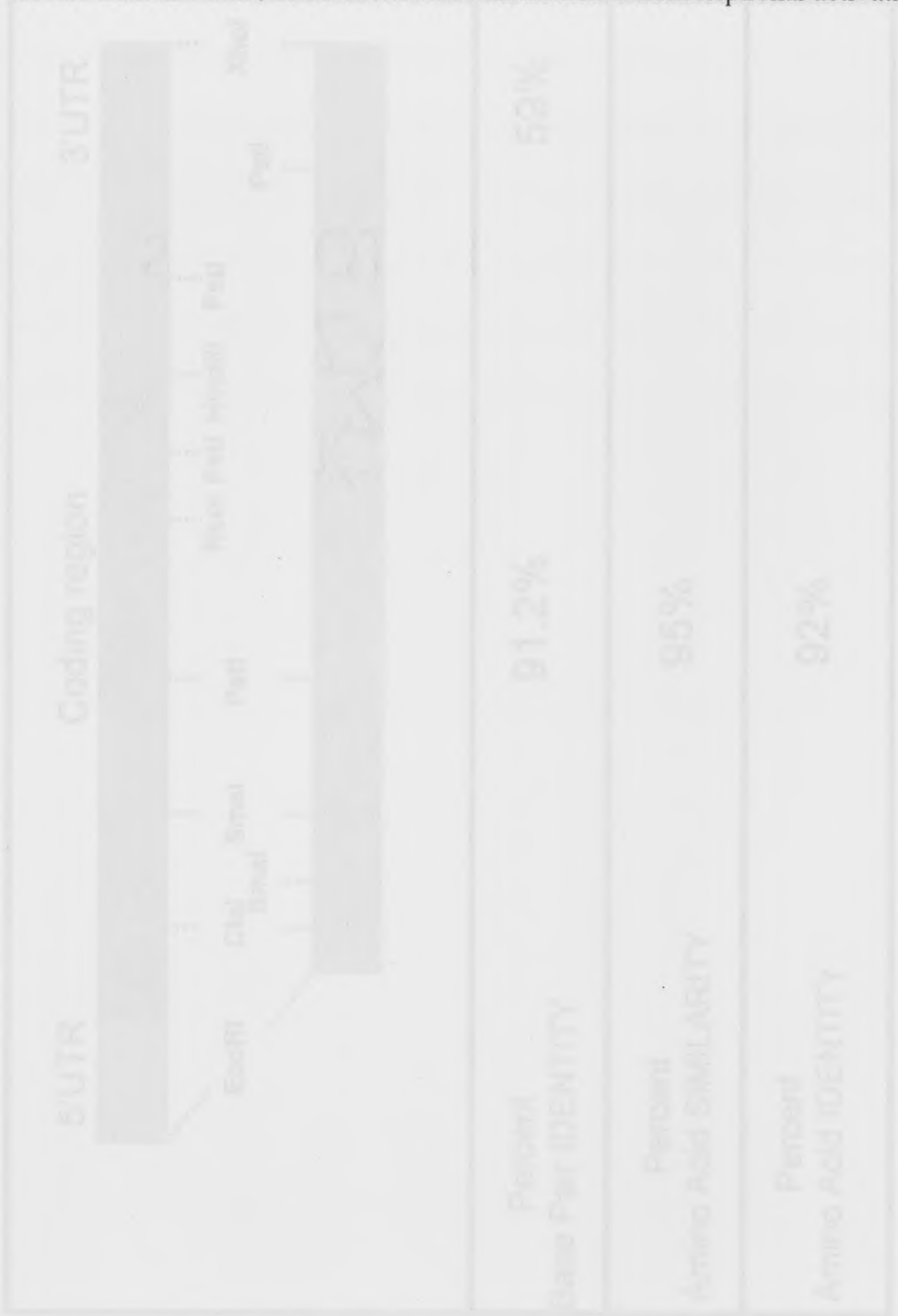


Figure 2-1: *xbmal-1a* and *xbmal1-b* are highly similar

Figure 2-1: *xbmal-1a* and *xbmal1-b* are highly similar

Schematic representation of the alignment between the *xbmal1-a* and *xbmal1-b* clones. The alignment was generated with MacVector. (Oxford Molecular). The top shows the two cDNA clones and the unique restriction sites in each one. EcoRI, ClaI, SmaI, PstI, and XhoI are present in both clones. The red area represents the coding region, the gray area represents the 5'UTR and the light blue the 3'UTR. The bottom shows the percent base-pair identity in the coding region and the 3'UTR and the percent similarity and identity between the deduced amino acid sequences for xBMAL1-a and xBMAL1-b.

Figure 2-2: xBMAL1, mBMAL and zebrafish BMAL1 and 2 (zfBMAL1/2) are highly homologous.

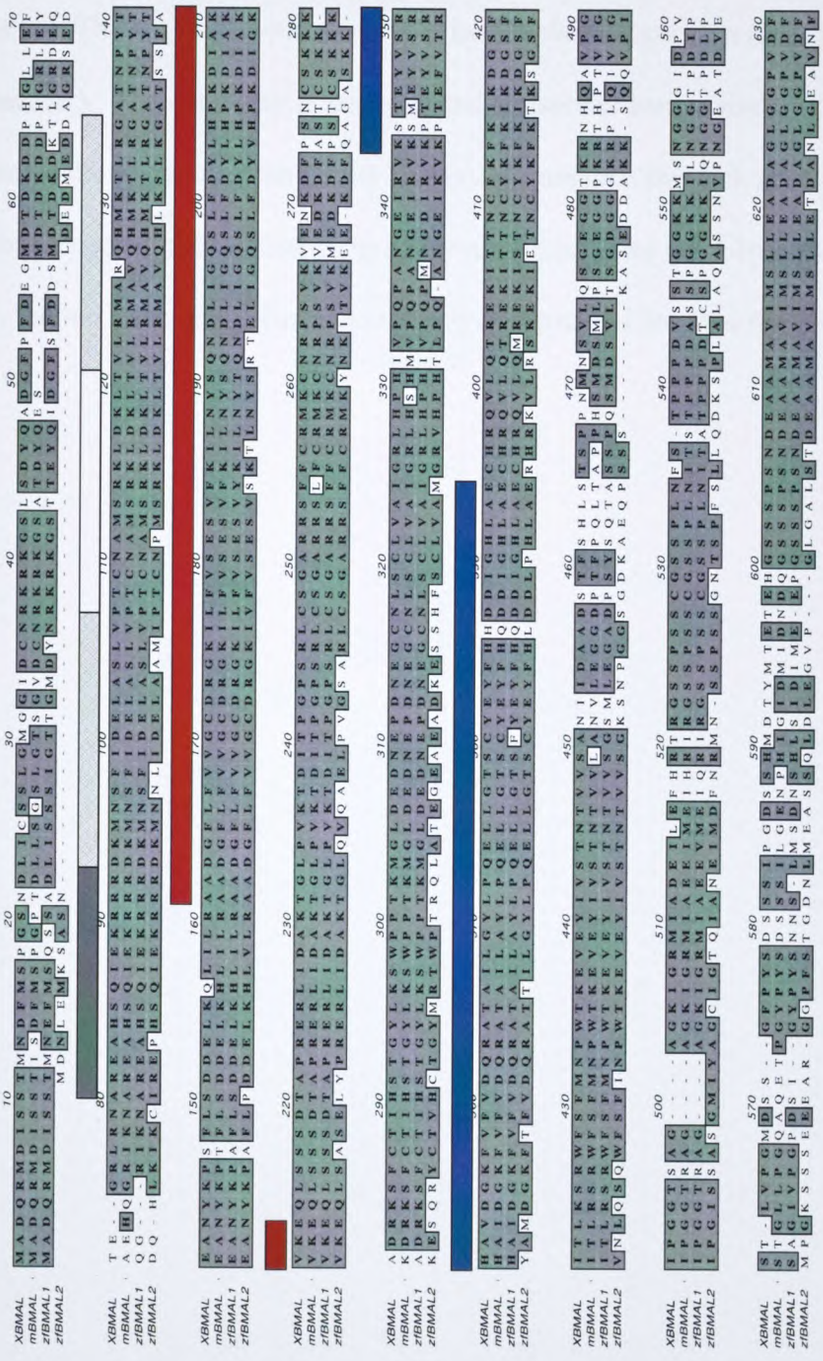


Figure 2-2: xBMAL1, mBMAL and zebrafish BMAL1 and 2 (zfBMAL1/2) are highly homologous.

Protein alignment between xBMAL1 (was xBMAL1-a), mBMAL1 and zfBMAL1 and 2. The alignment was generated by ClustalW algorithm (MacVector, Oxford Molecular). The dark gray areas indicated conserved amino acids. The rectangles above the sequences denote the conserved domains: the dark gray rectangle denotes the basic region, the gray/white/gray rectangles indicate helix-loop-helix domains respectively, the red rectangle defines the PAS-A domain, and the blue the PAS-B domain.

Figure 2-3: xBMAL1 is particularly conserved across the basic, HLH and PAS domains.

Figure 2-3: xBMAL1 (xBMAL1-a) is particularly conserved across the basic, HLH and PAS domains.

Schematic representation of the percent identity (top) and percent similarity (bottom) between xBMAL1, mBMAL1 and zfBMAL1 and 2. Numbers represent percentage.

Figure 2-4: *xbmal1* is expressed in the photoreceptor layer of the *Xenopus* retina.

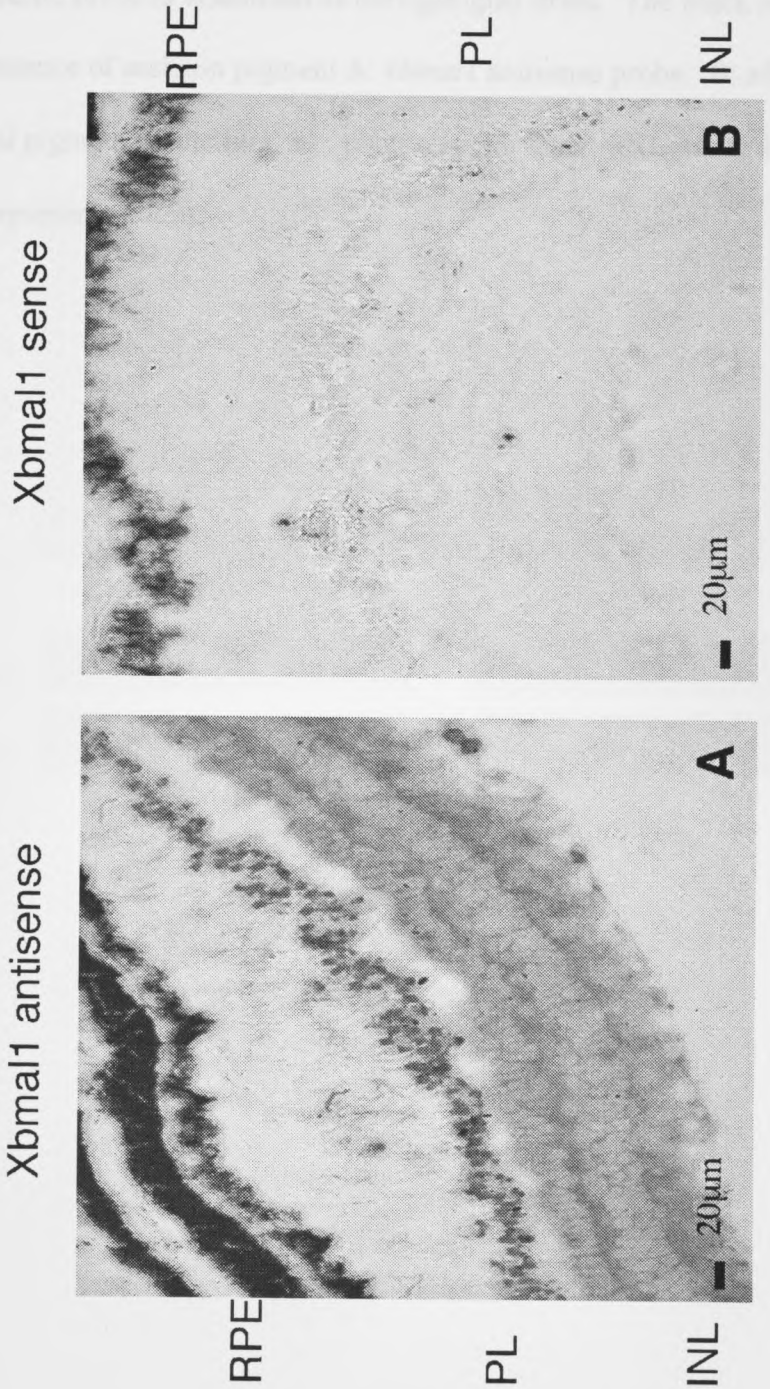


Figure 2-4: *xbmal1* is expressed in the photoreceptor layer of the *Xenopus* retina.

In situ hybridization of *xbmal1* in the *Xenopus* retina. Hybridization of the *xbmal1*-specific probe is visualized as the light-gray areas. The black regions in the RPE indicate presence of melanin pigment **A**: *xbmal1* antisense probe. **B**: *xbmal1* sense probe. RPE: retinal pigment epithelium. PL: photoreceptor layer. INL: inner nuclear layer. The black bar represents 20μm.

Figure 2-5: ***xbmal1*** **is strongly rhythmic in the retina of** ***Xenopus laevis*** **in LD and DD.**

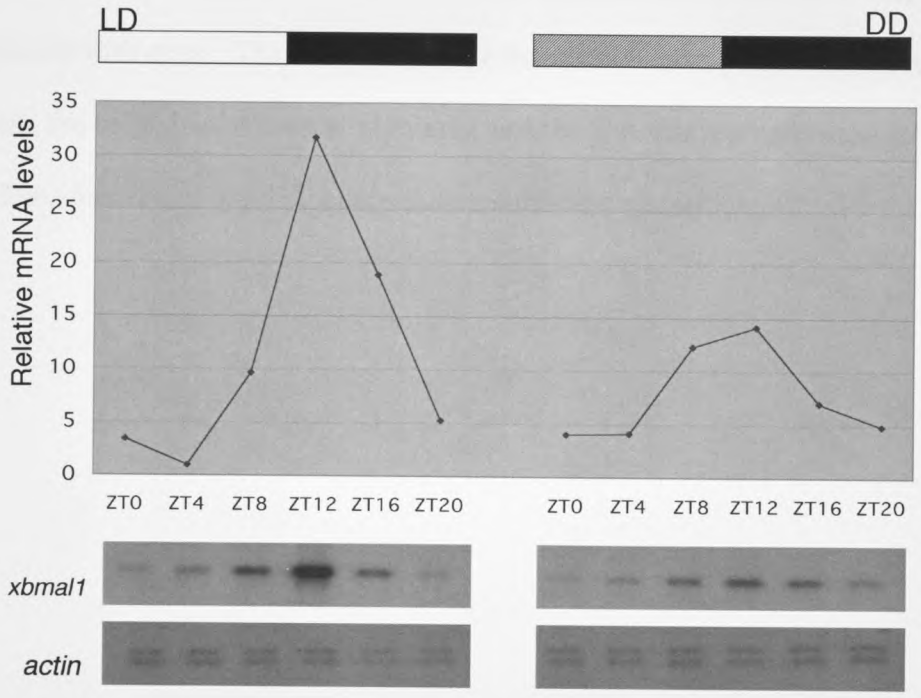


Figure 2-5: *xbmal1* is strongly rhythmic in the retina of *Xenopus laevis* in LD and DD.

Northern blot hybridization of *xbmal1* of retinal mRNA collected at the indicated ZT and CT times. Relative mRNA levels were quantified on a phospho imager and normalized with *actin*. The white bar above the mRNA quantification represents daytime, the black bars represent nighttime and the gray bar represents the subjective day. The *xbmal1* and *actin* blots are shown below the quantification.

Figure 2-6: xBMAL1 protein is rhythmic in the retina of *Xenopus laevis*.

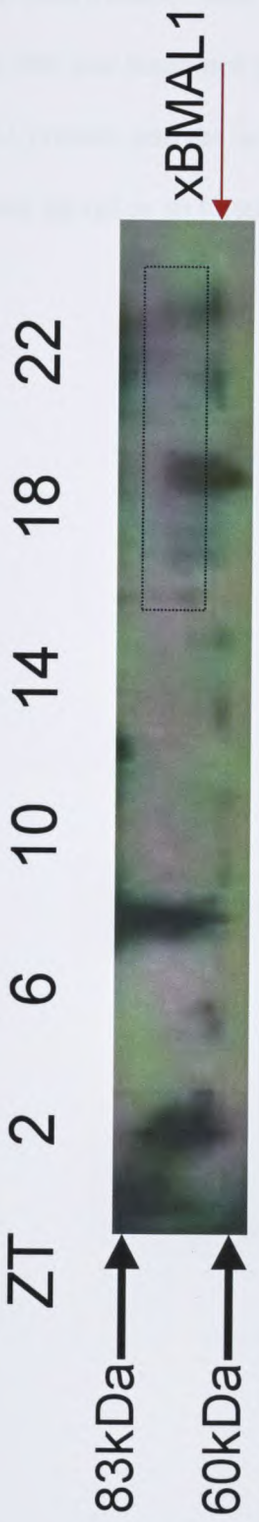


Figure 2-6: xBMAL1 protein is rhythmic in the retina of *Xenopus laevis*.

Western blot of total retinal lysates collected at the indicated ZT times. The Western blot was incubated with anti-BMAL1 antibodies. The gray rectangle indicates xBMAL1 protein, and the red arrow points to a lower molecular weight unspecific band, which also serves as an internal loading control.

Figure 2-7: *xbmal1* is not rhythmic in the tissues of *Xenopus laevis*.

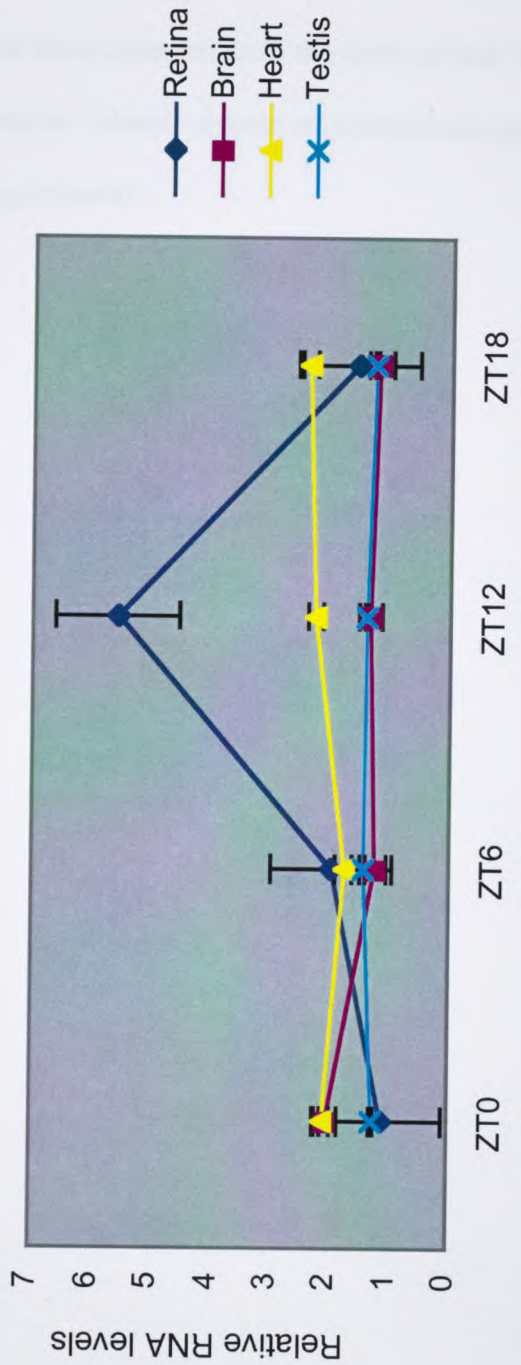


Figure 2-7: *xbmal1* is not rhythmic in the tissues of *Xenopus laevis*.

Quantification of a Northern blots of *xbmal1* from retina, brain, heart and testis of *Xenopus*. The tissues were collected from the same animal at the indicated times and used for mRNA isolation. *xbmal1* signal was normalized against actin. Values represent three independent experiments.

Figure 2-8: ***xbmal1*** **activates transcription when co-transfected with** ***xClock*****.**

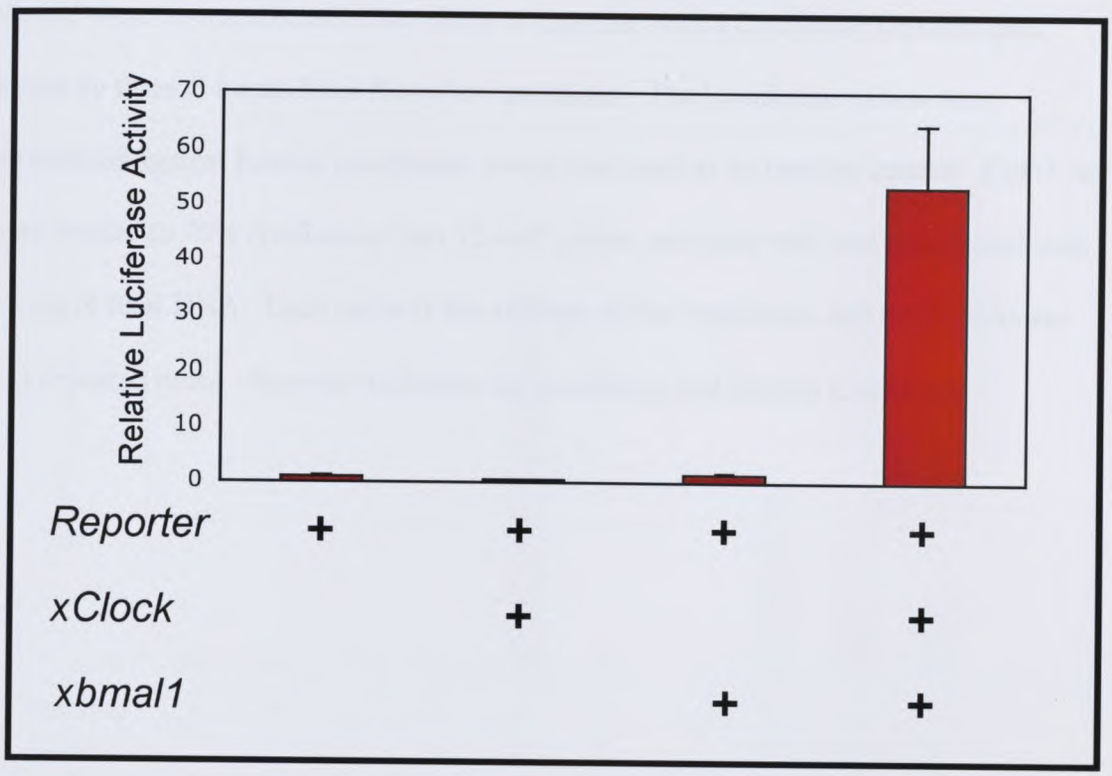


Figure 2-8: *xbmal1* activates transcription when co-transfected with *xClock*.

Relative Luciferase activity from transient transfection experiments. *xClock* and *xbmal1* were co-transfected either alone or together with a Luciferase reporter gene driven by three E-boxes from the *mPer1* promoter. The Luciferase values were normalized against Renilla Luciferase, which was used as an internal control. Cos-7 cells were seeded to 70% confluency into 12-well plates, and each well was transfected with 1.5 μg of total DNA. Each value is the average of four replicates, and the experiment was repeated twice. *Reporter* indicates the Luciferase and Renilla Luciferase.

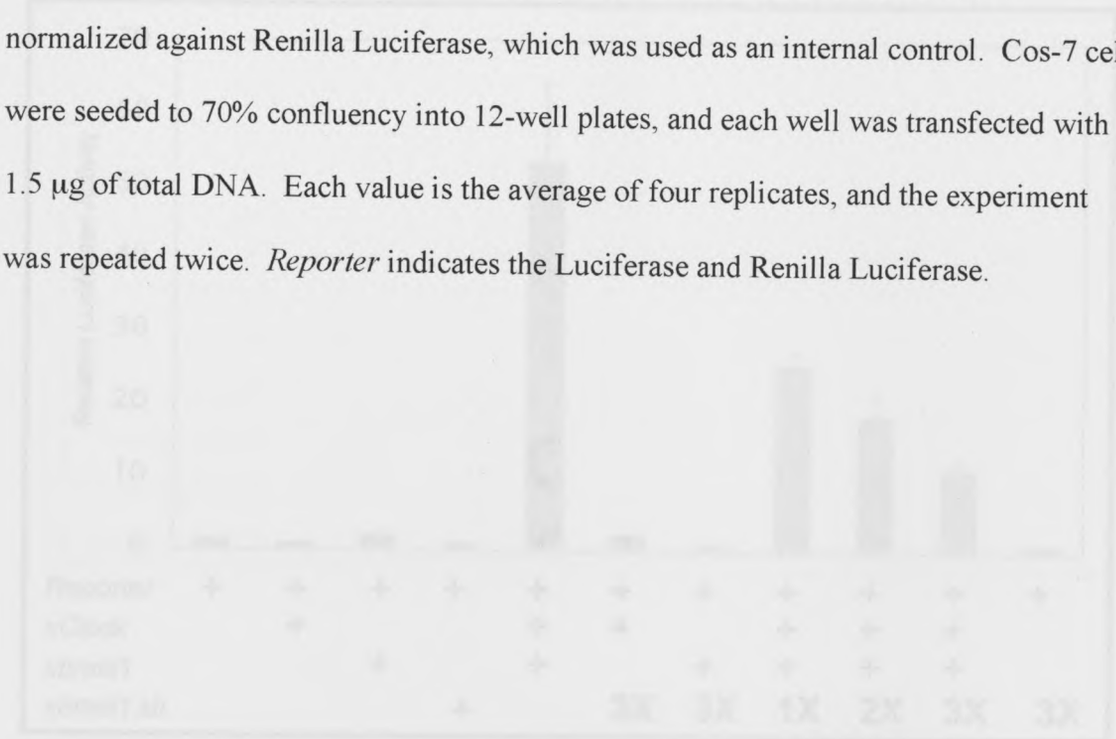
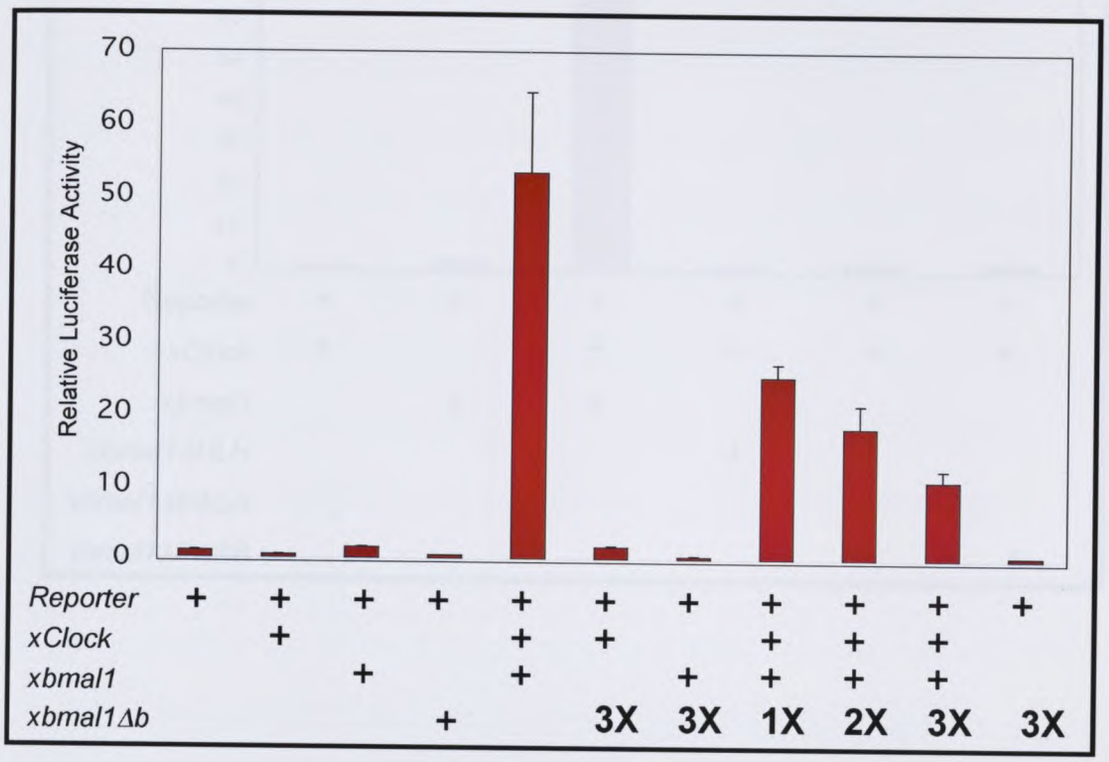


Figure 2-9: Deletion of the basic, HLH or PAS domain abolishes xBMAL1 transcriptional activation.

A



B

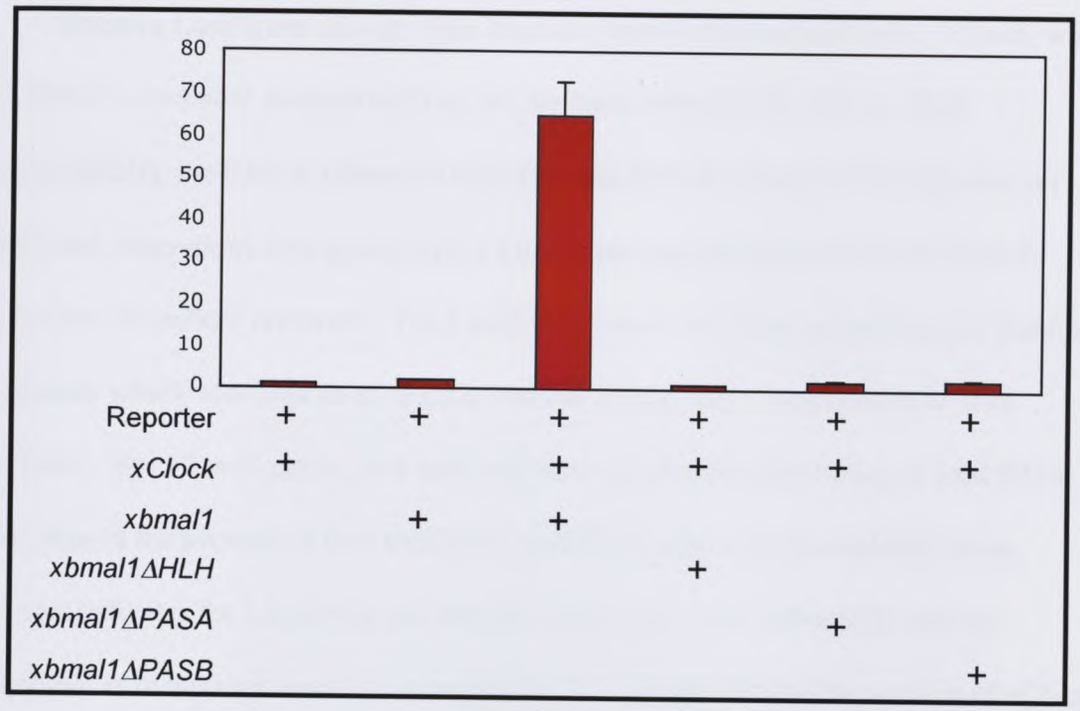

80
70
60
50
40
30
20
10
0
Reporter + + + + + +
xClock + + + + +
xbmal1 + +
xbmal1ΔHLH +
xbmal1ΔPASA +
xbmal1ΔPASB +

Figure 2-9: Deletion of the basic, HLH or PAS domain abolishes xBMAL1 transcriptional activation.

Relative Luciferase activity from transient transfection experiments. *xClock,* wild type *xbmal1* or *xbmal1* mutants lacking (**A**) the basic (*xbmal1Δb*), (**B**) the HLH (*xbmal1ΔHLH*), the PAS-A (*xbmal1ΔPAS-A*) or the PAS-B (*xbmal1ΔPAS-B*) were co-transfected either alone or together with a Luciferase reporter gene driven by three E-boxes from the *mPer1* promoter. The Luciferase values were normalized against Renilla Luciferase, which was used as an internal control. Cos-7 cells were seeded to 70% confluency into 12-well plates, and each well was transfected with 1.5 μg of total DNA. Each value is the average of four replicates, and the experiment was repeated twice. *Reporter* indicates the Luciferase and Renilla Luciferase. In **A**, *xbmal1Δb* was co-transfected in increasing amounts, respectively 1X, 2X and 3X than the amount of *xClock* and wild type *xbmal1*. In **B**, the same amount of wild type and ***xbmal1*** mutants was used (0.15 μg). 0.15 μg of each *xClock* and *xbmal1* was used in each experiment.

Figure 2-10: Wild type and mutant *xbmal1* are expressed at comparable amounts in Cos-7 cells.

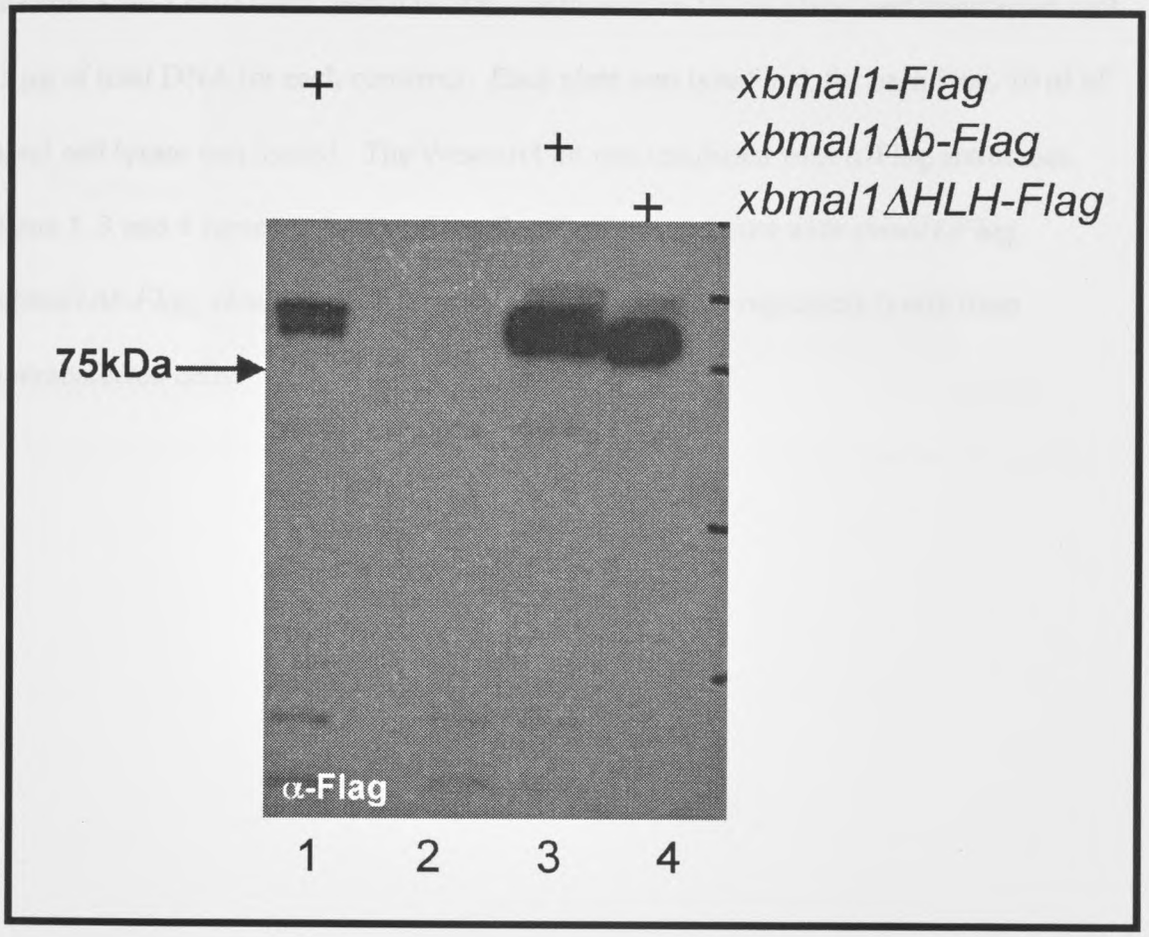
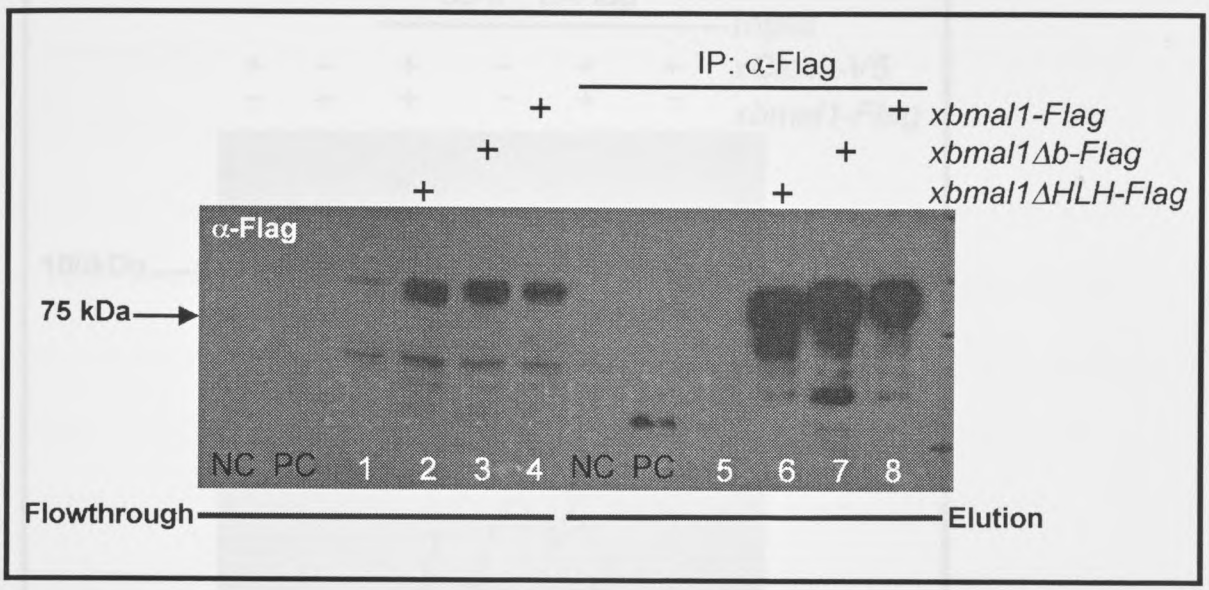


Figure 2-10: Wild type and mutant *xbmal1* are expressed at comparable amounts in Cos-7 cells.

Cos-7 cells were transfected with the indicated wild type or mutant *xbmal1-Flag* constructs. Each *xbmal1* construct is fused to the Flag peptide in the pCMV-Tag2b vector. Cos-7 cells were seeded to 70% confluency in 10-cm plates and transfected with 3 μg of total DNA for each construct. Each plate was lysed and, for each lane, 10 μl of total cell lysate was loaded. The Western blot was incubated with α-Flag antibodies. Lane 1, 3 and 4 represent lysates from Cos-7 cells transfected with *xbmal1-Flag*, *xbmal1Δb-Flag*, *xbmal1ΔHLH-Flag* respectively. Lane 2 represents lysate from untransfected cells.

Figure 2-11: xCLOCK and xBMAL1 co-precipitate *in vitro*.

A



B

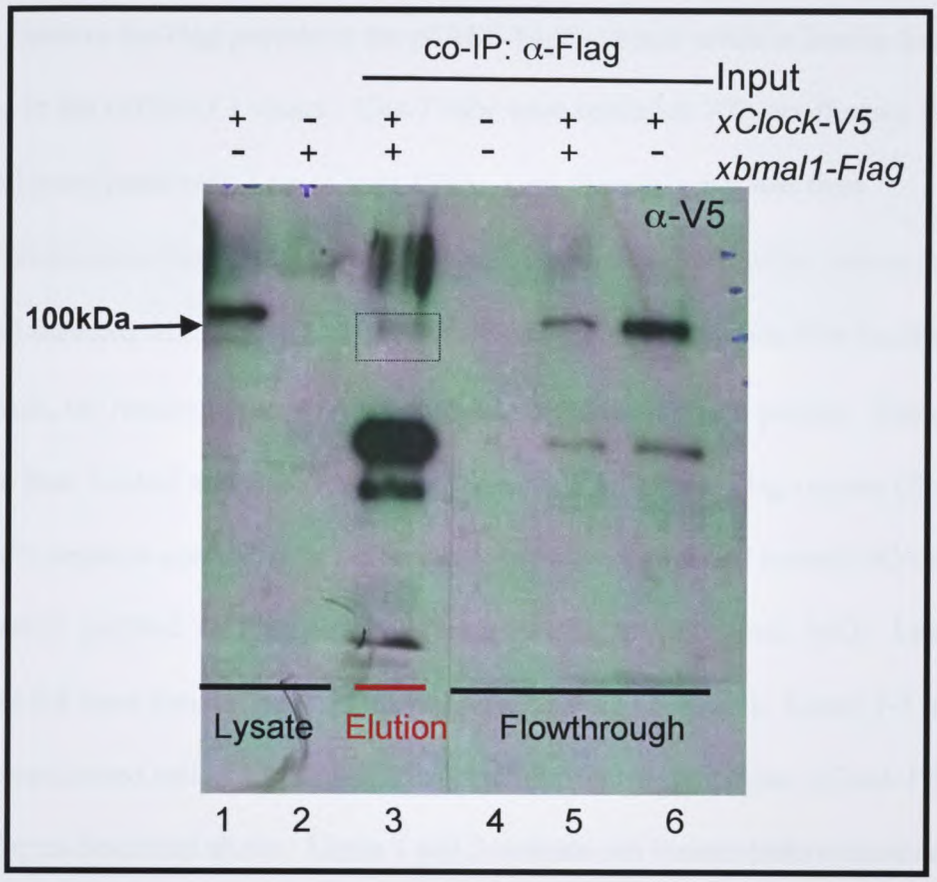

co-IP: α-Flag
Input
+ - + - + +
- + + - + -
xClock-V5
xbmal1-Flag
α-V5
100kDa
Lysate
Elution
Flowthrough
1 2 3 4 5 6

Figure 2-11: xCLOCK and xBMAL1 co-precipitate *in vitro*.

Cos-7 cells were transfected with the indicated constructs. Each *xbmal1* construct is fused to the Flag peptide in the pCMV-Tag2b vector, while *xClock* is fused to the V5 tag in the pcDNA3.1 vector. Cos-7 cells were seeded to 70% confluency in 10-cm plates and transfected with 3 μg of total DNA. 1 ml of each lysate was then immunoprecipitated (IP) with resin-conjugated α-Flag antibodies, run on a Western blot and detected with α-Flag (**A**) or α-V5 (**B**) antibodies. After the first incubation with the beads, the remaining lysate (Flowthrough) contains unbound protein. The α-Flag beads are then washed and eluted with a peptide containing three Flag repeats (Elution). (**A**) NC= negative control: lysis buffer only (same amount as cell lysate). PC= positive control: purified 3X Flag peptide (Sigma-Aldrich Inc., St. Louis, MO). Lanes 2-6, 3-7 and 4-8 were transfected with the indicated *xbmal1* constructs. Lanes 1-5 indicate untransfected cells. (**B**) Cos-7 cells were transfected with either *xClock-V5* or *xbmal1-Flag* as described above. Lanes 1 and 2 indicate cell lysates before immunoprecipitation. Lane 4 indicates lysate of untransfected cells after binding to the α-Flag beads, and lanes 5 and 6 indicate lysate of cells transfected either with both *xClock* and *xbmal1* (5) or with *xClock* alone (6) after binding to the α-Flag beads. Lane 3 indicates eluted protein from the α-Flag beads. Grey rectangle indicates co-immunoprecipitated xCLOCK.

Figure 2-12: xBMAL1 is phosphorylated.

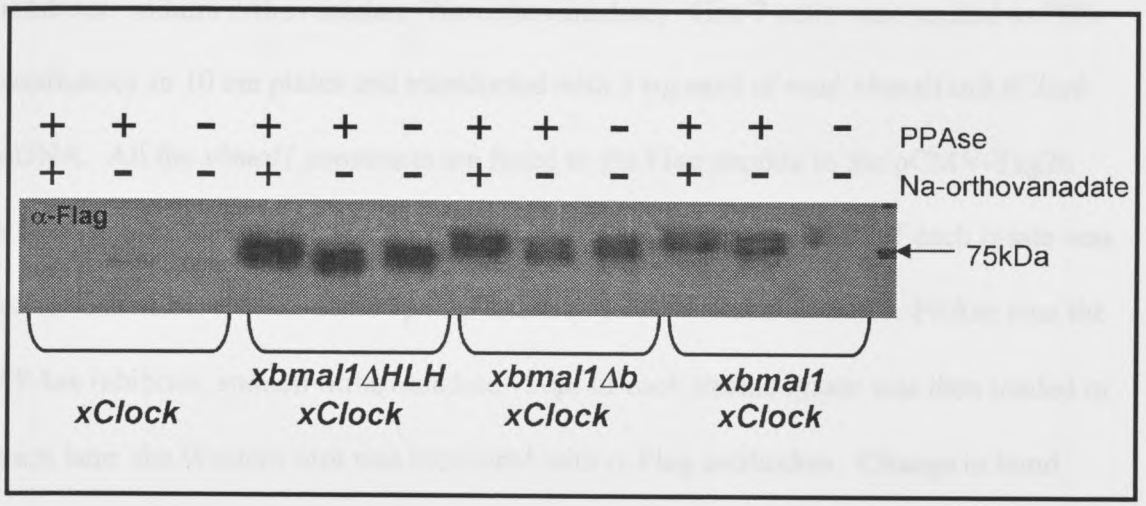


Figure 2-12: xBMAL1 is phosphorylated.

Western blot of cell lysates treated with λ-phosphatase (PPAse) and the phosphatase inhibitor, sodium orthovanadate (Na-orthovanadate). Cos-7 cells were seeded to 70% confluency in 10 cm plates and transfected with 3 μg each of total *xbmal1* and *xClock* cDNA. All the *xbmal1* constructs are fused to the Flag peptide in the pCMV-Tag2b vector; *xClock* is fused to the V5 tag in the pcDNA3.1 vector. 20 μl of each lysate was either untreated, treated with λ-phosphatase (λ-PPAse) alone or with λ-PPAse plus the PPAse inhibitor, sodium orthovanadate. 10μl of each treated lysate was then loaded in each lane: the Western blot was incubated with α-Flag antibodies. Change in band mobility indicates a change in the phosphorylation state of the protein.

Figure 2-13: xBMAL1 induces the proteolytic degradation of xCLOCK.

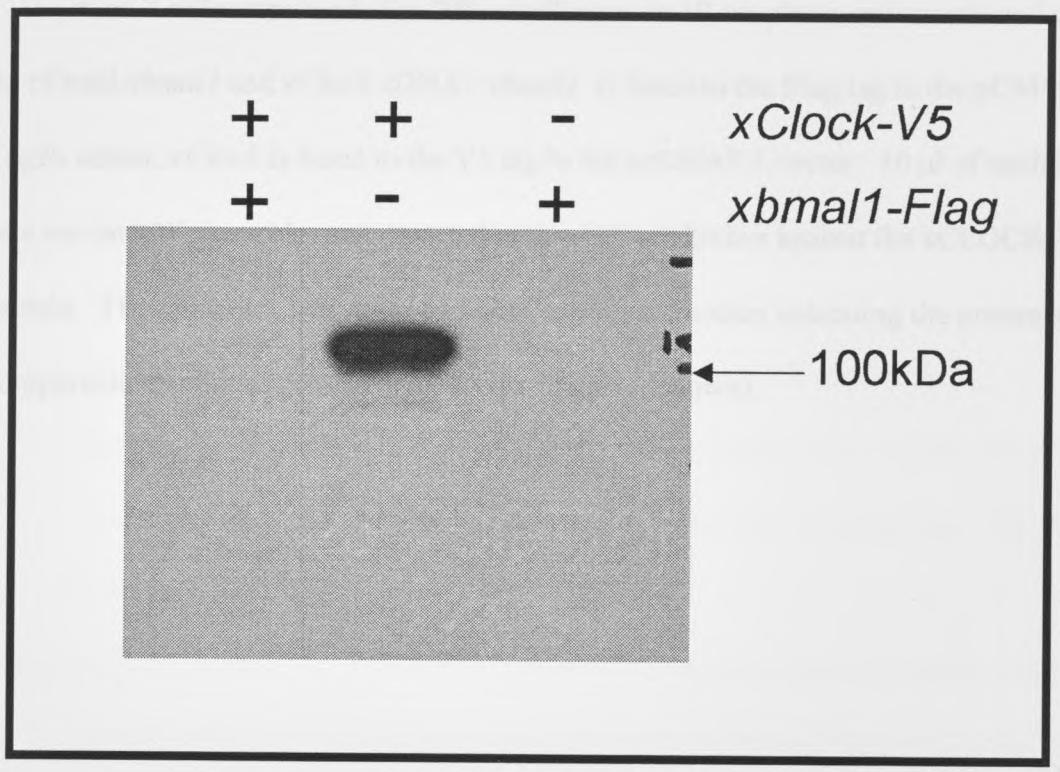


Figure 2-13: xBMAL1 induces the proteolytic degradation of xCLOCK.

Cos-7 cells were seeded to 70% confluency in 10 cm plates and transfected with 3 μg of total *xbmal1* and *xClock* cDNA. *xbmal1* is fused to the Flag tag in the pCMV-Tag2b vector; *xClock* is fused to the V5 tag in the pcDNA3.1 vector. 10 μl of each lysate was run on a Western blot and detected with α-V5 antibodies against the xCLOCK-V5 protein. The same blot was re-probed with α-Flag antibodies indicating the presence of comparable amount of protein in each lane (data not shown).

References

Abe, H., Honma, S., Namihira, M., Tanahashi, Y., Ikeda, M. et al (1998) Circadian rhythm and light responsiveness of BMAL1 expression, a partner of mammalian clock gene *Clock*, in the suprachiasmatic nucleus of rats. *Neurosci. Lett.* , **258**: 93 96.

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

Burbach, K.M., Poland, A., Bradfield, C.A. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci.*, **89**: 8185-8189.

Cahill, G.M. and Besharse, J.C. (1989) A D-2 dopamine receptor agonist resets the phase of a circadian clock in cultured eyecups from *Xenopus laevis*. *Soc. Neurosci. Abstr.*, **15**: 24.

Cahill, G.M. and Besharse, J.C. (1990) Circadian regulation of melatonin in the retina of *Xenopus laevis*: limitation by serotonin availability. *J. Neurochem.*, **54**: 716-719.

Cahill, G.M. and Besharse, J.C. (1991) Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. *J. Neurosci.*, **11**: 2959-2971.

Cahill, G.M. and Besharse, J.C. (1993) Circadian clock functions localized in *Xenopus* retinal photoreceptors. *Neuron*, **10**: 573-577.

Cahill, G.M., and Besharse, J.C. (1995) Circadian rhythmicity in vertebrate retinas: Regulation by a photoreceptor oscillator. *Prog. Retin. Eye Res.*, **14**:267-291.

Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. Kirschner, M.W. (1980) Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. *Cell*, **20**: 95-105.

Coumailleau, P., Poellinger, L., Gustafsson, J.-A., Whitelaw, M.L. (1995) Definition of a minimal domain of the dioxin receptor that is associated with hsp90 and maintains wild type ligand binding affinity and specificity. *J. Biol. Chem.*, **270**: 25291 25300.

Crews, S.T. (1998) Control of cell lineage-specific development and transcription of bHLH-PAS proteins. *Genes and Development*, **12**: 607-620.

Dennis, M., Cuthill, S., Wikstrom, A.C., Poellinger, L., Gustafsson, J.A. (1988) Association of the dioxin receptor with the Mr 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.*, **155**: 801-807.

Dolwick, K.M., Swanson, H.I., Bradfield, C.A. (1993) In vitro analysis of Ah receptor domains involved in ligand-activated DNA recognition. *Proc. Natl. Acad, Sci.*, **90**: 8566-8570.

Eide, E.J., Vielhaber, E.L., Hinz, W.A., Virshup, D.M. (2002) The circadian regulatory protein BMAL1 and Cryptochromes are substrates of casein kinase Iε. *J. Biol. Chem.*, **277**: 17248-17245.

Ema, M., Sogawa, K., Watanabe, N., Chuioj, Y., Matsushita, N., Gotoh, O., Funae, Y., Fujii-Kuriyama, Y. (1992) cDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.*, **184**: 246-253.

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

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

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

Green, C.B., Besharse, J.C. (1994) Tryptophan hydroxylase expression is regulated by a circadian clock in Xenopus laevis retina. *J. Neurochem.*, **62**: 2420-2428.

Green, C.B., Liang, M.-Y., Steenhard, B.M., and Besharse, J.C. (1999) Ontogeny of circadian and light regulation of melatonin release in *Xenopus laevis* embryos. *Brain Res. Dev. Brain Res.*, **117**:109-116.

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

Harkinson, O. (1995) The Aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.*, **35**: 307-340.

Hoffman, E.C., Reyes, H., Chu, F.F., Sander, F., Conley, L.H., Brooks, B.A., Hankinson, O. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science*, **252**: 954-958.

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

Honma, S., Ikeda, M., Abe, H., Tanahashi, Y., Namihira, M. et al (1998) Circadian oscillation of *BMAL1*, a partner of a mammalian gene *Clock*, in the rat suprachiasmatic nucleus. *Biochem. Biophys. Res. Commun.*, **250**: 83-87.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., Honma, K. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature*, **419**: 841-844.

Hosoda, H., Motohashi, J., Kato, H., Masushige, S., Kida, S. (2004) A BMAL1 mutant with arginine 91 substituted with alanine acts as a dominant negative inhibitor. *Gene*, **338**: 235-241.

Huang, Z.J., Edery, I., Rosbash, M. (1993) PAS is a dimerization domain common to Drosophila period and several transcription factors. *Nature*, **364**: 259-262.

Huang, J.-D., Dubincoff, T., Liaw, G.-J., Bai, Y., Valentine, S., Shirokawa, J., Lengyel, J.A., Courey, A.J. (1995) Binding sites for transcription factor NTF-1/Elf-1 contribute to the ventral repression of *decapentaplegic*. *Genes and Dev.*, **9**: 3177 3189.

Ikeda, M., Nomura, M. (1997) cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively

spliced variants with alternative translation initiation site usage. *Biochem. Biophys. Res. Commun.*, **233**: 258-264.

Jackson, F.R., Bargiello, T.A., Yun, S.H., Young, M.W. (1986) Product of per locus of Drosophila shares homology with proteoglycans. *Nature*, **320**: 185-188.

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

Kadesch, T. (1993) Consequences of heteromeric interactions among helix-loop-helix proteins. *Cell Growth Differ.* **4**: 49-55.

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

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Hotz Vitaterna, M., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S. (1997b) Positional cloning of the mouse circadian *Clock* gene. *Cell*, **89**:641-653.

Kondratov, R.V., Chernov, M.V., Kondratova, A.A., Gorbacheva, V.Y., Gudkov, A.V., Antoch, M.P. (2003) BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. *Genes and Development*, **17**: 1921-1932.

Kroll, K.L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*, **122**: 3173-3183.

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

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., Whitelaw, M.L. (2002) Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. *Science*, **295**: 858-861.

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

Lindebro, M.C., Poellinger, L., Whitelaw, M.L. (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.*, **14**: 3528-3539.

Matsushita, N., Sogawa, K., Ema, M., Yoshida, A., Fujii-Kuriyama, Y. (1993) A factor binding to the xenobiotic responsive element (XRE) of P-4501A1 gene consists of at least two helix-loop-helix proteins, Ah receptor and Arnt. *J. Biol. Chem.*, **268**: 21002-21006.

Murre, C., Bain, G., van Dijk, M.A., Engel, I., Furnari, B.A., Massari M.E., Matthews J.R., Quong M.W., Rivera R.R., Stuiver M.H. (1994) Structure and function of the helix-loop-helix proteins. *Biochem. Biopys. Acta.* **1218**: 129-135.

Nambu, J.R., Lewis, J.O., Wharton, K.A., Crews, S.T. (1991) The Drosophila singleminded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell*, **67**: 1157-1167.

Oishi, K., Sakamoto, K., Okada, T., Nagase, T., Ishida, N. (1998) Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem. Biophys. Res. Commun.*, **253**: 199-203.

Oishi, K., Fukui, H., Ishida, N. (2000) Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and pheripheral tissues. *Biochem. Biophys. Res. Commun.*, **268**: 164-171.

Perdew, G.H. (1988) Association of the Ah receptor with the 90-kDa heat-shock protein. *J. Biol. Chem.*, **263**: 13802-13805.

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

Reyes, H., Reisz-Porszasz, S., Hankinson, O. (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science*, **256**: 1193-1195.

Rowlands, J.C., and Gustafsson, J.A. (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Critic. Rev. Toxicol.* **27**: 109-134.

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

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

Sanada, K., Okano, T., Fukada, Y. (2001) Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *J. Biol. Chem.*, **277**: 267-271.

Sato, F., Kawamoto, T., Fujimoto, K., Noshiro, M., Honda, K.K., Honma, S., Honma, K., Kato, Y. (2004) Functional analysis of the basic helix-loop-helix transcription factor DEC1 in circadian regulation. Interaction with BMAL1. *Eur. J. Biochem.*, **271**: 4409-4419.

Semenza, G.L. (2000) HIF-1 and human disease: one highly involved factor. *Genes Dev.*, **14**: 1983-1991.

Schaeren-Wiemers N., Gerfin-Moser A. (1993) A single protocol to detect transcripts of various types and expression levels in neural tissues and cultured cells: *in situ* hybridization using digoxygenin-labeled cRNA probes. *Histochemistry*, **100**: 431 -440.

Schibler, U., Ripperger, J.A., Brown, S.A. (2001) Chronobiology – Reducing time. *Science*, **293**: 437-438.

Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., van der Horst, G.T., Hastings, M.H., Reppert, S.M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science*, **12**: 943 -944.

Steenhard, B.M., Besharse, J.C. (2000) Phase shifting and retinal circadian clock: xPer2 mRNA induction by light and dopamine. *J. Neurosci.*, **20**: 8572-8577.

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

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

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

Welsh, D.K., Yoo, S.-H., Liu, A.C., Takahashi, J.S., Kay, S.A. (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.*, **14**: 2289-2295.

Whitlock, J.P., Okino, S.T., Dong, L., Ko, H.P., Clarke-Katzenberg, R., Ma, Q., Li, H. (1996) Induction of cytochrome P450IA1: a model for analyzing mammalian gene transcription. *FASEB*, **10**: 809-818.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H. (2002) Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, **288**: 682-685.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.O., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveal

persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences, USA*, **101**: 5339-5346.

Zhu, H., and Green, C.B. (2001) A putative flavin electron transport pathway is differentially utilized in *Xenopus* CRY1 and CRY2. *Current Biology*, **11**: 1945 -1949.

Chapter III

Nuclear Localization and Transcriptional Repression are Confined to Separable Domains in the Circadian Protein CRYPTOCHROME

Note:

The work presented in this chapter was previously published as:

***Zhu, H., *Conte, F.E. and Green, C.B.** (2003) Nuclear localization and transcriptional repression are confined to separable domains in the circadian protein CRYPTOCHROME. *Current Biology*, **13**: 1653-1658.

The text of this chapter is adapted from the above-mentioned paper.

* These authors contributed equally to this work. Generation and preliminary functional analysis of the *xcry* mutants was carried out by Haisun Zhu. I completed the functional and immunocytochemical analyses and wrote the complete manuscript.

Summary

CRYPTOCHROME (CRY) proteins are critical components of the circadian clock and, in vertebrates, they repress the activity of the transcription factor heterodimer CLOCK/BMAL1. Unlike the homologous DNA repair enzyme 6-4 PHOTOLYASE, CRYs have extended carboxyl-terminal tails and cannot repair DNA damage. Unlike mammals, *Xenopus laevis* contains both CRYs (xCRYs) and 6-4 PHOTOLYASE (xPHOTOLYASE), providing an excellent comparative tool to study CRY repressive function. We can extend findings to CRYs in general because xCRYs share high sequence homology with mammalian CRYs. We have generated xCRYs C-terminus-truncation mutants and examined their repressive activity and subcellular localization by transient transfection.

We show here that deletion of xCRYs' C-terminal domain produces proteins that are, like xPHOTOLYASE, unable to suppress CLOCK/BMAL1 activation. However, these truncations also cause the proteins to be cytoplasmically localized. An heterologous nuclear localization signal (NLS) restores the truncation mutants' nuclear localization and repressive activity. These results demonstrate that the CRYs' C-termini are essential for nuclear localization but not necessary for the suppression of CLOCK/BMAL1 activation, indicating that these two functions reside in separable domains. Furthermore, the functional differences between CRYs and PHOTOLYASE can be attributed to the few amino acid changes in the conserved portions of these proteins.

Introduction

In vertebrates, Cryptochromes (CRYs) are the transcriptional repressors of CLOCK and BMAL1, and they are essential to maintain clock function (Kume *et al.*, 1999; Griffin *et al.*, 1999; Zhu and Green, 2001b). The main body of the protein is highly conserved amongst CRYs in different organisms and between the CRYs and PHOTOLYASE, a blue-light activated DNA repair enzyme (Hitomi *et al.*, 1997). This region also binds the cofactors flavin adenine dinucleotide (FAD) and pterin. In plants and insects, CRYs act as photoreceptors, and are thought to absorb photons and signal via these cofactors. In mammals and *Xenopus*, CRYs act as transcriptional repressor, and this activity has been abolished by mutating the FAD binding domain in *Xenopus* (Zhu and Green, 2001b) but not in mouse (Froy *et al.*, 2002). It is intriguing that proteins whose sequences are so similar (CRYs and PHOTOLYASE) can have extremely different functions. In fact, CRYs cannot repair DNA (Lin *et al.*, 1995; Hsu *et al.*, 1996), and PHOTOLYASE cannot repress CLOCK/BMAL1 mediated transcriptional activation (Zhu, Conte and Green, 2003). CRY's failure to bind and repair DNA is partly explained by the recent unveiling of the crystal structure of CRY1 from plants, which shows that while the surface region of PHOTOLYASE is positively charged (therefore allowing DNA binding), the surface region of *Arabidopsis* CRY1 is negatively charged (Brautigam *et al.*, 2004).

CRYs' C-terminal region, on the other hand, is missing in PHOTOLYASE, and is highly variable between CRYs. As discussed in the introduction, its functions has recently been demonstrated in plants, insects and, here, in *Xenopus*. In *Arabidopsis*, the

C-terminal domain is inactive in the dark, and is possibly being repressed by the core domain. Light absorption by the core domain causes activation of the tail domain, and subsequent activation of the phototransduction pathway (Yang *et al*, 2001). In *Drosophila*, the tail functions by inhibiting the core domain: absorption of photons by the core domain relieves the repression by the C-terminus, and allows the core region to bind and cause the degradation of PER and TIM (Dissel *et al*., 2004; Busza *et al*., 2004).

In order to explore the differences between CRY and PHOTOLYASE and the possible mechanism by which CRY represses transcription, I have investigated the role of the carboxyl terminus in *Xenopus laevis*. This animal is an excellent model system, since it is one of the few organisms to contain both CRYs –xCRY1 and xCRY2b- (Zhu and Green, 2001a) and PHOTOLYASE (Hitomi *et al*., 1997).

Here, I will show that the C-termini of both *Xenopus* CRY1 and CRY2b are essential for nuclear localization, but are not necessary for repression of CLOCK/BMAL1-mediated transcriptional activation. Therefore, these two functions reside in separable domains. This also demonstrates that the major functional difference between xCRYs and xPHOTOLYASE is due to a few amino acid changes in the core region of the proteins.

Methods

Cloning and alignment

Cloning of *Xenopus* cryptochromes was previously described in Zhu and Green, 2001a. *xClock* and *xBmal1* are expressed in the pVAX1 vector (Invitrogen, Carlsbad, CA), while *xPhotolyase*, *xCryptochrome1* and *xCryptochrome2b* are cloned into the pCMV-Tag2b expression vector, which adds a N-terminal Flag tag (Stratagene, La Jolla, CA). The Luciferase reporter gene contains three tandem repeats of the E-box sequence from the mouse *Per1* gene promoter (Gekakis *et al.*, 1998; Hida *et al.*, 2000), which was cloned upstream of the rat prolactin basal promoter (pRL vector, Promega, Madison, WI) (Dr. Richard Day, University of Virginia, unpublished data). The Renilla reporter gene is cloned into the pRL vector (Promega, Madison, WI), and driven by a basal promoter. The alignment in Figure 1 was generated by ClustalW algorithm (MacVector, Oxford Molecular). Accessions numbers for cryptochrome are as follows: *xCry1* (AY049033) and *xCry2b* (AY049035). Refer to Hitomi *et al.*, 1997, for xPHOTOLYASE. Truncations were generated with the Quick-change Mutagenesis PCR kit (Stratagene, La Jolla, CA).

Cloning of NLS constructs:

To generate the constructs, two complementary oligonucleotides (GCG GAT CCC CCC CAA AAA AGA AGA GAA AGG TAG AAG GGG AAT TCG A, TCG AAT TCC CCT TCT AAC TTT CTC TTC TTT TTT GGG GGG GAT CCG C) were synthesized by MWG Biotech (High Point, NC). These oligonucleotides contain a sequence, which encodes a previously reported (Kalderon *et al.*, 1987) NLS peptide

(PPKKKRKVEGEF). The two oligonucleotides were annealed together, and digested with BamHI and EcoRI. The resulting fragment was then cloned into the pCMV-Tag2b vector (Stratagene, La Jolla, CA), resulting in a vector containing an in-frame NLS peptide right after the FLAG tag.

Transient transfection

The transient transfection protocol used here was previously described in Zhu and Green, 2001b. Cos-7 cells were seeded at about 60-70% confluency into 6-well plates, and transfected with one microgram of total DNA per each well. The FuGENE (Roche, Basel, Switzerland) delivery system was used for each transfection. The clones used for these assays are described above. The *per E-box-Luc* reporter construct was a gift from Dr. Richard Day. The promoter contains three direct repeats of the *mper1* E-box (Gekakis *et al.*, 1998) and 87 base pairs of the rat prolactin basal promoter (Dr. Richard Day, unpublished data). The reporter constructs were cotransfected with expression plasmids containing the x*Clock,* x*bmal1* and *xPhotolyase*, *xCry1*, *xCry2b* and the *xCry1*/*xCry2b* truncation mutants cDNAs as indicated below each measurement. 0.15 μg of *xClock*, *xBmal1*, *xPhotolyase* or *xCrys* was transfected (unless indicated otherwise); 0.2 μg of the Luciferase reporter gene was transfected; 0.015 μg of the Renilla reporter gene was transfected. Total amount of transfected DNA was kept constant at 1 μg. Transcriptional activity was assessed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) by measuring Luciferase activity from cell lysates and normalizing to Renilla Luciferase. Each value is an average of four replicas (+/- SEM) and each experiment was repeated at least three times with similar results.

Western Blotting and antibody detection

Cos-7 cells seeded in 10 cm plates to 60-70% confluency and transfected the following day with the FuGENE (Roche, Basel, Switzerland) delivery system according to the manufacturer's recommendations. The cells were incubated for two days and lysed. The cell lysates were then run on a 10% SDS-PAGE gel and blotted onto a PVDF membrane (BIO-RAD, Hercules, CA). Proteins were detected with the Flag tag detection kit (Stratagene, La Jolla, CA). The blot was incubated with a 1:5000 dilution of M2 anti-Flag primary antibody and with a 1:5000 dilution of goat anti-mouse alkaline phosphatase (AP) conjugated secondary antibody following manufacturer's protocol. 5μl of lysate was loaded in each lane.

Immunocytochemistry

Cos-7 cells were seeded in 6-well plates containing sterile cover slips and transfected as described above. 3 μl of FuGENE was incubated with 1 μg of each DNA following manufacturer's recommendations (Roche, Basel, Switzerland). The cells were grown overnight in DMEM + 10%FBS in 5% CO_2 at 37° C. The following day, the cells were rinsed twice in PBS and fixed for 15 min with ice-cold methanol. They were rinsed again in PBS and stained with antibodies.

Anti-Flag primary antibody was diluted 1:1000 in blocking solution: 0.3% Triton, 1% protease free BSA, o.25% λ-carrageenan, 0.1% Sodium Azide in 1X TBS, pH 7.6. Anti-digoxygenin (DIG) conjugated goat anti-mouse secondary antibody was diluted 1:1000 in blocking solution and rhodamine conjugated rabbit anti-DIG tertiary antibody

was diluted 1:1000 in blocking solution. Cells were stained for 5 minutes in Hoechst's dye H33258 (0.5 μg/ml in PBS).

The slides were viewed under an Olympus inverted EPI-fluorescent microscope (IX-70), and the images were taken using Optronics CCD camera (Optronics, Goleta, CA). The slides were randomly numbered and about 200 cells were counted for each construct transfected.

Eyes from transgenic tadpoles were dissected and fixed in 4% paraformaldehyde-PBS overnight. After rinsing in PBS, the eyes were cryoprotected with 30% sucrose and embedded in O.C.T compound (Ted Pella, Redding, CA). After cryosectioning, the sections were stained with α-Flag antibody as described above.

Anti-xCRY1 antibodies were raised to the following peptide: QSVGPKVQRQSTP (Research Genetics, Hunstville, AL). The antibody (90132) was affinity purified and then characterized on Western blots by Dr. H. Zhu (data not shown). The α-xCRY1 antibody was diluted 1:500 in blocking solution (see above). The secondary antibody, Cy-3 conjugated affinity purified donkey α-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA), was diluted 1:750 in blocking solutions (see above).

Transgenesis

Xenopus laevis adults were purchased from NASCO (Fort Atkinson, WI), and egg and sperm were obtained from the adult frogs. Transgenic tadpoles expressing the pCMVTagxCry1 and the pCMVTagxCry1N506* were generated by restriction enzyme-mediated integration (REMI). The constructs were linearized with SalI and

phenol/chloroform purified. The transgene was then stably inserted into the sperm genome and the eggs were fertilized with the sperm nuclei as described in Kroll and Amaya, 1996, but with the following changes. The REMI reaction was prepared by mixing 1 μl of linearized plasmid (150 ng/ μl) with 4 μl of sperm nuclei (~4 X 10^4 nuclei). After 5 min at room temperature, 1 μl of 100 mM $MgCl_2$, 0.5 μl of a 1:10 dilution of SalI (10 U/ μl), 2 μl of oocyte extract (preheated at 80°C) and 9 μl of sperm dilution buffer (250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride , 0.2 mM spermidine tetrahydrochloride) were added and incubated for 10min at room temperature. The reaction was then diluted in MOH (10 mM KPO, 125 mM K-gluconate, 5 mM NaCl, 0.5 mM $MgCl_2$, 25 mM sucrose, 0.25 mM spermidine, 0.125mM spermine) for a final concentration of about 3 sperm nuclei/5 nl. The nuclei were then injected into the dejellied eggs at a rate of about 5 nl/sec using glass needles and a syringe pump (Model 1111, Harvard Apparatus, Holliston, MA). This flow rate was established to deliver one functional sperm nucleus to the majority of eggs. The resulting embryos were grown for 2-3 weeks and genotyped.

Genotyping

The tip of the tadpoles' tail was cut and genomic DNA was isolated using the DNeasy Tissue kit (Quiagen, Valencia, CA). PCR to detect the animals carrying the transgene was then carried out with AmpliTaq (PE Applied Biosystems, Foster City, CA) and xCry1-specific primers. A 750 base pair product was amplified by using the T7 primer (detecting the pCMV-Tag2b vector) and the Cry1Cterm9-16F primer (detecting the xCry1 C-terminus). The sequence of the Cry1Cterm9-16 primer is:

5’ GCTGCCGCAGAATTCGGCAGATCTCTCTCGAGGGAGAAGTGCTTCCTGGG

ACTTCAGG

Results

The core domain is conserved between xCRY1, xCRY2b and xPHOTOLYASE

The *Xenopus* CRYs and PHOTOLYASE are very well conserved throughout the core region, where they share over 85% homology (Figure 1). xPHOTOLYASE lacks the extended C-terminal domain present in both CRYs. It is therefore possible that the C-terminal domain might play a role in the difference in function between the CRYs and PHOTOLYASE.

xPHOTOLYASE cannot suppress CLOCK/BMAL1-mediated transcriptional activation

Unlike PHOTOLYASE, CRYs have no DNA damage repair activity (Lin *et al.*, 1995; Hsu *et al.*, 1996), even though their core region is very well conserved. We asked if xPHOTOLYASE has transcriptional repressive activity like the CRYs by testing if it could repress CLOCK/BMAL1-mediated transcriptional activation.

We cloned *Xenopus 6-4 photolyase* into the pCMV-Tag 2b vector, and tested its repressive activity by transient transfection in Cos-7 cells. Briefly, the CLOCK/BMAL1 binding site is cloned in front of the Luciferase reporter gene, and Luciferase activity is measured. Co-transfection of *xClock* and *xbmal1* correlates with high luciferase activity, while co-transfection of the *Crys* correlates with low luciferase activity. Despite its high homology with the CRYs, xPHOTOLYASE cannot repress CLOCK/BMAL1-mediated transcriptional activation (Figure 2).

In plants and flies CRYs act as photoreceptors (Cashmore *et al.*, 1999); therefore, we tested if light has an effect on repression of CLOCK/BMAL1-mediated transcriptional activation. We observed no difference on xCRY1 repression if cells were

kept in constant light (LL) or in constant dark (DD), indicating that xCRYs have no photoreceptive activity. In addition, there was no difference in lack of repression by PHOTOLYASE in the light versus the dark (Figure 2).

xCRYs C-terminal truncations affect the repressive activity

To investigate if the C-terminal domain of CRY is required for repression of CLOCK and BMAL1, we generated C-terminal deletions of both xCRY1 and xCRY2b by introducing stop codons at specific amino acid sites via site-directed mutagenesis (see Figure 1). We introduced four stop codons for each CRY. xCRY1-Q490* and xCRY2b-Q494* introduced stop codons at the end of the region of homology between xCRYs and PHOTOLYASE. xCRY1-N506* and xCRY2b-C510* generated mutants truncated at the end of homology between xCRY1 and xCRY2b. xCRY1-E550* and xCRY2b-K531* and xCRY1-E602* and xCRY2b-R557* generated mutants truncated at two locations within the remaining sequence. All the truncations mutants were confirmed by sequencing and their sizes were verified by Western blot analysis (Figure 3).

We then tested all mutants in the Luciferase reporter gene transfection assay. As expected, co-transfection of the Luciferase reporter with *xClock* and *xbmal1* lead to high relative Luciferase levels (Figure 4 A, B). Co-transfection with *xClock/xbmal1* and the wild type *Crys* lead to low Luciferase activity, and therefore repression of CLOCK/BMAL1-mediated transcriptional activation. The shortest C-terminal truncation mutants for both xCRY1 and xCRY2b (xCRY1-Q490* and N506* and xCRY2b-Q494* and C510*) displayed the most severe loss of repressive activity; in fact, these mutants are almost completely unable to repress CLOCK/BMAL1 mediated transcriptional

activation. Truncation of xCRY1 at amino acid E550* also resulted in complete loss of repressive activity, while truncation at E602* had no effect, indicating that the region between E550 and E602 is essential for proper repressive activity. Truncation of xCRY2b at K531* resulted in a less severe loss of function, and truncation at R557* had no measurable effect.

I observed partial deficiency in repression in the xCRY2b-K531* truncation mutant at both saturating and sub-saturating doses (Figure 5). I will explore possible reasons for the partial loss in repression later in this Chapter and in Chapter IV.

These results show that if the C-terminus is lost in xCRY1 or xCRY2b, repression is severely affected. The key regions contributing to this effect lie between amino acids E550 and E602 for xCRY1, and between amino acids K531 and R557 for xCRY2b. This effect is not due to lower protein levels in the mutants since, as shown in Figure 3, the total levels of wild type and mutant proteins are comparable.

Truncation mutants have altered intracellular localizations *in vitro*

In order to understand the function of the C-terminal regions in the CRYs, we have examined more closely the amino acid sequences between residues 550 and 602 in xCRY1 and 531 and 557 in xCRY2b to possibly identifying conserved domains. The xCRY1 C-terminal region does not resemble any known conserved domain, but it does contain amino acids that could be involved in signaling (serines) or nuclear localization (lysines, arginines and histidines) (Figure 1, black dotted line). In xCRY2b, on the other hand, the region between K510 and R557 contains six positively charged amino acids

arranged in a **K**X_9**KRK**X_{10}**KXR** pattern. This pattern resembles a known nuclear localization signal (NLS) (Kalderon *et al.*, 1984) (Figure 1, red solid line).

Because of this evidence, we hypothesized that these regions might be involved in the subcellular localization of the CRYs, and we tested this by examining the subcellular localization of xCRY proteins transfected in Cos-7 cells. As shown in figure 6, wild type xCRY1 (Figure 6, A, Table 1) and xCRY2b (Figure 6, B, Table 1) show mostly nuclear subcellular localization. Notably, the mutants displaying lack of repressive activity (xCRY1-Q490*, xCRY1-N506*, xCRY1-E550*; xCRY2b-Q494*, xCry2b-C510*, xCry2b-K531*) also showed loss of nuclear localization (Figure 6, A and B, Table 1). On the other hand, the mutants with the less severe truncations, which showed intact repressive activity, still localized to the nucleus (xCRY2b-R557*, Figure 6, B, Table 1) or to both the nucleus and cytoplasm (xCRY1-E602*, Figure 6, A, Table 1). As a positive control we used xPHOTOLYASE, which localizes to the nucleus as expected. The xCRY2b-K531* lacks the putative NLS and is mostly cytoplasmic (Figure 6, B, Table 1), yet it displays partial repressive activity. I will show data that possibly explains this apparent discrepancy in the following chapter.

Truncation mutants have altered intracellular localizations *in vivo*

In order to test if the CRY mutants have intracellular localization *in vivo* as well as *in vitro*, I generated transgenic *Xenopus laevis* tadpoles by using the transgenic technique (Kroll and Amaya, 1996). This technique has been successfully used in our laboratory to study the effects of mutants on the molecular clock *in vivo* (Hayasaka *et al.*, 2002). Tadpoles were generated to over-express wild type xCRY1 (in addition to

endogenous xCRY1) or truncated xCRY1 (xCRY1-N506*), both fused to an N-terminal Flag tag. I chose the xCRY1-N506* truncation mutant because it displayed severe loss of repressive activity and nuclear localization in tissue culture. I grew the tadpoles until two weeks of age, at which point they were sacrificed and cryosectioned for the immunocytochemical analysis.

I detected the over-expressed and endogenous wild type proteins with rabbit anti-xCRY antibodies and anti-rabbit Cy3-conjugated antibodies. To distinguish between endogenous xCRY1 and truncated xCRY1-N506*, I detected only the mutant protein by using mouse anti-Flag and anti-mouse Cy3-conjugated antibodies. Results show that while the over-expressed wild type protein is present in the nucleus and cytoplasm of photoreceptor cells in the tadpole retinas, the over-expressed truncation mutant is present only in the cytoplasm of the photoreceptor cells (Figure 7). This result is analogous to the results I obtained in tissue culture cells, and it shows that the mutants are defective in their nuclear localization *in vitro* and *in vivo.*

Addition of an heterologous NLS rescues nuclear localization and repressive activity

We hypothesized that the truncation mutants still had full repressive activity, but that they could not carry it out because of impaired subcellular localization. We therefore cloned the mutants into a vector containing an in-frame heterologous NLS (PPKKKRKVEGEF) (Kalderon *et al.*, 1984). We then tested the truncated mutants now containing an heterologous NLS at the N-terminus for subcellular localization and repressive activity in Cos-7 cells. As expected, results show that all xCry1 and xCry2b mutant constructs containing the heterologous NLS localize to the nucleus (Figure 7, A,

Table 2). Furthermore, acquired nuclear localization renders all truncation mutants fully capable of suppressing CLOCK/BMAL1-mediated transcriptional activation (Figure 7, B). The rescue of repressive activity occurs over a range of doses, demonstrating that it is not an artifact of saturating protein amounts (Figure 8).

Discussion

Even though xCRYs and xPHOTOLYASE are well conserved in the core region of the protein, they display different functions. xPHOTOLYASE is a blue-light activated DNA repair enzyme (Hitomi *et al.*, 1997), while the vertebrate CRYs are crucial for maintaining clock function (Kume *et al.*, 1999; Griffin *et al.*, 1999). In plants and insects, the CRYs have been shown to function as circadian photoreceptors (Somers *et al.*, 1998; Cashmore *et al.*, 1999; Stanewsky et al, 1998). Intriguingly, both families of proteins bind to flavin adenine dinucleotide and pterin. In PHOTOLYASE, these cofactors are required for the UV-induced redox reaction, which leads to DNA repair (Sancar and Sancar, 1988; Feng Li *et al.*, 1991). In plants and insects, the cofactors and the residues that are part of the electron transport chain mediate the light response required for entrainment of the circadian oscillator (Payne et al., 1987; Lin *et al.*, 1995; Park *et al.*, 1995; Stanewsky *et al.*, 1998; Emery *et al.*, 1999). In mammals and frogs, where the CRYs function as transcriptional repressors, the function of the cofactors is not known. In frogs, mutating the flavin-binding domain leads to loss of repressive activity (Zhu and Green, 2001) nevertheless, this is not the case for mouse CRY (Froy *et al.*, 2002).

Today, much of the study of CRY in vertebrates has focused on the role of the core region by studying the similarities and differences between CRY and PHOTOLYASE (Hirayama *et al.*, 2003), and on the role of the C-terminus, which seems to have different functions in plants, insects and vertebrates (reviewed in Green, 2004). I have shown here that, in frogs, the C-terminus for xCRY1 and xCRY2b functions as a nuclear localization signal. Deletion of the C-terminus leads to loss of repression not

because the repressive function is affected, but because the protein is now localized in the cytoplasm instead of the nucleus. In addition, only the core domain is required for repressive function, because when the truncation mutants are driven into the nucleus by an heterologous NLS, their repressive function is intact. It is still unclear if nuclear import of xCRYs is achieved through importin binding or thought a more indirect mechanism.

Nuclear localization of CRY is likely to be a regulated and crucial step in the loop, and it most likely affects the length of the circadian period. Lee *et al.* (2001) have shown that in mouse liver presence of CRY and PER in the nucleus is strongly rhythmic, more so than overall protein levels. In fact, mCRY1 and mCRY2 are present in the cytoplasm at comparable levels throughout the circadian cycle, while their nuclear presence is strongly rhythmic. In addition, *mCry1* or *mCry2 b* single knockout mice display opposite phenotypes. While, in constant conditions, wild type mice' locomotor activity has a period very close to 24 hours, *mCry1*$^{-/-}$ mice have a period shorter than 24 hours, while *mCry2*$^{-/-}$ mice have a period longer than 24 hours. *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ double knockout mice are completely arrhythmic in constant conditions (van der Horst *et al*, 1999). This indicates the two *Crys* do not have a redundant role but that, instead, they possibly compliment each other to maintain the 24 hour period stable.

The C-termini of xCRY1 and xCRY2b have very different amino acid sequences. xCRY1 has no discernable conserved domain, while xCRY2b contains a sequence resembling a conserved NLS (Kalderon *et al.*, 1984). This might indicate a different nuclear import pathway regulating the two proteins (possibly the importin pathway for xCRY2b and unknown binding proteins for xCRY1), which could explain, at least in

part, the opposite phenotype of the knockout mice. Considering the evidence from plants, where the C-terminus functions by activating the phototransduction pathway (Yang *et al*, 2001), and from insects, where the C-terminus inhibits the core region (Dissel *et al.*, 2004; Busza *et al.*, 2004), and now from *Xenopus*, indicates that the C-terminus might have a modulatory role in CRY's function. Since mCRYs bind to mPER/CKIε complexes in the cytoplasm (Kume *et al.*, 1999; Lee *et al.*, 2001) and are necessary for nuclear accumulation of mPER (Lee *et al.*, 2001), it is possible that nuclear localization of CRY also dictates the subcellular localization of the complex itself.

These studies are important not only to understand CRY function and regulation, but also to understand the evolutionary difference between CRY and PHOTOLYASE, two proteins so closely related yet with such different functions. Understanding CRY's function in Cos-7 cells allows a diminutive approach, but analysis of the CRY mutants in real clock cells is essential to understand the role that the C-terminally-directed nuclear localization plays within the clock. Unlike Cos-7 cells (Yagita *et al.*, 2000) NIH3T3 mouse fibroblast cells (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004) and rat-1 fibroblast cells (Yagita *et al.*, 2001; Welsh *et al.*, 2004) have been shown to contain the clock components and to cycle in constant conditions. They would therefore be ideal to study the phenotype of the CRYs truncation mutants. Possibly, endogenous levels of wild type CRY, which could mask the mutants' phenotype, could be diminished to undetectable levels via RNA interference, which has recently been shown to be also very effective in cell lines (Fire *et al.*, 1998; Shinomiya *et al.*, 2004).

Nevertheless, cell lines are only representatives of the situation *in vivo*. More in depth studies in transgenic *Xenopus laevis* retinas will be needed to unveil phenotypes

impossible to discern through transfection assays (Kroll and Amaya, 1996). I have begun those studies by investigating the subcellular localization of overexpressed xCRY1 truncation mutants in transgenic *Xenopus laevis'* retinas. Our laboratory has successfully used the transgenic technique to study the effect of mutants on the clock (Hayasaka *et al.*, 2002).

Biochemically, more studies will be needed to show that the regions of xCRY1 and xCRY2b, shown here to regulate the subcellular localization of the proteins, can function as NLS when isolated. To test this hypothesis, I will clone the C-termini in frame with a cytoplasmic protein and observe the subcellular localization of the fusion protein (see Chapter IV). It will also be necessary to show that the C-terminus of xCRY2b indeed binds importin. Canonical NLSs have been shown to function via the importin pathway (Gorlich *et al.*, 1994), the most likely candidate for xCRY2b's nuclear import. This will have to be demonstrated via co-immunoprecipitation studies. For xCRY1's C-terminus, a binding partner will have to be identified via yeast-two-hybrid or via co-immunoprecipitation screens.

The xCRY2b-K531* truncation mutant represents a particularly interesting case. Even though the protein is mostly cytoplasmic, it still displays about 80% repression. This could be due to two factors:

1) Most of the detectable protein is cytoplasm, but very low levels sufficient for partial repression still enter the nucleus.

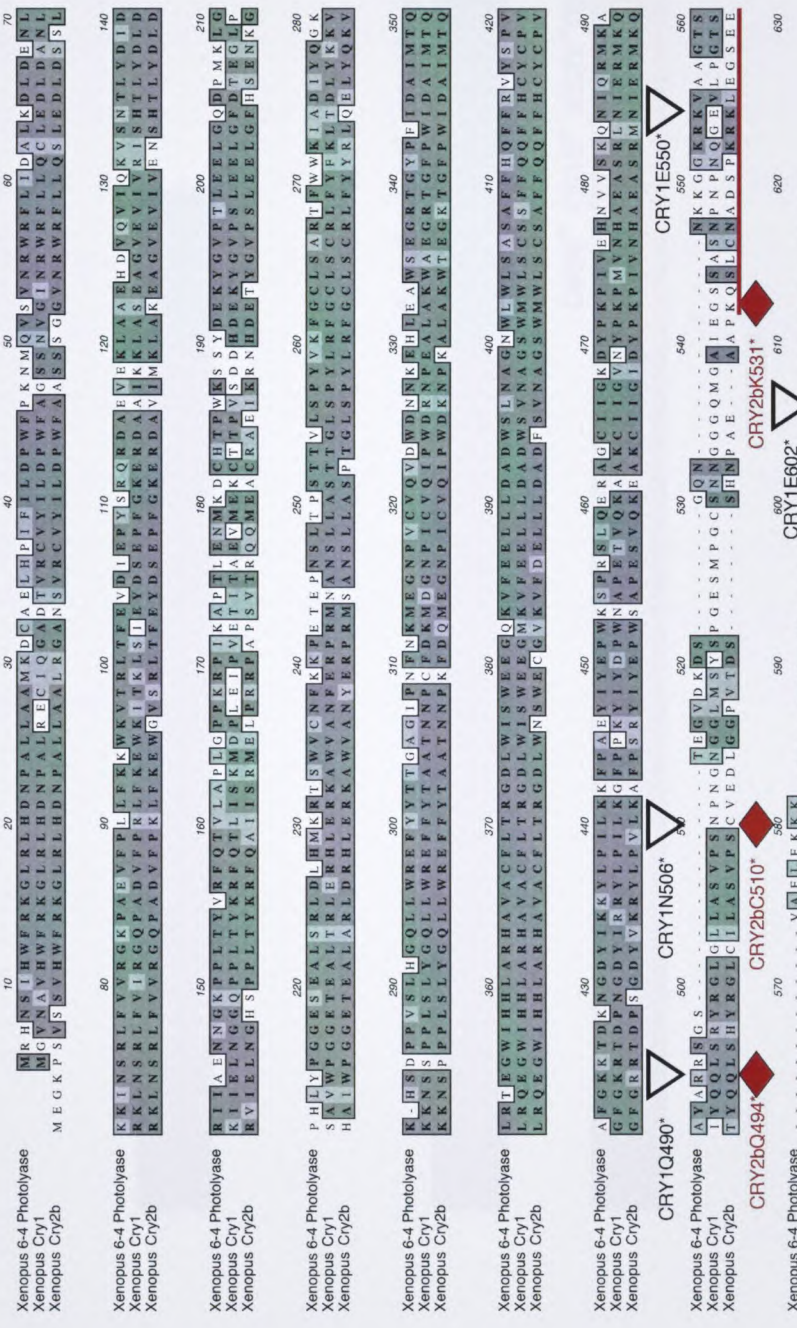
2) All of the immunocytochemistry experiments were carried out by transfecting only the *xCrys*, while all the repression assays were carried out by co-transfecting the *xCrys* with

xClock and *xbmal1*. This discrepancy could lead to the possibility that CLOCK or BMAL1 might affect the subcellular localization of xCRY2b.

Experiments designed to distinguish between these two possibilities will be presented in the following chapter.

Figure 3-1: xPHOTOLYASE and xCRYs are conserved across the core region of the proteins, but differ in the C-termini.

A



B

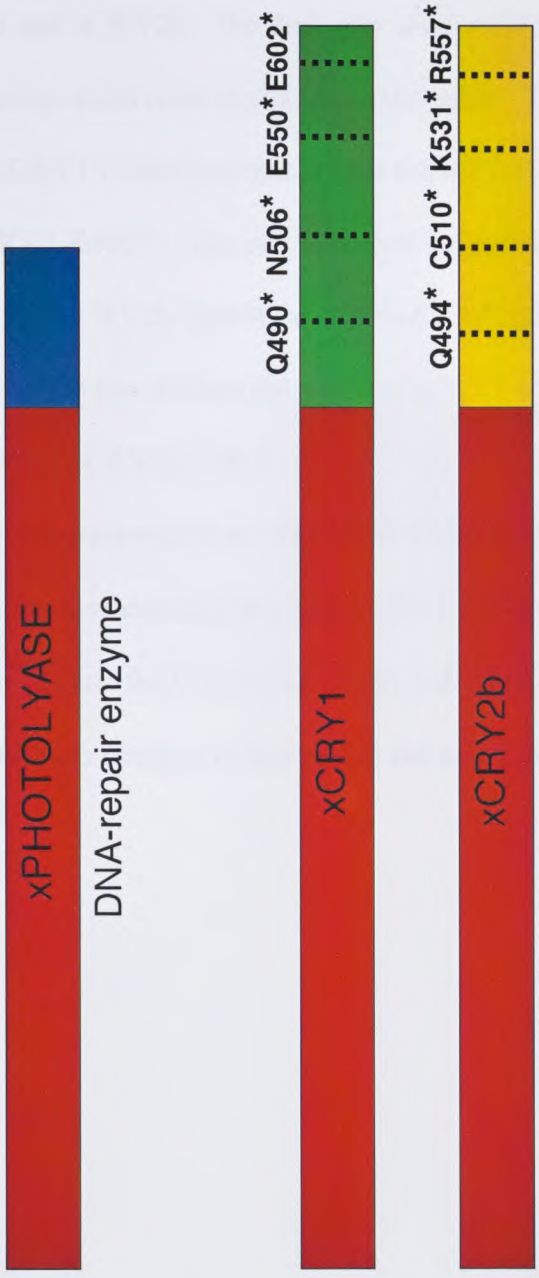

xPHOTOLYASE
DNA-repair enzyme
Q490* N506* E550* E602*
xCRY1
Q494* C510* K531* R557*
xCRY2b

Figure 3-1: xPHOTOLYASE and xCRYs are conserved across the core region of the proteins, but differ in the C-termini.

A: Alignment of *Xenopus* 6-4 PHOTOLYASE and of the deduced amino acid sequence of xCRY1 and xCRY2b. The dark grey areas refer to identical amino acids, and the light gray amino acids refer to similar amino acids. The white triangles indicate the position of the xCRY1 C-terminal truncations (xCRY1-Q490*, xCRY1-N506*, xCRY1-E550*, xCRY1-E602*). The red diamonds indicate the positions of the xCRY2b C-terminal truncations (xCRY2b-Q494*, xCRY2b-C510*, xCRY2b-K531*, xCRY2b-R557*). The black dotted line defines the putative xCRY1's NLS, while the solid red line defines the putative xCRY2b's NLS.

B: Schematic representation of xPHOTOLYASE (the core region is depicted in red and C-terminal region is depicted in blue), xCRY1's C-terminal truncations (the core region is depicted in red and the C-terminal region is depicted in green) and xCRY2b's C-terminal truncations (core region is depicted in red and C-terminal region is depicted in yellow).

Figure 3-2: xPHOTOLYASE cannot repress CLOCK/BMAL1-mediated transcriptional activation.

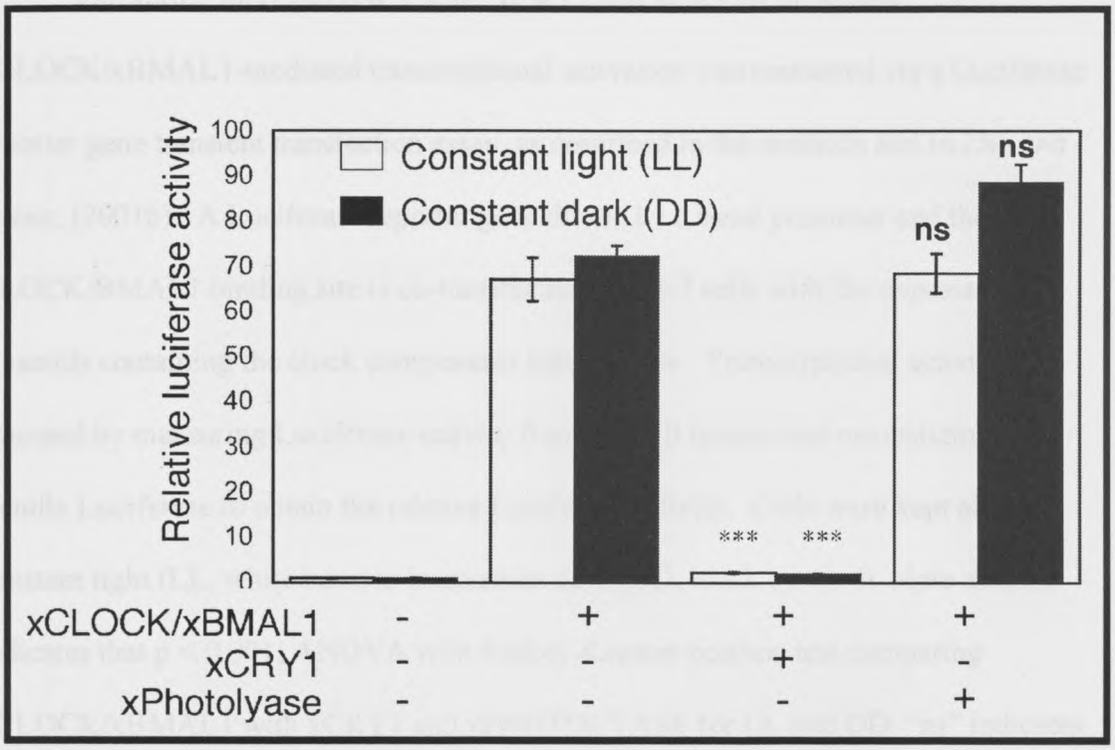


Figure 3-2: xPHOTOLYASE cannot repress CLOCK/BMAL1-mediated transcriptional activation.

The ability of xPHOTOLYASE, xCRY1 and xCRY2b to repress xCLOCK/xBMAL1-mediated transcriptional activation was measured via a Luciferase reporter gene transient transfection assay, as described in the methods and in Zhu and Green, (2001b). A Luciferase reporter gene driven by a basal promoter and the CLOCK/BMAL1 binding site is co-transfected in Cos-7 cells with the expression plasmids containing the clock components listed below. Transcriptional activity was assessed by measuring Luciferase activity from the cell lysates and normalizing it to Renilla Luciferase to obtain the relative Luciferase activity. Cells were kept either in constant light (LL, white bars) or in constant dark (DD, black bars). A triple asterisk indicates that $p < 0.001$; ANOVA with Turkey-Kramer posthoc test comparing xCLOCK/xBMAL1 with xCRY1 and xPHOTOLYASE for LL and DD. "ns" indicates "not significant".

Figure 3-3: Wild type and truncated xCRY1 are expressed at comparable levels.

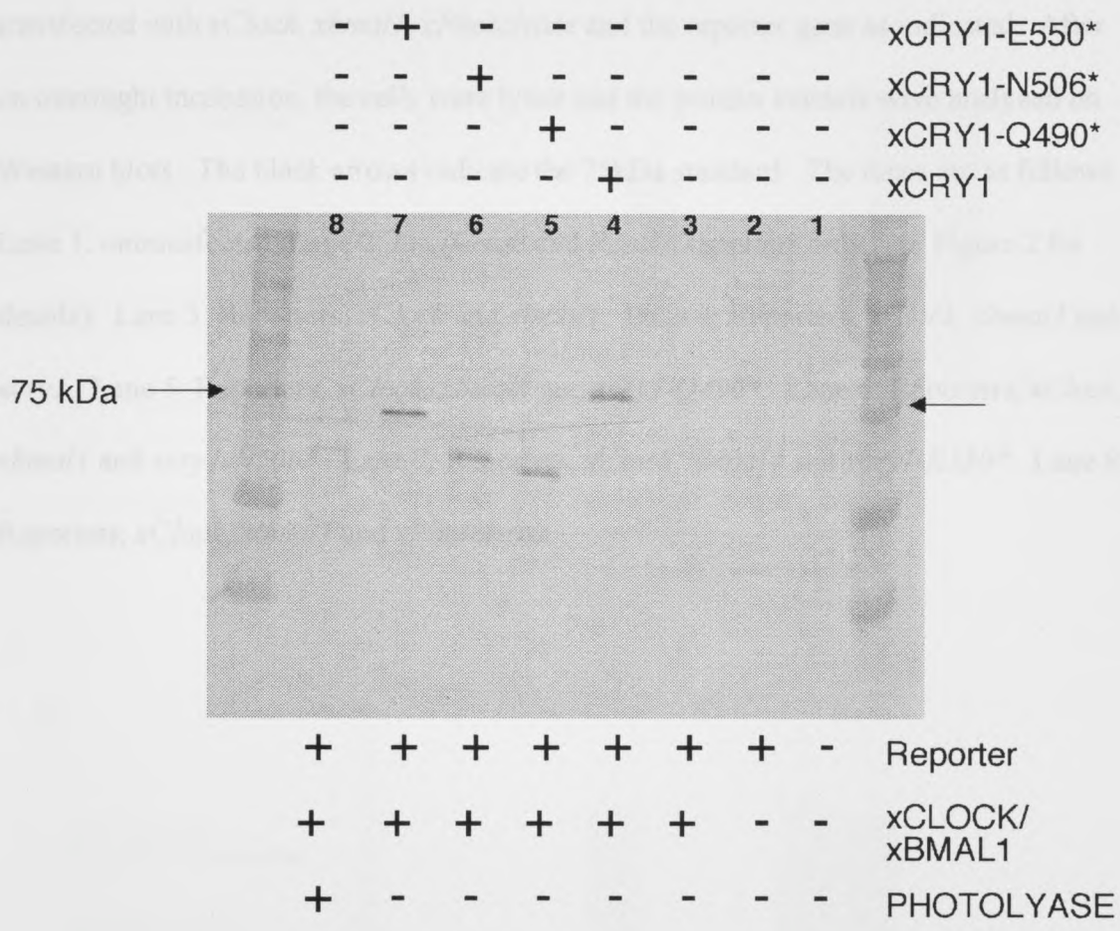


Figure 3-3: Wild type and truncated xCRY1 are expressed at comparable levels.

Western blot showing protein levels of wild-type xCRY1 and the truncation mutants xCRY1-Q490*, xCRY1-N506* and xCRY1-E550*. Cos-7 cells were co-transfected with *xClock*, *xbmal1*, *xPhotolyase* and the reporter gene as indicated. After an overnight incubation, the cells were lysed and the protein extracts were analyzed on Western blots. The black arrows indicate the 75kDa standard. The lanes are as follows. Lane 1: untransfected. Lane 2: *Luciferase* and *Renilla* reporters only (see Figure 2 for details). Lane 3: Reporters, *xClock* and *xbmal1*. Lane 4: Reporters, *xClock*, *xbmal1* and *xcry1*. Lane 5: Reporters, *xClock*, *xbmal1* and *xcry1-Q490**. Lane 6: Reporters, *xClock*, *xbmal1* and *xcry1-N506**. Lane 7: Reporters, *xClock*, *xbmal1* and *xcry1-E550**. Lane 8: Reporters, *xClock*, *xbmal1* and *xPhotolyase*.

Figure 3-4: xCRY C-terminal truncation mutants lose repressive ability.

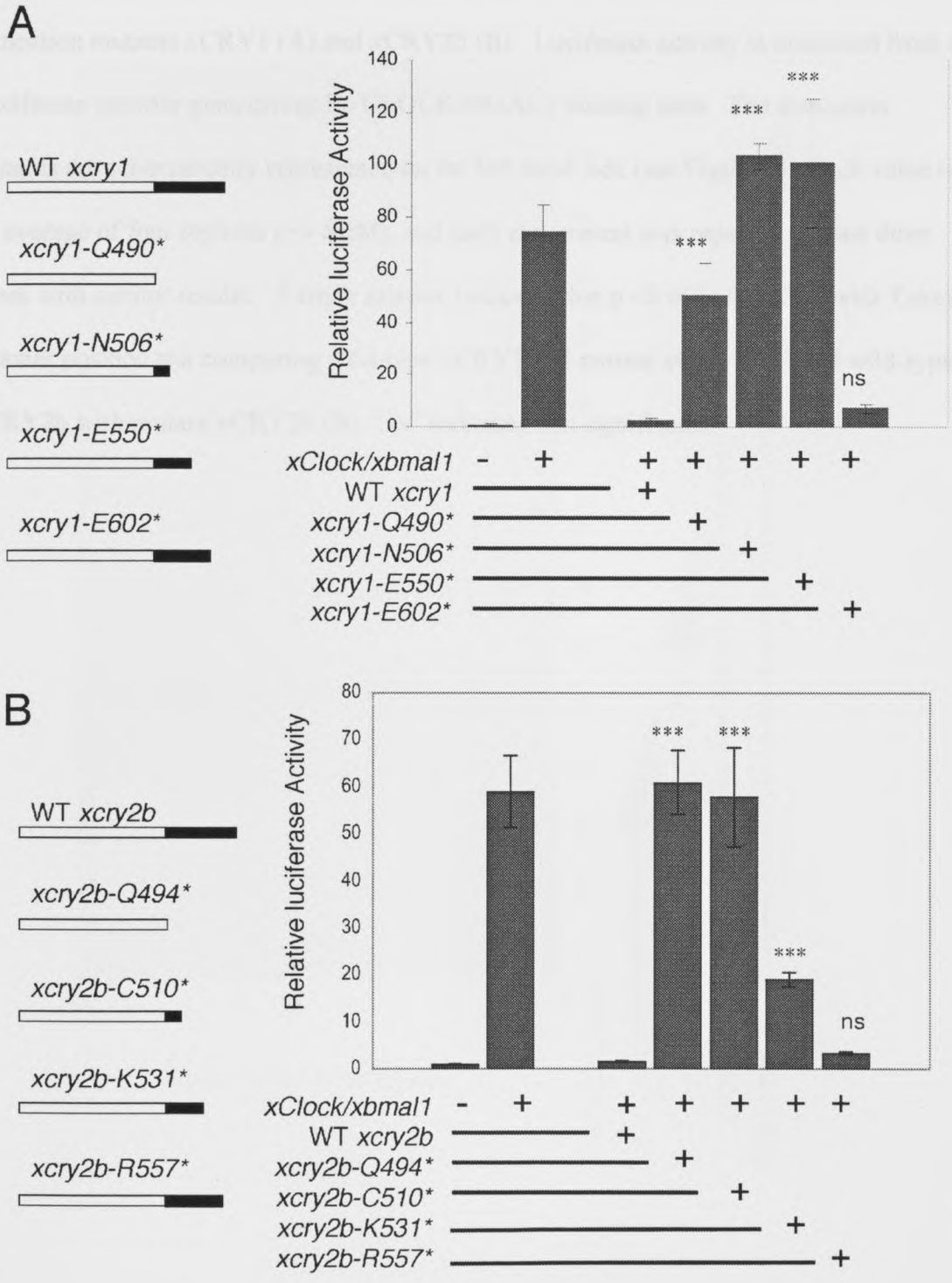


Figure 3-4: xCRY C-terminal truncation mutants loose repressive ability.

Transient transfection assay to test the repressive ability of wild type and truncation mutants xCRY1 (A) and xCRY2b (B). Luciferase activity is measured from a Luciferase reporter gene driven by CLOCK/BMAL1 binding sites. The truncation mutants are schematically represented on the left hand side (see Figure 1). Each value is an average of four replicas (+/- SEM), and each experiment was repeated at least three times with similar results. A triple asterisk indicates that $p < 0.001$; ANOVA with Tukey-Kramer posthoc test comparing wild-type xCRY1 and mutant xCRY1 (A) and wild-type xCRY2b with mutant xCRY2b (B). "ns" indicates non significant.

Figure 3-5: Deficiency in repression by xCRY2b-K531* is seen at both saturating and sub-saturating doses.

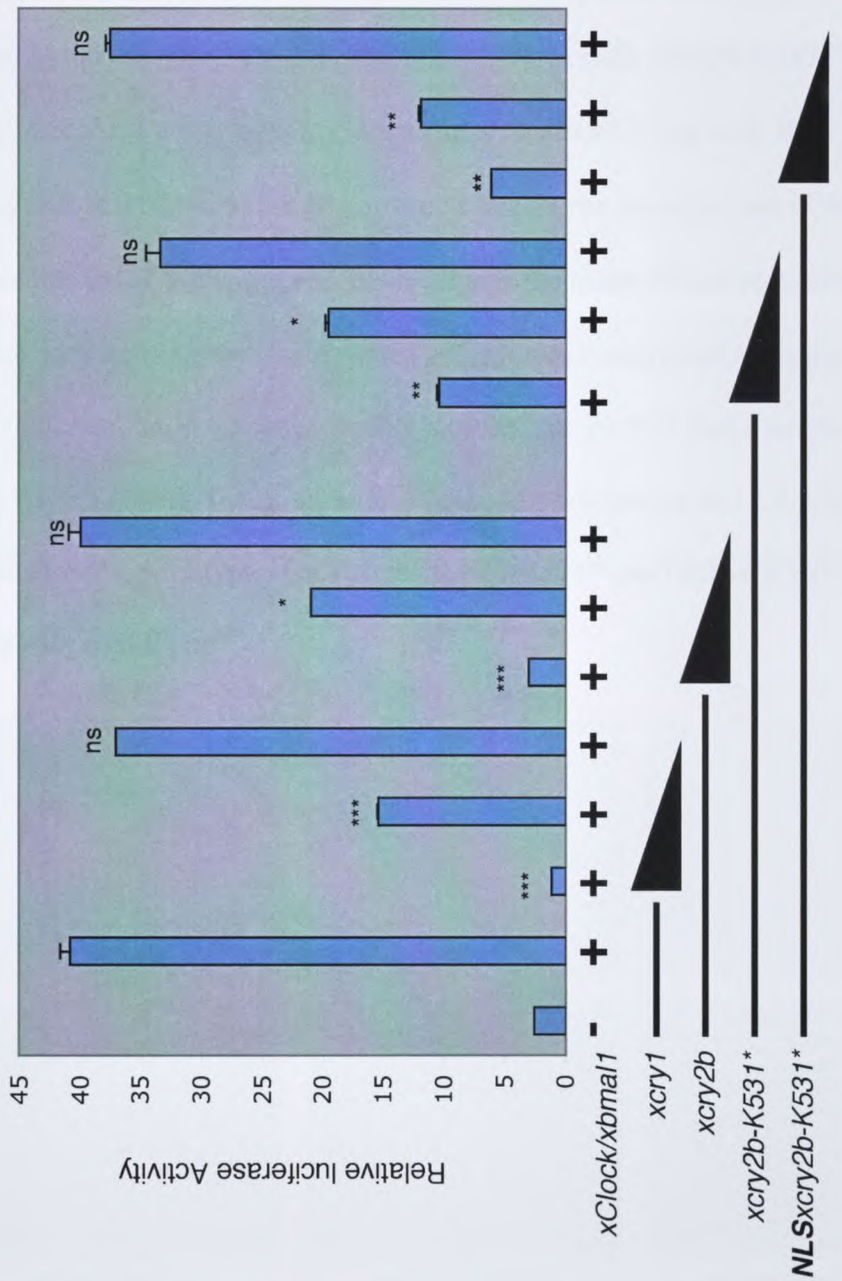


Figure 3-5: Deficiency in repression by xCRY2b-K531* is seen at both saturating and sub-saturating doses.

Repression activity was measured in a transient transfection assay in Cos-7 cells as described in the methods. DNA constructs (x*cry1, xcry2b, xcry2b-K531* and NLSxcry2b-K531**) are present at decreasing amounts (0.15 μg/well, 0.05 μg/well and 0.015 μg/well respectively) for all constructs except the reporters and *xClock* and *xbmal1*, which are present at 0.15 μg/well. Each value is the mean of two independent wells (+/- SEM) and each experiment was repeated at least twice with similar results. A triple asterisk indicates that $p < 0.001$, a double asterisk that $p < 0.01$ and a single asterisk that $p < 0.05$; ANOVA with Tukey-Kramer posthoc test comparing xCLOCK/xBMAL1 with wild type xCRY1, wild type xCRY2b, xCRY2b-K531* and NLSxCRY2b-K531*. "ns" indicates "not significant".

Figure 3-6: Truncation mutants have altered intracellular localization.

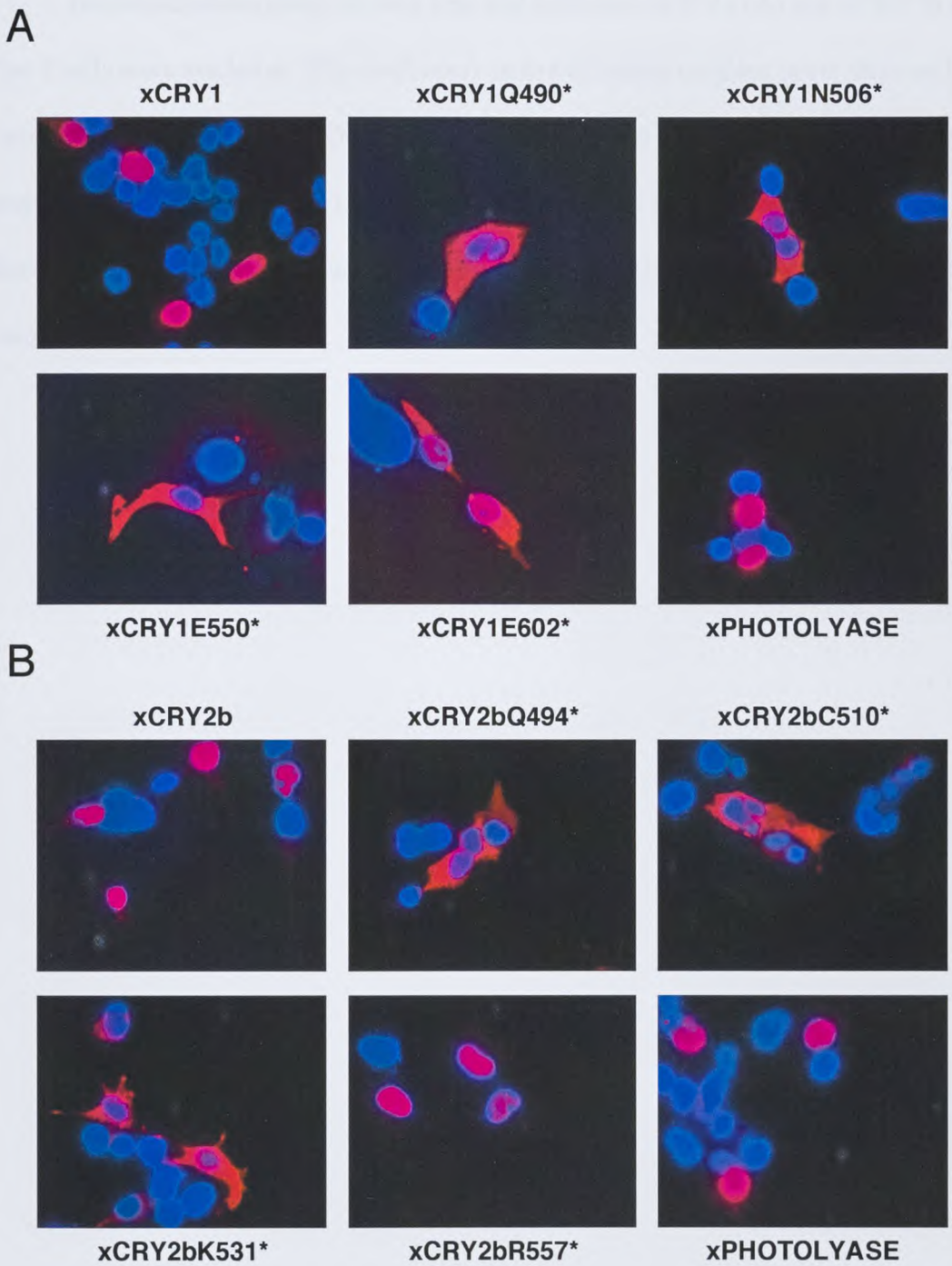


Figure 3-6: Truncation mutants have altered intracellular localization.

Immunocytochemistry of wild type and truncated xCRY1 (A) and xCRY2b (B). Cos-7 cells were seeded at 70% confluency in 6-well plates on glass cover slips and transfected with 1 μg of total DNA. Proteins are detected with anti-Flag and rhodamine-conjugated antibodies. Anti-Flag staining detecting CRY is shown in red. Nuclear Hoechst's stain is shown in blue. Overlay and co-localization of CRY and Hoechst's stain is shown in purple.

Table 3-1: Intracellular localization of xCRY1 and xCRY2b truncation mutants

	Nuclear	Cytoplasmic	Both
WTxCRY1	**94.8%**	0.7%	4.5%
xCRY1Q490*	2.8%	**91.7%**	5.5%
xCRY1N506*	0	**98.8%**	1.2%
xCRY1E550*	1.8%	**91.7%**	6.5%
xCRY1E602*	17.1%	2.7%	**80.2%**
WTxCRY2b	**72.2%**	1.1%	26.7%
xCRY2bQ494*	1.4%	**95.1%**	3.5%
xCRY2bC510*	0	**96.9%**	3.1%
xCRY2bK531*	1.7%	**98.3%**	0
xCRY2bR557*	**87.8%**	4.1%	8.1%
xPHOTOLYASE	**91.3%**	3.1%	5.6%

Table 3-1: quantification of the immunocytochemistry results for wild type and truncation mutants xCRY1 and xCRY2b. The slides were randomly numbered and about 200 cells were counted for each construct transfected .

Figure 3-7: Truncation mutants are expressed in the cytoplasm of photoreceptor cells in the retina of *Xenopus* tadpoles.

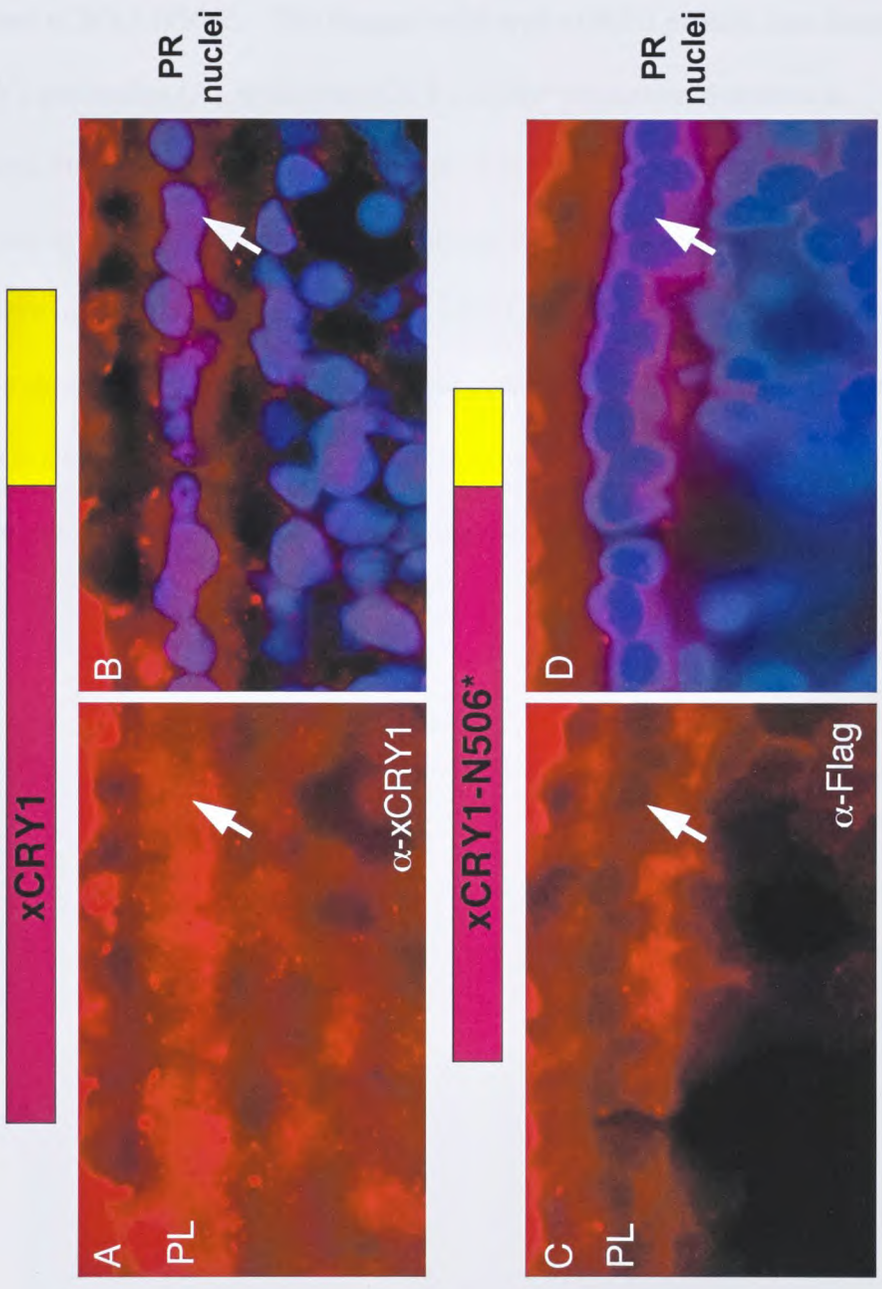


Figure 3-7: Truncation mutants are expressed in the cytoplasm of photoreceptor cells in the retina of *Xenopus* tadpoles.

Transgenic tadpoles were generated by overexpressing wild-type xCRY1 and truncation mutant xCRY1-N506*. The flagged wild-type xCRY1 protein was detected with anti-xCRY1 antibodies (A), while the xCRY1-N506* truncation mutant was detected with anti-Flag antibodies (C). Full length xCRY1 detected with anti-CRY antibody is shown in red in A, and overlay between xCRY1 and nuclear Hoechst's stain is shown in purple in B. The truncation mutant xCRY1-N506* detected with the anti-Flag antibody is shown in red in C, and overlay between xCRY1-N506* and nuclear Hoechst's stain is shown in purple in D. The white arrow indicates the nuclei of the cells in the photoreceptor layer (PL). PR nuclei= photoreceptor nuclei.

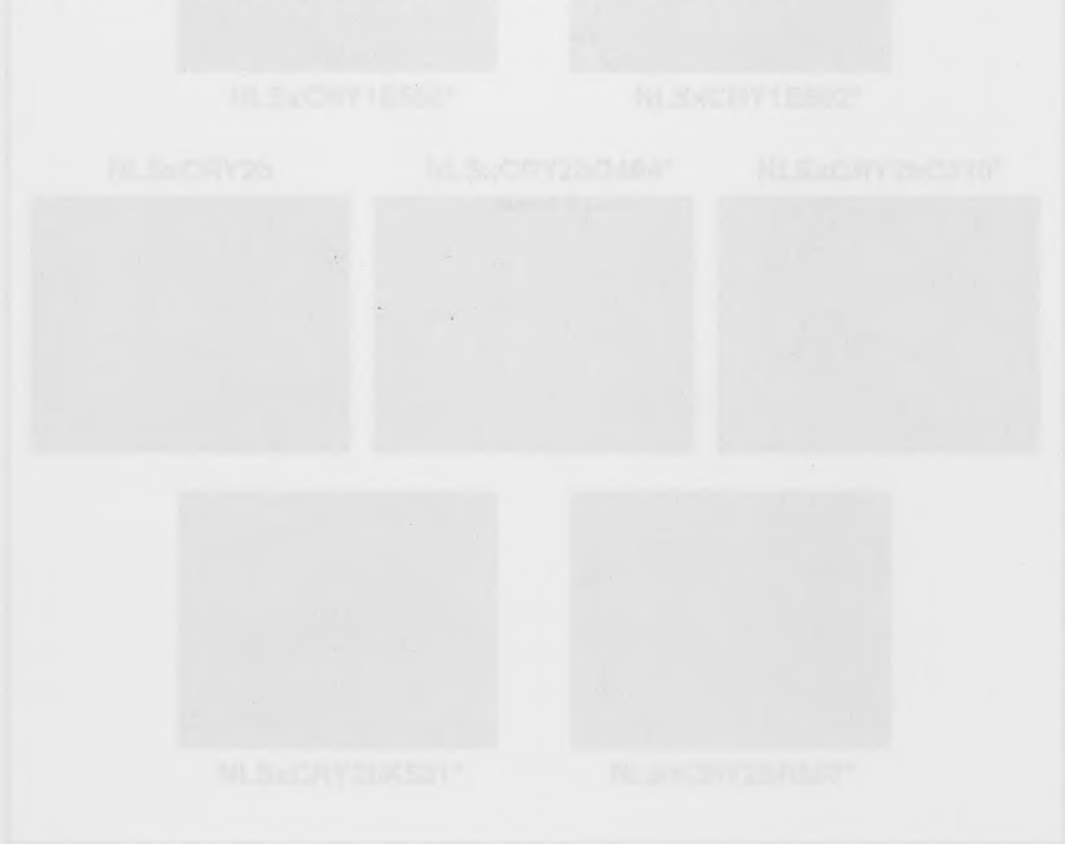
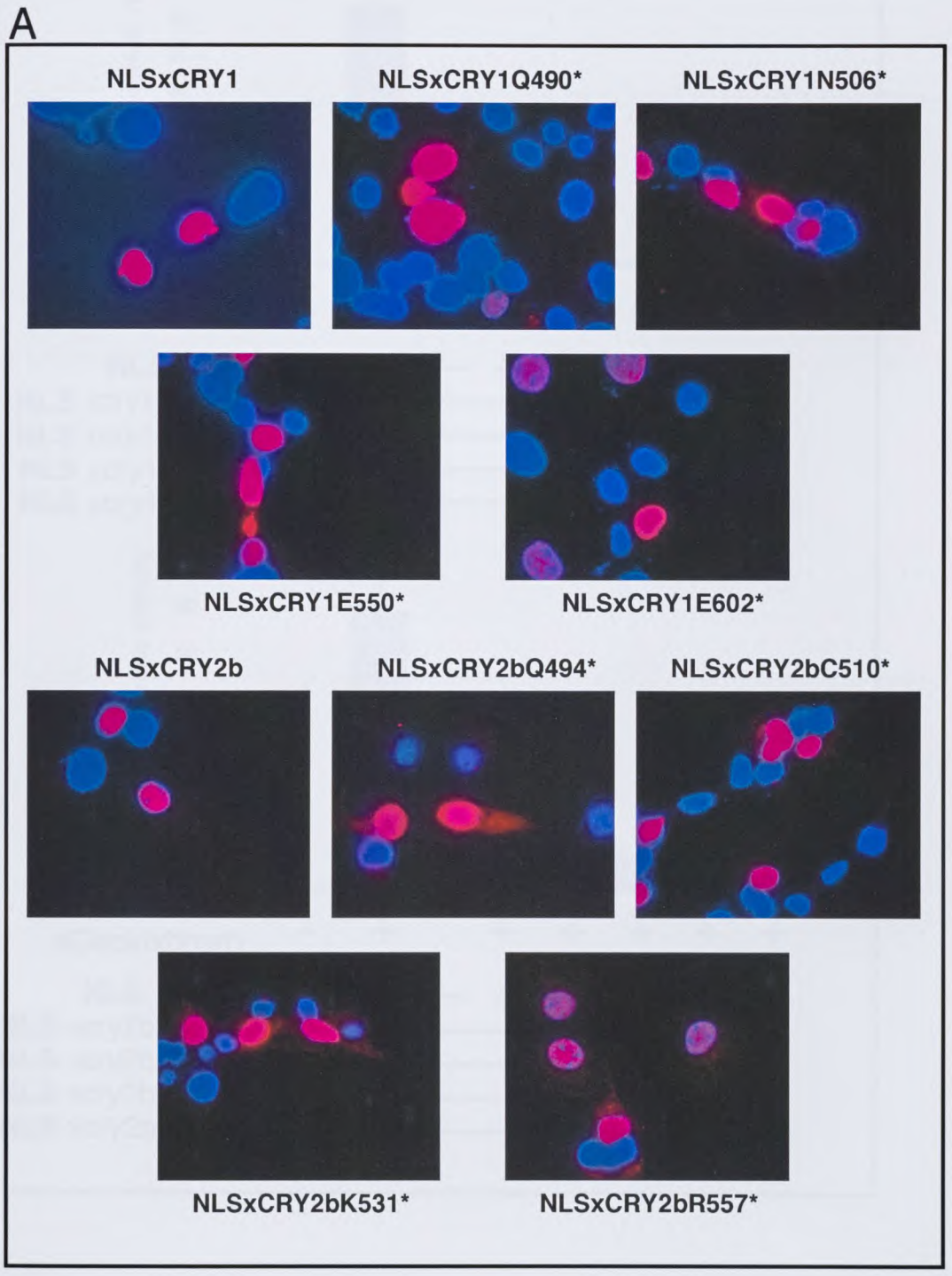


Figure 3-8: Addition of an NLS rescues nuclear localization and repressive function.



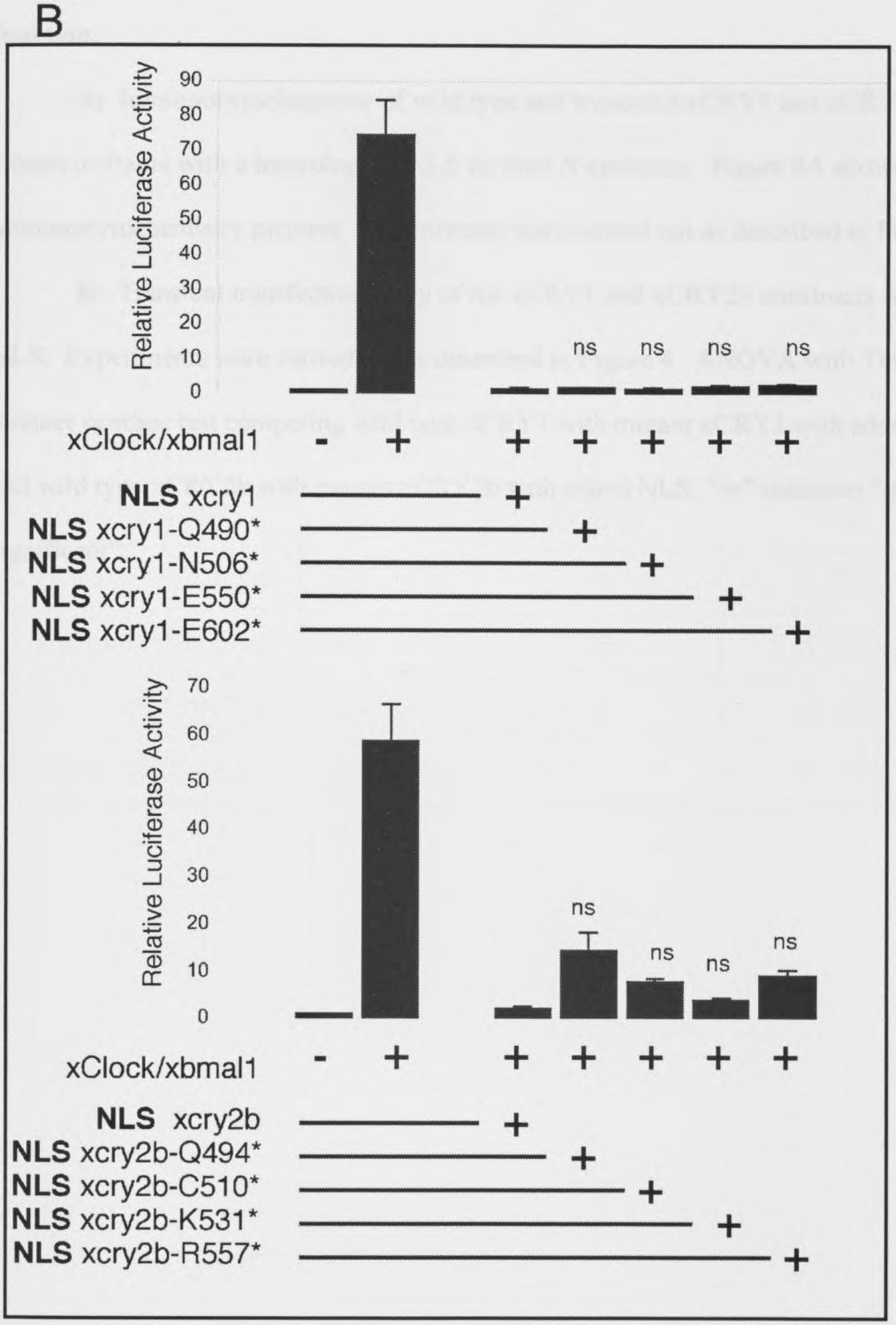

B
Relative Luciferase Activity
90
80
70
60
50
40
30
20
10
0
ns
ns
ns
ns
xClock/xbmal1
- + + + + + +
NLS xcry1
NLS xcry1-Q490*
NLS xcry1-N506*
NLS xcry1-E550*
NLS xcry1-E602*
Relative Luciferase Activity
70
60
50
40
30
20
10
0
ns
ns
ns
ns
xClock/xbmal1
- + + + + + +
NLS xcry2b
NLS xcry2b-Q494*
NLS xcry2b-C510*
NLS xcry2b-K531*
NLS xcry2b-R557*

Figure 3-8: Addition of an NLS rescues nuclear localization and repressive function.

A: Immunocytochemistry of wild type and truncated xCRY1 and xCRY2b cloned in-frame with a heterologous NLS on their N-terminus. Figure 8A shows sample immunocytochemistry pictures. Experiments were carried out as described in Figure 6.

B: Transient transfection assay of the xCRY1 and xCRY2b constructs with added NLS. Experiments were carried out as described in Figure 4. ANOVA with Tukey-Kramer posthoc test comparing wild type xCRY1 with mutant xCRY1 with added NLS and wild type xCRY2b with mutant xCRY2b with added NLS. "ns" indicates "not significant".

Table 3-2: Intracellular localization of xCRY1 and xCRY2b truncation mutants with added NLS

	Nuclear	Cytoplasmic	Both
NLSxCRY1	**97.5%**	0	2.5%
NLSxCRY1Q490*	**92.1%**	1.6%	6.3%
NLSxCRY1N506*	**86.6%**	1.9%	11.5%
NLSxCRY1E550*	**94.9%**	0	5.1%
NLSxCRY1E602*	**89.6%**	0	10.4%
NLSxCRY2b	**92.2%**	0.8%	7%
NLSxCRY2bQ494*	**95.5%**	0	4.5%
NLSxCRY2bC510*	**95.1%**	0	4.9%
NLSxCRY2bK531*	**87.4%**	0	12.6%
NLSxCRY2bR557*	**81.4%**	0	18.6%

Table 3-2: quantification of the immunocytochemistry results for wild type and truncation mutants xCRY1 and xCRY2b with added NLS. The slides were randomly numbered and about 200 cells were counted for each construct transfected .

Figure 3-9: Rescue of the repressive phenotype by the truncation mutants with added NLS occurs over a range of doses.

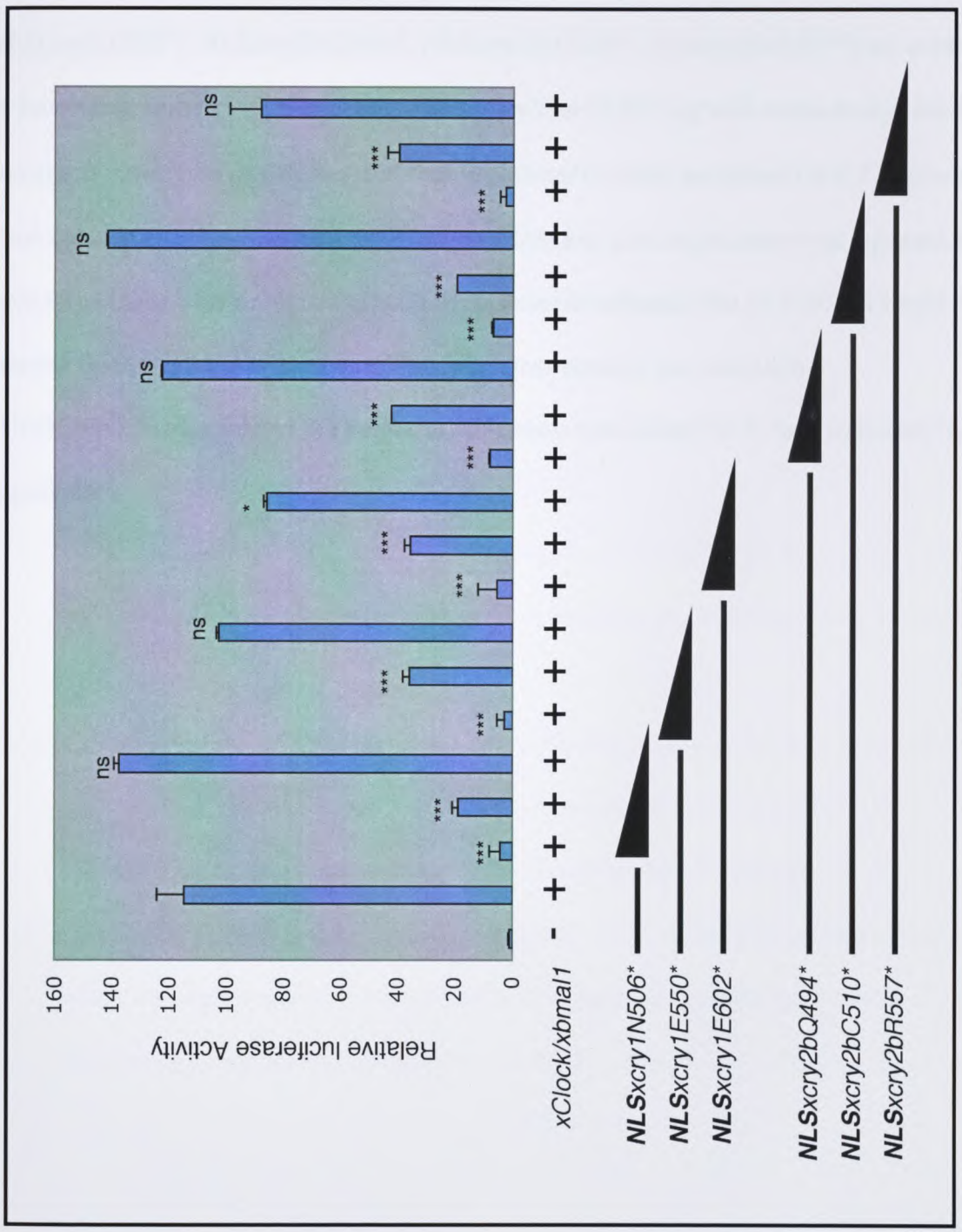


Figure 3-9: Rescue of the repressive phenotype by the truncation mutants with added NLS occurs over a range of doses.

Repression activity was measured in a transient transfection assay in Cos-7 cells as described in Figure 4. DNA constructs (*NLSxcry1-N506*, NLSxcry1E550*, NLSxcry1-E603*, NLSxcry2b-Q494*, NLSxcry2b-G510*, NLSxcyr2b-R557**) are present at decreasing amounts (0.15 μg/well, 0.05 μg/well and 0.015 μg/well respectively) for all constructs except the reporters and *xClock* and *xbmal1*, which are present at 0.15 μg/well. Each value is an average of two replicas (+/- SEM) and each experiment was repeated at least three times with similar results. A triple asterisk indicates that $p < 0.001$, a single asterisk that $p < 0.05$; ANOVA with Tukey-Kramer posthoc test comparing xCLOCK/xBMAL1 with xCRYs truncation mutants with added NLS. "ns" indicates "not significant".

References

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

Brautigam, C.A., Smith, B.S., Zhiquan, M., Palnitkar, M., Tomchick, D.R., Machius, M., Deisenhofer, J. (2004) Structure of the photolyase-like domain of cryptochrome 1 form *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.* **101**: 12142-12147.

Busza, A., Emery-Le, M., Rosbash, M. and Emery, P. (2004) Role of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science*, **304**: 1503-1506.

Cashmore, A.R., Jarillo, J.A., Wu, Y., Liu, D (1999) Cryptochromes: blue-light receptors for plants and animals. *Science*, **284**: 760-765.

Dissel, S., Codd, V., Fedic, R., Garner, K.J., Costa, R., Kyriacou, C.P., Rosato, E. (2004). A constitutively active cryptochrome in *Drosophila melanogaster*. *Nat. Neurosci.* **7**: 834-840.

Feng Li, Y., Heelis, P.F., Sancar, A. (1991) Active site of DNA photolyase: Tryptophan-306 is the intrinsic hydrogen atom donor essential for flavin radical photoreduction and DNA repair in vitro. *Biochem.*, **30**: 6322-6329.

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

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. (1998) Potent and specific genetic interference by double stranded RNA in *Caenorhadbtis elegans*. *Nature*, **19**: 806-811.

Froy, O., Chang, D.C., Reppert, S.M. (2002) Redox potential: different roles in dCRY and mCRY functions. *Curr. Biol.*, **12**: 147-152.

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

Gorlich, D., Prehn, S., Laskey, R.A., Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**: 767-778.

Green, C.B. (2004) Cryptochromes: Tail-ored for distinct functions. *Curr. Biol.*, **14**: 847-849.

Griffin E.A. Jr, Staknis D., and Weitz C.J. (1999) Light independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science*, **286:** 768-71.

Hayasaka, N., LaRue, S.L., Green, C.B. (2002) In vivo disruption of *Xenopus* CLOCK in the retinal photoreceptor cells abolishes circadian melatonin rhythmicity without affecting its production levels. *J. Neurosci.*, **22**: 1600-1607.

Hida, A., Koike, N., Hirose, M., Hattori, M., Sakaki, Y., Tei, H. (2000) The human and mouse Period1 genes: five well-conserved E-boxes additively contribute to the enhancement of mPer1 transcription. *Genomics*, **65**: 224-233.

Hirayama J., Nakamura, H., Ishikawa, T., Kobayashi, Y., Todo, T. (2003) Functional and structural analyses of Cryptochrome. *J. Biol. Chem.*, **278**: 35620-35628.

Hitomi, K., Kim, S.T., Iwai, S., Harima, N., Otoshi, E., Ikenaga, M., Todo, T. (1997) Binding and catalytic properties of *Xenopus* (6-4) photolyase. *J. Biol. Chem.*, **272**: 32591-32598.

Hsu D.S., Zhao S., Kazantsev A., Wang R.P., Todo T., Wei Y.F., Sancar A. (1996) Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. *Biochemistry*, 3**5:** 13871-7.

Kalderon D., Roberts B.L., Richardson W.D., Smith A.E.. (1984) A short amino acid sequence able to specify nuclear localization. *Cell*, **39: 4**99-509.

Kroll, K.L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*, **122**:3173-3183.

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

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

Lin C., Robertson D.E., Ahmad M., Raibekas A.A., Jorns M.S., Dutton P.L., Cashmore A.R. (1995) Association of flavin adenine dinucleotide with the A*rabidopsis* blue light receptor CRY1. *Science*, 2**69:** 968-970.

Lin, C., and Shalitin, D. (2003) Cryptochrome Structure and Signal Transduction. *Annu. Rev. Plant Biol.*, **54**: 469-496.

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

Park, H.W., Kim, S.T., Sancar, A., Deisenhofer, J. (1995) Crystal structure of DNA photolyase from *Escherichia coli*. *Science*, **268**: 1866-1872.

Payne, G., Heelis, P.F., Rohrs, B.R., Sancar, A. (1987) The active form of *Escherichia coli* DNA photolyase contains a fully reduced flavin and not a flavin radical, both *in vivo* and *in vitro*. *Biochemistry*, **26**: 7121-7127.

Reppert S.M., Weaver D.R. (2002) Coordination of circadian timing in mammals. *Nature*, **418**: 935-941.

Sancar, A., Sancar, G.B. (1988) DNA repair enzymes. *Annu. Rev. Biochem.*, **57**: 29-67.

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, *et al* (2000) Interacting molecular loops in the mammalian circadian clock. *Science*, **288:** 1013-1019.

Shinomiya, N., Gao, C.F., Cie, Q., Gustafson, M., Waters, D.J., Zhang, Y.W., Vande Woude, G.F. (2004) RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. *Cancer Res.*, **64**: 7962 -7970.

Somers, D.E., Delvin, P.E., Kay, S.A. (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science*, **282**: 1488-1490.

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

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

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., *et al.* (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc. Natl. Acad. Sci. USA*, 9**6:**12114-12119.

Welsh, D.K., Yoo, S.H., Liu, A.C., Takahashi, J.S., Kay, S.A. (2004) Bioluminescence imaging of individual fibroblasts reveal persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.*, **14**: 2289-2295.

Yagita, K., Yamaguchi, S., Tamanini, F., van der Horst, G.T.J., Hoeijmakers, J.H.J., Yasui, A., Loros, J.J., Dunlap, J.C., Okamura, H. (2000) Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes and Dev.*, **14**: 1353-1363.

Yagita, K., Tamanini, F., van der Horst, G.T.J., Okamura, H. (2001) Molecular mechanisms of the biological clock in cultured fibroblasts. *Science*, **292**: 278-281.

Yang, H.Q., Tang, R.H., Cashmore, A.R. (2001) The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. *Plant Cell*, **13**: 2573-2587.

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

Zhu, H., and Green, C.B. (2001a) Three cryptochromes are rhythmically expressed in *Xenopus laevis* retinal photoreceptors. *Mol. Vis.*, **7**: 210-215.

Zhu, H., and Green, C.B. (2001b) A putative flavin electron transport pathway is differentially utilized in *Xenopus* CRY1 and CRY2. *Current Biology*, **11**: 1945-1949.

Zhu, H., Conte, F.E. and Green, C.B. (2003) Nuclear localization and transcriptional repression are confined to separable domains in the circadian protein CRYPTOCHROME. *Curr. Biol.*, **13**: 1653-1658.

Chapter IV

xCRY1 and xCRY2b Nuclear Import is Differentially Regulated

Summary

Vertebrate CRYs exhibit mostly nuclear localization in culture, and in mouse liver their nuclear entry is highly regulated. We have previously shown that the C-termini of xCRY1 and xCRY2b are important for their nuclear localization because truncated xCRYs are retained in the cytoplasm. Here, I describe the isolation and characterization of the NLSs in the C-termini of xCRY1 and xCRY2b. Via mutagenesis studies I have identified the amino acids necessary for NLS function and demonstrated that phosphorylation regulates the function of xCRY2b's NLS.

I have found that the xCRY1 and xCRY2b NLSs are differentially regulated, which might in part explain the phenotypic difference between the *mCry1*$^{-/-}$ and the *mCry2b*$^{-/-}$ knockout mice. I have also shown that serine 539 (S539) preceding the NLS in xCRY2b is important for its regulation. Mutation of S539 to aspartate prevents nuclear entry of the mutant construct. I have also shown that BMAL1 might be involved in the nuclear import of xCRY2b, possibly via direct binding. Therefore, the nuclear import mechanisms of xCRY1 and xCRY2b are differentially regulated.

Introduction

The period of circadian activity is very close to 24 hours. While the phase of activity is quite variable, the period is an intrinsic property of the organism itself (reviewed in Menaker, 1969; Dunlap, 1999). In vertebrates, a few mutants with altered period length have been isolated (Ralph and Menaker, 1988; Vitaterna *et al.*, 1994; van der Horst *et al.*, 1999). In *Drosophila*, where much has been discovered about how period is regulated, a sustainable molecular rhythm is achieved via a number of delays affecting message stability (Suri *et al.*, 1998), protein stability (Price *et al.*, 1998) and regulation of subcellular localization (Saez and Young, 1996).

In mammals maintenance of a 24-hour period is not well understood, because many steps are likely to be involved. Not all of the clock components are rhythmic: for example, *mClock* is not rhythmic in the SCN or in the liver (Lee *et al.*, 2001). Likewise, although CLOCK and BMAL1 play key roles in sustaining the circadian loop, the loop itself is not driven by rhythmicity of these proteins. Evidence from the liver demonstrates that the CLOCK/BMAL1 heterodimer is bound to the E-box in the liver throughout the day (Lee *et al.*, 2001). On the other hand, nuclear entry of the repressors, CRYs and PERs, is strongly rhythmic in the liver (Lee *et al.*, 2001), suggesting that their nuclear entry is crucial for period maintenance. These experiments describe a scenario where a number of delays are responsible for a sustainable molecular rhythm.

The *tau* mutant is an excellent example of how posttranscriptional modifications regulate the circadian period. The *tau* mutant hamsters display a shortened circadian period (Ralph and Menaker, 1988). The phenotype is caused by a deficiency in CK (casein kinase) Iε (Lowrey *et al.*, 2000). In wild type animals, CKIε binds and

phosphorylates PER1 protein, destabilizing it (Vielhaber *et al.*, 2000; Keesler *et al.*, 2000) and therefore introducing a period delay. Because of an arginine to cysteine substitution in the catalytic domain, the mutant CKIε in mutant *tau* animals still binds PER1, but phosphorylation is strongly reduced (Lowrey *et al.*, 2000).

Phosphorylation of PER and its interaction with CRY seems to regulate its stability and, as discussed later, timing of its nuclear entry. Binding of mCRYs to mPER2 seems to increase its stability by inhibiting the ubiquitylation and proteasome-mediated degradation (Yagita *et al.*, 2002), which counteracts the rapid degradation of PER following CKIε phosphorylation (Lowrey *et al.*, 2000; Takano *et al.*, 2000; Akashi *et al.*, 2002). Since the PERs, CRYs and CKIε are found in a complex during the repressive portion of the loop (Kume *et al.*, 1999; Lee *et al.*, 2001), it is possible that these mechanisms are in place to modulate and delay the period to 24 hours.

Nuclear entry of the repressive complex

While in mouse liver CLOCK protein is present in the nucleus and the cytoplasm at constant levels, BMAL1 protein displays a circadian change in nuclear abundance and is present at low but constant amounts in the cytoplasm throughout the circadian cycle. mPER1, mPER2, mCRY1, mCRY2 and CKIε are found in a large complex in the nucleus during the night and the repressive phase of the circadian cycle (Lee *et al.*, 2001). What is the mechanism regulating the timing of their nuclear entry?

As mentioned above, PER's (and possibly CRY's) localization is regulated to delay repression (Lowrey and Takahashi, 2000). When expressed alone in tissue culture, each protein exhibits different and sometimes contrasting subcellular localization,

depending on the conditions. While PER1 is both nuclear and cytoplasmic in NIH3T3 and Cos-7 cells (Kume *et al.*, 1999), in HEK293 cells it is only nuclear (Vielhaber *et al.*, 2000). Vertebrate CRY1 and CRY2 exhibit mostly nuclear localization in cell culture (Kume *et al.*, 1999; Miyazaki *et al.*, 2001; Hirayama *et al.*, 2003; Zhu *et al.*, 2003), while CKIε is mostly cytoplasmic when transfected alone and mostly nuclear when co-transfected with other clock proteins (Vielhaber *et al.*, 2000; Lee *et al.*, 2001; Yagita *et al.*, 2002). These results emphasize the importance of *in vivo* studies, and they also depict a scenario where the interaction between the CRYs, the PERs, CKIε and possibly other binding partners are crucial for nuclear entry, repression and therefore the period of the clock.

The interactions among these proteins are complicated, and they change depending upon the binding partners. In cell lines, mCRY1 and mCRY2 can localize the PERs to the nucleus (Kume *et al.*, 1999). Although these data seem to indicate that PERs nuclear localization depend on the CRYs, data from mouse embryonic fibroblasts (MEF) argues against this. PER2 is found in the nucleus of MEFs lacking CRY1 and CRY2 protein (Yagita *et al.*, 2002). Furthermore, mCRY1 and mCRY2 are cytoplasmic in *Per1*$^{-/-}$ *Per2*$^{-/-}$ mice, suggesting that CRY nuclear localization *in vivo* depends on PER (Lee *et al.*, 2001). Although these results seem contradictory, they might simply indicate that *in vivo* CRYs and PERs depend upon each other in order to enter the nucleus and that the molecular form entering the nucleus at the correct time for repression is only the complex as a whole (Lee *et al.*, 2001).

Protein/protein interaction is not the only factor regulating the entry of the repressor complex. mPER1 and mPER2 contain nuclear localization signals (NLS), and

mPER2 also contains a nuclear export sequence (NES). mPER1's NLS is masked by CKIε phosphorylation, retaining mPER1 in the cytoplasm (Takano *et al.*, 2000). mPER2 can shuttle between the nucleus and cytoplasm using its own NLS and NES, but mCRY1 can bind to the C-terminal region of mPER2 and retain it in the nucleus (Yagita *et al.*, 2002).

CRYs also have nuclear localization signals, and this has been shown in zebrafish (Hirayama *et al.*, 2003), *Xenopus laevis* (Zhu *et al.*, 2003) and mice (Kobayashi *et al.*, 1998). In zebrafish, where six CRY genes have been cloned (*zcry1a, zcry1b, zcry2a, zcry2b, zcry3, zcry4*) and two do not have repressive function (Kobayashi *et al.*, 2000), Hirayama *et al.* identified a bipartite NLS in zCRY1a (Hirayama *et al.*, 2003). Both regions comprising the NLS (RD-1 and RD-2b) are in the N-terminal region of the protein: RD-2b is conserved in mice and renders mCRY1 cytoplasmic when mutated (Hirayama *et al.*, 2003). The C-terminus of mCRY2 contains a putative NLS (Kobayashi *et al.*, 1998), which is consistent with our data in *Xenopus* CRY1 and CRY2b (Zhu *et al.*, 2003).

Nuclear import and period regulation

As mentioned above, the nuclear entry of the repressive complex including CRYs, PERs and CKIε is a highly regulated process. It is not known if nuclear entry is regulated by one key factor or, more likely, by the completion of several steps, such as PER phosphorylation and complex formation. The discovery that both PER and CRYs contain functional NLSs (see above for references) introduces more complexity. Nevertheless, the mouse model indicates that the mammalian CRYs, as opposed to the PERs, play

necessary roles in repression (van der Horst *et al.*, 1999) and are therefore also key players in complex formation and nuclear entry. The accessory role of the PERs is not fully understood (reviewed in Lowery and Takahashi, 2000).

The majority of nuclear import and export is carried out by members of the importin β family. Cargoes containing a nuclear localization signal are bound by importin β via direct binding or via an adaptor protein, such as importin α. A few of these signals have been identified, and they appear to be extremely variable, but the best characterized is the canonical lysine-rich NLS (Kalderon *et al.*, 1984). The importin α/β heterodimer binds the cargo and shuttles it to the nuclear pore complex (NPC), where it is translocated into the nucleus (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). Regulation of this process must be tightly regulated, since many critical processes, such as cell division, depend upon it.

Here, I propose that a similar mechanism controls xCRYs subcellular localization and consequently the subcellular localization of the repressor complex and ultimate regulation of period. As described in Chapter III, xCRY1 and xCRY2b have different NLSs. xCRY2b contains a canonical lysine-rich NLS, and it is therefore possibly regulated by the importin pathway (Zhu *et al.*, 2003). The NLS is immediately preceded by a serine and a proline (539**Ser-Pro-Lys-Arg-Lys**). NLSs are known to be regulated by phosphorylation of serines or threonines immediately preceding the conserved basic amino acids: often, these phosphopeptide motifs also contain a proline (Aitken *et al.*, 1995; Muslin *et al.*, 1996; Yaffe *et al.*, 1997; Sekimoto *et al.*, 2004).

Here, I will demonstrate that the C-terminal regions shown to be important for xCRY1 and xCRY2b subcellular localization (Zhu *et al.*, 2003) function as NLSs when

fused to a normally cytoplasmic protein. In addition, xCRY1 and xCRY2b's NLS are differentially regulated. xCRY1's NLS does not resemble any conserved signal sequence, and it possibly functions as a protein/protein interaction domain. xCRY2b's NLS resembles a canonical basic NLS (Kalderon *et al.*, 1984), and nuclear localization is lost when the basic residues are deleted. Nuclear localization of xCRY2b is also lost when S539 is mutated to an aspartate, mimicking the phosphorylated state. This indicates that phosphorylation of S539 prevents NLS function, retaining xCRY2b in the cytoplasm. In addition, xBMAL1 promotes xCRY2b's nuclear localization in tissue culture: this might represent a further level of regulation of xCRY2b's subcellular localization *in vivo*.

Methods

Amplification and cloning of *xCry1* and *xCry2b* C-terminus

Full length *xCry1* and *xCry2b* are cloned into the pCMV-Tag4C vector (Stratagene, La Jolla, CA). Accession numbers for *cryptochrome* are as follows: *xCry1* (AY049033) and *xCry2b* (AY049035).

I have amplified the regions of *xCry1* and *xCry2b* which, when deleted, cause loss of nuclear localization (amino acids E550 to R596 for *xCry1*; amino acids G515 to R556 for *xCry2b*. See Chapter II and III for repression assay). The regions were cloned by PCR amplification.

To amplify the *xCry1* C-terminal region I used the following primers: Cry1CtermF (GCTGCCGCAGAATTCGGCAGATCTGCTCTCGAGGGAGAAGTGC TTCCTGGGACTTCAGG); Cry1CtermR (TACAACTCTAGAGGCGGATCCTGCAA GCTTACCTCGCTTGCCTGAAGATACCAC).

To amplify the *xCry2b* C-terminal region I used the following primers: Cry2bCtermF (GCTGCCGCAGAATTCGGCAGATCTGCTCTCGAGGGAGGAGGT CCCGTAACAGACTCCAGCC); Cry2bCtermR (TACAACTCTAGAGGCGGATCCT GCAAGCTTACCCCTGACCTTCACGTGCGACGC). The primers contain an EcoRI, BglII and XhoI restriction sites on the 5' end and XbaI, BamHI, HindIII restriction sites on the 3' end. Each site is separated by an Ala or Gly to assure complete digestion.

The *xCrys* C-terminal regions were then cloned in-frame between enhanced GFP (EGFP) and *Nocturnin* (*Noc*) in the pEGFP-NOC vector. This vector was constructed by cloning *Noc* in-frame with EGFP into the pEGFP-C2 vector (BD Biosciences, Clontech) in the EcoRI restriction site. Both the *xCry1* and *xCry2b* C-termini were cloned into the

5' BglII site and the 3' HindIII site between EGFP and *Nocturnin* and confirmed by sequencing.

Cloning of *xCry1* and *xCry2b* mutants

I have generated serial deletions of *xCry1* C-terminus with the Quick-change Mutagenesis PCR kit (Stratagene, La Jolla, CA). Site-directed mutagenesis was carried out on the pEGFP-xCry1Cterm-NOC vector. By mutagenizing 2 or 3 bases I have inserted EcoRV restriction sites in the *xCry1* C-terminus: no amino acid was changed. For each *xCry1* C-terminus deletion mutant, two EcoRV sites were inserted: the clone was then digested and re-ligated with EcoRV, deleting a portion of the C-terminus. Four deletion mutants were generated: pEGFP-xCry1C-termΔ1-NOC is missing the amino acids between E550 and G564. pEGFP-xCry1C-termΔ2-NOC is missing the amino acids between S565 and D574. pEGFP-xCry1C-termΔ1-2-NOC is missing the amino acids between E550 and D574. pEGFP-xCry1C-termΔ2-3-NOC is missing the amino acids between S565 and R596 (See Figure 2).

To generate the mutants, the following primers were used. Each deletion mutant was obtained with two sets of primers: for each construct, the smaller number indicates the 5' EcoRV site, while the larger number indicates the 3'EcoRV site (example: Cry1EcoRV#1F/ Cry1EcoRV#1R: 5'EcoRV site. Cry1EcoRV#2F/ Cry1EcoRV#2R: 3' EcoRV site).

Clone:

pEGFP-xCry1CtermΔ1-NOC

Primers:

Cry1EcoRV#1F (CCGGCCGGACTGATATCTCTCTCGAGGGAG),

Cry1EcoRV#1R (CTCCCTCGAGAGAGATATCAGTCCGGCCGG).

Cry1EcoRV#2F (GGACTTCAAGGATATTGGCAAGATATCAGCATTCTACATT ACAGC).

Cry1EcoRV#2R (GCTGTAATGTAGAATGCTGATATCTTGCCAATATCCTTGAAG TCC).

Clone:

pEGFP-xCry1CtermΔ2-NOC

Primers:

Cry1EcoRV#2F (GGACTTCAAGGATATTGGCAAGATATCAGCATTCTACATTAC AGC).

Cry1EcoRV#2R (GCTGTAATGTAGAATGCTGATATCTTGCCAATATCCTTGAAG TCC).

Cry1EcoRV#3F (GCCACAGTGATATCCAGCAATCTTAC).

Cry1EcoRV#3R (GTAAGATTGCTGGATATCACTGTGGC).

Clone:

pEGFP-xCry1CtermΔ1-2-NOC

Primers:

Cry1EcoRV#1F (CCGGCCGGACTGATATCTCTCTCGAGGGAG),

Cry1EcoRV#1R (CTCCCTCGAGAGAGATATCAGTCCGGCCGG).

Cry1EcoRV#3F (GCCACAGTGATATCCAGCAATCTTAC).

Cry1EcoRV#3R (GTAAGATTGCTGGATATCACTGTGGC).

Clone:

pEGFP-xCry1CtermΔ2-3-NOC

Primers:

Cry1EcoRV#2F (GGACTTCAAGGATATTGGCAAGATATCAGCATTCTACATTAC AGC).

Cry1EcoRV#2R (GCTGTAATGTAGAATGCTGATATCTTGCCAATATCCTTGAAG TCC).

Cry1EcoRV#4F (GGCAAGCGAGGTGATATCTTCGAATTCGGC).

Cry1EcoRV#4F (GCCGAATTCGAAGATATCACCTCGCTTGCC).

xCry2b mutant constructs were generated by using the Quick-change Mutagenesis PCR kit (Stratagene, La Jolla, CA). I mutated specific basic residues of the *xCry2b* C-terminus in the pEGFP-xCry2bCterm-NOC vector and in the full-length clone (pCMVTag2b-xCry2b). Specifically, the following amino acids were changed: K531I, KRK541-543NAN and KVR554-556QGQ.

Clone:

pEGFP-xCry2bCtermK531I-NOC and pCMVTag2b-xCry2bK531I

Primers:

Cry2bK531IF (CCCAGCGGAAGCAGCTCCTATACAGTCTCTGTGCAATGCC).

Cry2bK531IR (GGCATTGCACAGAGACTGTATAGGAGCTGCTTCCGCTGGG).

Clone:

pEGFP-xCry2bCtermKRK541-543NAN-NOC and pCMVTag2b-xCry2bKRK541-543NAN

Primers:

Cry2b541F (GCAATGCCGACTCCCCCTTCGCAAACCTGGAGGGGTCAGAAGAA

GCG).

Cry2b541R (CGCTTCTTCTGACCCCTCCAGGTTTGCGTTGGGGGAGTCGGCAT

TGC).

Clone:

pEGFP-xCry2bCtermKRK554-556QGQ-NOC

Primers:

Cry2b554FNoc (GCGTCGCACGTGCAAGGTCAGGGTGAAAGCTTCGAATTCG).

Cry2b554RNoc (CGAATTCGAAGCTTTCACCCTGACCTTGCACGTGCGACGC)

Clone:

pCMVTag2b-xCry2bKRK554-556QGQ

Primers:

Cry2b554F2b (GCGTCGCACGTGCAAGGTCAGGTGCGGAGTGTCCCGGTG).

Cry2b554R2b (CACCGGGACACTCCGCACCTGACCTTGCACGTGCGACGC).

Clone:

pEGFP-xCry2bCtermS539A-NOC and pCMV Tag-xCry2bS539A

Primers:

xCry2bS-AF (GCAATGCCGACGCCCCCAAACGC).

xCry2bS-AR (GCGTTTGGGGGCGTCGGCATTGC).

pEGFP-xCry2bCtermS539D-NOC and pCMV Tag-xCry2bS539D

xCry2bS-DF (GCAATGCCGACGACCCCAAACGC).

xCry2bS-D (GCGTTTGGGGTCGTCGGCATTGC).

Immunocytochemistry

Cos-7 cells were transfected the same day as the seeding. 3 μl of FuGENE were incubated with 1 μg of each DNA following manufacturer's recommendations (Roche, Basel, Switzerland). The cells were then split, seeded in 6-well plates containing sterile cover slips at 60-70% confluency and the DNA-FuGENE mixture was immediately added. The cells were grown overnight in DMEM + 10%FBS in 5% CO_2 at 37° C. The following day, the cells were rinsed twice in PBS and fixed for 15 min with ice-cold methanol. They were rinsed again in PBS and either mounted with Fluoromount (Electron Microscopy Sciences, Hatfield, PA) for EGFP viewing or stained with antibodies.

Anti-Flag primary antibody was diluted 1:1000 in blocking solution: 0.3% Triton, 1% protease free BSA, 0.25% λ-carrageenan, 0.1% Sodium Azide in 1X TBS, pH 7.6. Anti-digoxygenin (DIG) conjugated goat anti-mouse secondary antibody was diluted 1:1000 in blocking solution and rhodamine conjugated rabbit anti-DIG tertiary antibodies was diluted 1:1000 in blocking solution. Cells were stained for 5 minutes in Hoechst's dye H33258 (0.5 μg/ml in PBS).

The slides were viewed under an Olympus inverted EPI-fluorescent microscope (IX-70), and the images were taken using Optronics CCD camera (Optronics, Goleta, CA). The slides were randomly numbered and about 200 cells were counted for each construct transfected.

Transient transfection

The transient transfection protocol used here was previously described in Zhu and Green, (2001). Cos-7 cells were seeded at about 60-70% confluency into 6-well plates, and transfected with one microgram of total DNA per each well. The FuGENE (Roche, Basel, Switzerland) delivery system was used for each transfection. The clones used for these assays are described above. The *per E-box-Luc* reporter construct was a gift from Dr. Richard Day. The promoter contains three direct repeats of the *mper1* E-box (Gekakis *et al.*, 1998) and 87 base pairs of the rat prolactin basal promoter (Day, R., unpublished). The reporter constructs were cotransfected with expression plasmids containing the x*Clock,* x*bmal1*, *xCry1*, *xCry2b* and the *xCry1*/*xCry2b* C-terminal mutants cDNAs as indicated below each measurement. 0.15 μg of *xClock*, *xbmal1*, *xCrys* was transfected (unless indicated otherwise); 0.2 μg of the Luciferase reporter gene was transfected; 0.015 μg of the Renilla reporter gene was transfected. Total amount of transfected DNA was kept constant at 1μg. Transcriptional activity was assessed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) by measuring Luciferase activity from cell lysates and normalizing to Renilla Luciferase. Each value is an average of four replicas (+/- SEM) and each experiment was repeated at least three times with similar results.

Results

The C-termini of CRY1 and CRY2b function as nuclear localization signals

I have previously shown that truncation of the C-terminus of *xCry1* and *xCy2b* causes loss of nuclear localization and therefore repression (Zhu *et al.*, 2003). Therefore, the C-terminus of the xCRYs is required for nuclear localization. While the C-terminus of xCRY2b contains a canonical NLS, the xCRY1 C-terminus does not include any recognizable signal sequence (see previous chapter).

To test the hypothesis that the C-termini of CRY1 and CRY2b function as NLSs, I have cloned them in frame with Enhanced Green Fluorescent Protein and *Nocturnin* (EGFP-NOC). *Nocturnin* has been extensively studied in our lab, and EGFP-NOC has been characterized as cytoplasmic. Specifically, the regions of CRYs amplified cause loss of nuclear localization of the CRYs when lost (see Chapter III). For xCRY1, this is the region between E550 and R596. No amplification was carried out beyond R596 because no basic amino acids are found between R596 and E602. For xCRY2b, the region amplified lies between G515 and R556, which contains the canonical NLS arranged in a **K**X_9**KRK**X_{10}**KXR** pattern (Kalderon *et al.*, 1984), with the last R being R556. Results showed that, while GFP-NOC was mostly cytoplasmic, fusion of the C-termini of xCRY1 or xCRY2b resulted in localization of the protein to the nucleus (Figure 1).

The entire xCRY1 C-terminus is required for nuclear localization

Since no canonical NLS was found in the xCRY1 C-terminus, I generated serial deletions of the area. *pEGFP-xCry1Cterminus Δ1-NOC* encoded a protein that lacked

the first 15 amino acids in the C-terminal region (E550 through G564), and *pEGFP-xCry1Cterminus Δ2-NOC* encoded a protein that lacked the middle region (S565 thought D574). *pEGFP-xCry1Cterminus Δ1-2-NOC* encoded a protein that lacked a larger portion of the 5' region (E550 through D574), while *pEGFP-xCry1Cterminus Δ2-3-NOC* encoded a protein that lacked a larger portion of the 3' end (S565 through R596) (Figure 2A).

Results demonstrate that, as previously shown, addition of the xCRY1 C-terminus re-localized GFP-NOC to the nucleus (with some nucleo-cytoplasmic expression). Deletion of any portion of the xCRY1 C-terminus caused loss of nuclear localization with varying degrees of severity (Figure 2A, B).

Mutation of the basic amino acids in the xCRY2b C-terminus causes loss of nuclear localization

As described previously, the xCRY2b C-terminus contains a canonical NLS: in this region, there are 6 positively charged amino acids arranged in a **K** (X_9) **KRK** (X_{10}) **K**(X)**R** pattern (NLS ref) (Figure 3A). In order to test the hypothesis that the basic residues are indeed involved in nuclear localization, I introduced single or triple amino acid mutations in the conserved basic residues (in the *pEGFP-xCry2bCterm-NOC* and *pCMVTag2b-xCry2b* constructs) (Figure 3A).

Mutation of the basic amino acids caused loss of nuclear localization. The Nocturnin fusion construct with the K531I mutation was now localized mostly in the cytoplasm, with some nucleo-cytoplasmic localization still present. Mutation of either KRK541-543 or KVR554-556 caused almost complete loss of any nuclear localization

(Figure 3B). These residues have been shown to be very important for binding to importin in other proteins containing this type of NLS sequence (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). Mutations in these residues in the full-length xCRY2b led to loss of nuclear localization (data shown below).

xCRY2b NLS is regulated by phosphorylation

Nuclear localization signals function by binding to importin, which then transports the protein into the nucleus across the nuclear pore (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). Often, they can be regulated by competitive binding of other factors, such as 14-3-3, that retain the protein in the cytoplasm by preventing importin binding (Igarashi, *et al.*, 2001; Sekimoto *et al.*, 2004).

Binding to competitive factors and therefore cytoplasmic retention are regulated by phosphorylation of serines (or threonines) immediately preceding the NLS: these conserved sequences also contain a proline between the phosphorylated and basic residues (Aitken *et al.*, 1995; Muslin *et al.*, 1996; Yaffe *et al.*, 1997; Sekimoto *et al.*, 2004). Phosphorylation of the regulatory serine causes cytoplasmic retention, while loss of the phosphate group induces shuttling into the nucleus (Sekimoto *et al.*, 2004) (Figure 4A).

In xCRY2b, S539 and P540 immediately precede K541, which is the beginning of the NLS. In order to test the hypothesis that phosphorylation of S539 is important for the regulation of xCRY2b's import into the nucleus, I mutated S539 to alanine and to aspartate in the *pEGFP-xCry2bCterminus-NOC* construct and in the full-length *xCry2b*. The S539A mutation simply destroys the site, while the S539D mutation mimics the

phosphorylated state. Because of its carboxyl group, aspartate carries a negative charge, which leads to a pseudo-phosphorylated state and therefore may result in binding of putative competitive factors and to cytoplasmic retention (Sekimoto *et al.*, 2004) (Figure 4A).

Results show that mutation of S539 to alanine had no effect on the subcellular localization of the NOC fusion protein (Figure 4B) or of the full-length xCRY2b (Figure 4C). Nevertheless, mutation of S539 to aspartate led to loss of nuclear localization in the NOC fusion construct (Figure 4B) and in the full-length xCRY2b (Figure 4C). While conversion of S539 to aspartate in the isolated C-terminus (pEGFP-xCry2bCterminus-S539D-NOC) led to severe loss of any nuclear localization (Figure 4B, table), the S539D mutation in the full-length *xCry2b* led to localization of the protein from only nuclear to both nuclear and cytoplasmic (Figure 4C, table). Therefore, while wild type xCRY2b was localized to the nucleus, in the majority of cases the S539D mutant was found in both the cytoplasm and the nucleus, therefore losing some but not completely abolishing nuclear localization as in the isolated C-terminus. As expected, there was no difference between the wild type and the S539A mutation in both the isolated C-terminus and the full length constructs.

Repression is not lost in the NLS and S539 mutants

As described above, mutation of S539 to D or alteration of the basic amino acids in the xCRY2b C-terminal NLS caused loss of nuclear localization. Therefore, repressive function should also be absent, because if the mutant xCRY2b is unable to enter the

nucleus, it is also unable to repress CLOCK/BMAL1-mediated transcriptional activation as previously shown for the xCRY2b C-terminal truncation mutants (Zhu *et al.*, 2003).

In order to test the hypothesis that mutating the basic amino acids in the NLS and S539 causes loss of repressive activity, I tested the full-length xCRY2b NLS and S539 mutants in the Luciferase reporter gene assay (Figure 5A, B). Briefly, this assay allows visualization of CLOCK and BMAL1 transcriptional activation and of its repression by CRY by using Luciferase (LUC) as a reporter gene. High LUC activity correlates with CLOCK/BMAL1 transcriptional activation, while low LUC activity correlates with CRY repression.

Mutation of K541 to I had no effect on repressive activity. This is expected, because while some nuclear localization was lost in the construct, it was not abolished (Figure 5A, right inset). However, mutation of KRK541-543 or KVR554-556 also had no effect on repression, since the construct could fully repress CLOCK/BMAL1 (Figure 5A, left). This is unexpected, because the subcellular localization of these constructs is mostly cytoplasmic (Figure 5A, right inset).

The S539 mutants displayed similar results. Both xCRY2- S593A and xCRY2b-S539D still fully repressed CLOCK/BMAL1 mediated activation (Figure 5B, left). This was not surprising, because although the S to D mutation caused some relocation of the construct into both the nucleus and the cytoplasm, it did not abolish presence of the protein in the nucleus (Figure 5B, right inset).

Cos-7 cells, used in these experiments, do not contain endogenous *Clock* and *bmal1* (Yagita *et al.*, 2000). To test the hypothesis that xCLOCK and/or xBMAL1

protein might re-localize the xCRY2b NLS (and S593) mutants to the nucleus, I carried out immunocytochemical studies in the presence of CLOCK or BMAL1.

BMAL1 re-localizes xCRY2b NLS and S539 mutants into the nucleus

In order to test the hypothesis that CLOCK or BMAL1 affect xCRY2b's subcellular localization, I co-transfected *xClock*, *xbmal1* and *xCry2bKRK541-543NAN* and *S539D* mutants and examined their subcellular localization. Immunocytochemistry results show that co-transfection of xCRY2b mutants with *xClock/xbmal1* changed their subcellular localization (Figure 6). While the majority of the xCRY2bS539D mutant was localized to both the nucleus and cytoplasm, co-transfection with *xClock/xbmal1* re-localized most of the protein to only the nucleus. Similarly, xCRY2bKRK541-543NAN was localized only to the cytoplasm, but co-transfection with *xClock* and *xbmal1* re-localized it to the nucleus as well (Figure 6).

In order to discern if both CLOCK and BMAL1 are needed for this effect, I repeated the immunocytochemistry studies by transfecting only *xClock* or only *xbmal1*. As shown in Figure 6B, xCLOCK had no effect on the subcellular localization of xCRY2bS539D or xCry2bKRK541-543NAN. On the other hand, xBMAL1 re-localized both mutant proteins to the nucleus. Therefore, it appears that BMAL1 can re-localize xCRY2b mutated in its own nuclear transport mechanism to the nucleus, either by direct binding or via an indirect mechanism.

BMAL1 might bind between C510 and K539

As previously shown (see Chapter III and Zhu *et al.*, 2003), deletion of the C-terminus caused loss of nuclear localization. Deletion of the whole NLS in *xCry2b* led to loss of nuclear localization (see Figure 4B, Chapter III) but the protein was still able to carry out repression. This indicates that, in the presence of xCLOCK and xBMAL1, the truncated construct could still enter the nucleus and repress. I have shown above that this effect might be due to xBMAL1 binding to xCRY2b with the deficient NLS and transporting it into the nucleus. Further deletion of the C-terminus in xCRY2bC510* also leads to loss of nuclear localization (see Figure 6B, Chapter III), but, most importantly, it also leads to complete loss of repression (see Figure 4B, Chapter III) (Figure 7, top). These results indicate that xCRY2bC510* cannot enter the nucleus, even in the presence of xBMAL1. We have shown that loss of repression in the xCRY2b C-terminal-truncation mutants is due to loss of nuclear localization and not to loss of repressive function itself, because addition of a heterologous NLS to the truncated xCRY2bC510* (and to all the other C-terminal truncation mutants) restored nuclear localization and repression (Figure 8B, Chapter III) (Figure 7, top). In conclusion, these results indicate that xBMAL1's effect on nuclear localization of xCRY2b depends on sequences between C510 and K539 (Figure 7, bottom), because while xBMAL1 could re-localize xCRY2b with a deficient NLS to the nucleus, it could not re-localize xCRY2bC510* , since this mutant was unable to carry out repression (Figure 7, top). It is unknown whether BMAL1's effect on xCRY2b localization is due to direct binding or to indirect effects.

Discussion

In the studies described here, I have shown that xCRY1 and xCRY2b contain signaling sequences that function as NLSs. In both proteins, these sequences are contained within the C-terminus of the protein. Recent evidence from plants, *Drosophila* and *Xenopus* shows that the C-terminus contributes to the function of CRY in these organisms in diverse ways (reviewed in Green, 2004). In *Arabidopsis*, where CRY functions as a circadian photoreceptor (Somers *et al.*, 1998), the C-terminus contacts and inhibits COP1, a protein that is downstream in the blue-light signaling pathway, following blue-light activation (Yang *et al.*, 2001). In the dark, the C-terminus is inhibited by the core domain, while in the light the C-terminus is activated by absorption of photons by the core domain (Yang *et al.*, 2000). In *Drosophila*, the C-terminus of dCRY inhibits the activity of the core domain in the dark, preventing it from interacting with dPER and dTIM (TIMELESS) (Dissel *et al.*, 2004; Busza *et al.*, 2004). Upon light exposure, repression by the C-terminus is relieved (Dissel *et al.*, 2004; Busza *et al.*, 2004), and the core domain binds to and leads to the degradation of dPER and dTIM (Ceriani *et al.*, 1999; Rosato *et al.*, 2001). As described in Chapter III and in Zhu *et al.* (2003) we have shown that the C-terminus of xCRY regulates its subcellular localization. Here, I have provided evidence that both xCRY1 and xCRY2b contain functional NLSs. The diverse functions of the C-terminus of CRY indicate that even though these proteins are conserved in plants, *Drosophila*, *Xenopus* and mammals, particularly across the core region, they probably evolved separately from photolyase (Cashmore *et al.*, 1999; Green, 2004). Recently, NLS sequences have also been found in zebrafish CRY1a (zCRY1a).

The NLS is localized to the N-terminus of zCRY1a, and it is separated in two regions, both sufficient to localize zCRY1a to the nucleus (Hirayama *et al.*, 2003).

I have described here how the NLSs in xCRY1 and xCRY2b differ. xCRY1 contains a non-canonical NLS. Several studies have identified nuclear targeting sequences that do not resemble any known NLS (Standiford and Richter, 1992; Smith and Greene, 1992). It is not known if this indicates the existence of yet unknown alternative receptors to the importin pathway, or if it indicates a high variability of the sequences recognized by the importins (Christophe *et al.*, 2000). Alternatively, the xCRY1 NLS might not bind to importin, but instead could function by binding to an adaptor protein, such as PER. Evidence from co-immunoprecipitation studies from mouse liver demonstrates that mCRY1 is present in a large complex that includes PER1 and PER2 (Lee *et al.*, 2001). The PER proteins are known to contain functional NLSs: mPER2 has a bipartite NLS between amino acids 596 and 916 (Yagita *et al.*, 2002), and an NLS has also been identified in mPER3 between amino acids 524 and 1077 (Yagita *et al.*, 2000). These results suggest that CRY1 might translocate into the nucleus only after binding to PER.

On the other hand, xCRY2b contains a canonical NLS (Kalderon *et al.*, 1984); these repeats of basic amino acids have been shown to work as binding sites for members of the importin family. The importin α/β heterodimer binds to proteins via an NLS and shuttles the cargo to the nuclear pore complex (NPC), where it is translocated into the nucleus (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). The NPC is one of the biggest macromolecular structures in the eukaryotic cell, consisting of a cylinder with 8-fold symmetry, which spans the nuclear envelope and creates a central

aqueous channel (reviewed in Fahrenkrog, *et al.*, 2001; Rout and Aitchison, 2001; Vasu and Forbes, 2001). Binding of cargo in the cytoplasm and its release in the nucleus is regulated by the Ran-GTP gradient. Importins bind their cargo in the cytoplasm and release their load upon Ran-GTP binding in the nucleus. The importin-RanGTP complex recycles back to the cytoplasm where GTP hydrolysis terminates the cycle; therefore, the critical feature of this mechanism is that GTP loading and GTP hydrolysis occur in different compartments (reviewed in Mattaj and Englmeier, 1998; Görlich, and Kutay, 1999; Macara, 2001; Weis, 2002). NLSs are known to be regulated by phosphorylation of serines or threonines immediately preceding the conserved basic amino acids: often, these phosphopeptide motifs also contain a proline (Aitken *et al.*, 1995; Muslin *et al.*, 1996; Yaffe *et al.*, 1997; Sekimoto *et al.*, 2004). Recently, S557 in mouse *Cry2*, which is conserved in *Xenopus* as S539, was shown to be phosphorylated by MAPK *in vitro*. The authors phosphorylated GST-mCry2 with phospho-MAPK (P-MAPK) *in vitro* and digested the protein with trypsin. The GST-mCry2 fragments were then subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), which identified S557 as one of the MAPK phosphorylation sites (Sanada *et al.*, 2004). These results indicate that the same site might be phosphorylated in *Xenopus*. In this study, I have demonstrated that phosphorylation regulates the subcellular localization of xCRY2b. When the serine preceding the NLS (S539) was converted to an aspartate to mimic the phosphorylated state, the protein was retained in the cytoplasm. Although preliminary, these results suggest that the nuclear entry of xCRY2b and possibly of the whole repressive complex, is a carefully timed and regulated process. Possibly, phosphorylation is one of the regulatory steps leading to a 24-hour period.

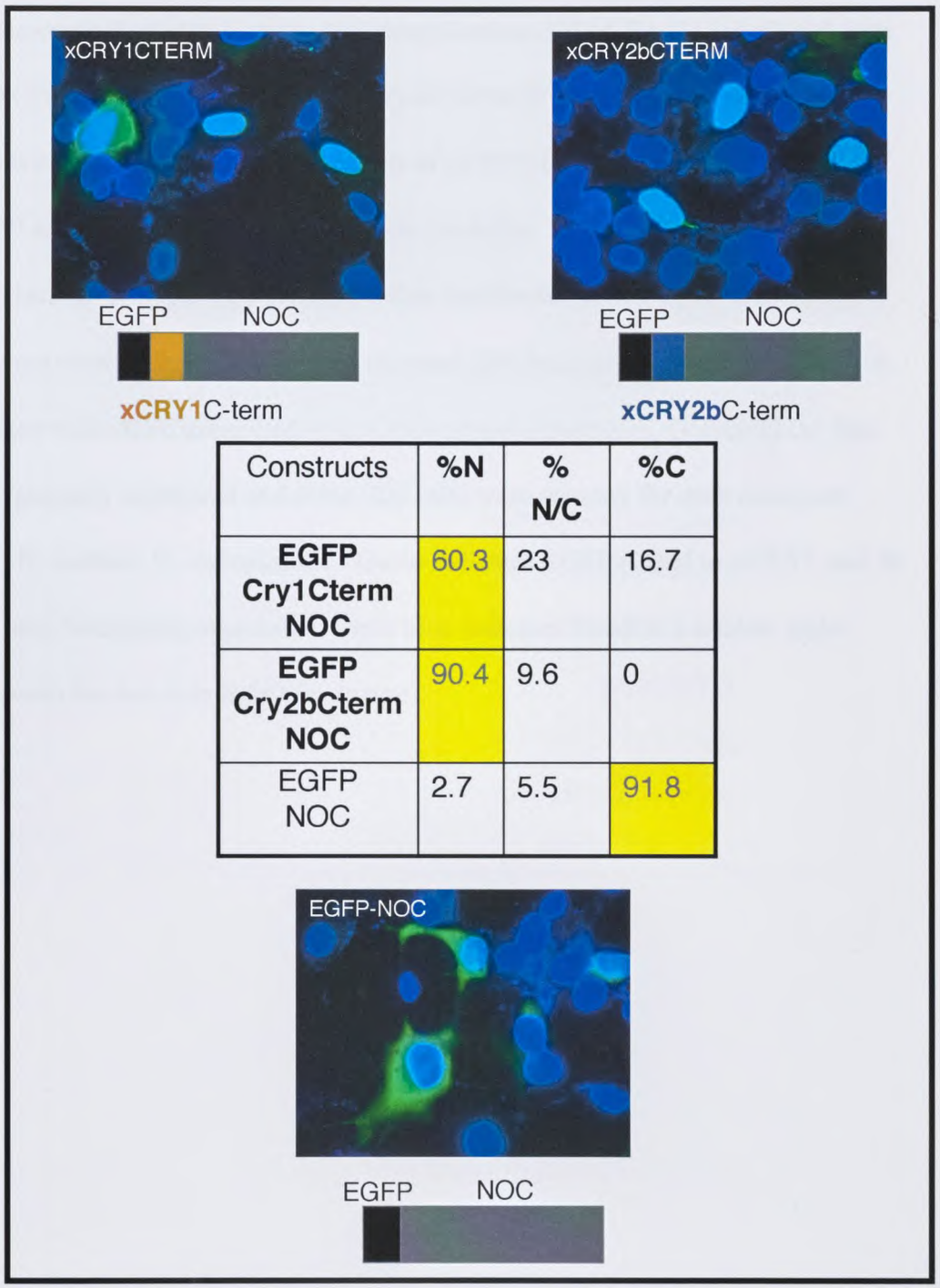
Phosphorylation has already been shown to be a crucial step in the maintenance of a 24-hour period. CKIε phosphorylation of PER leads to its rapid degradation via the ubiquitin-proteasome pathway (Akashi *et al.*, 1995; Lowrey *et al.*, 2000; Takano *et al.*, 2000). In the *tau* mutant hamster (Ralph and Menaker, 1988), CKIε has decreased kinase activity. This leads to increased PER stability and faster nuclear accumulation and therefore to faster repression which phenotypically causes a shorter period (Lowrey *et al.*, 2000).

These studies also suggest the existence of crosstalk between the circadian pathway and the MAPK pathway. It has been proposed that the MAPK cascade allows signal transduction of environmental information into the cell to produce changes in the phase of clock genes expression, consequently leading to phase-resetting (reviewed in Coogan and Piggins, 2004). MAPK phosphorylates BMAL1 in chicken (Sanada *et al.*, 2000) and mCRY1 and mCRY2 (Sanada *et al.*, 2004). Here, I hypothesize that MAPK also phosphorylates xCRY2b and regulates the subcellular localization of the repressive complex. As more is understood about the signal transduction that regulates circadian rhythms, researchers will be allowed to formulate further hypotheses on how crosstalk between the circadian and other pathways leads to cellular homeostasis.

These studies also raise the possibility that cytoplasmic xBMAL1 binds to xCRY2b and contributes to its subcellular localization. If this turns out to be the case, entry of the nuclear complex could be regulated by the phosphorylation of the PERs and CRYs, regulation of the NLSs in the CRYs and PERs and possibly binding to BMAL1. In the mouse liver, BMAL1 has been shown to shuttle between the cytoplasm and nucleus in a circadian manner, which supports the findings described here (Lee *et al.*,

2001). More experiments will be needed not only in other cell lines, but also *in vivo*. Studies in cell lines are very informative, but they also can lead to potentially artificial results. Many of the cell lines used in these experiments derive from different tissue and animals, such as mouse fibroblasts (NIH3T3), monkey kidneys (Cos-7), or human embryonic kidney (HEK293). They also display very different circadian genes expressions; while NIH3T3 cells are rhythmic (Yagita *et al.*, 2001), Cos-7 cells have low expression of clock genes (Yagita *et al.*, 2000). For example, while in HEK293 cells CKIε masks mPER1's NLS, in Cos-7 cells CKIε drags mPER1 and mPER3 into the nucleus (Takano *et al.*, 2000). These discrepancies reinforce the importance of repeating these studies *in vivo*, for example in transgenic *Xenopus* retinas.

Figure 4-1: The C-termini of xCry1 and xCry2b function as NLSs



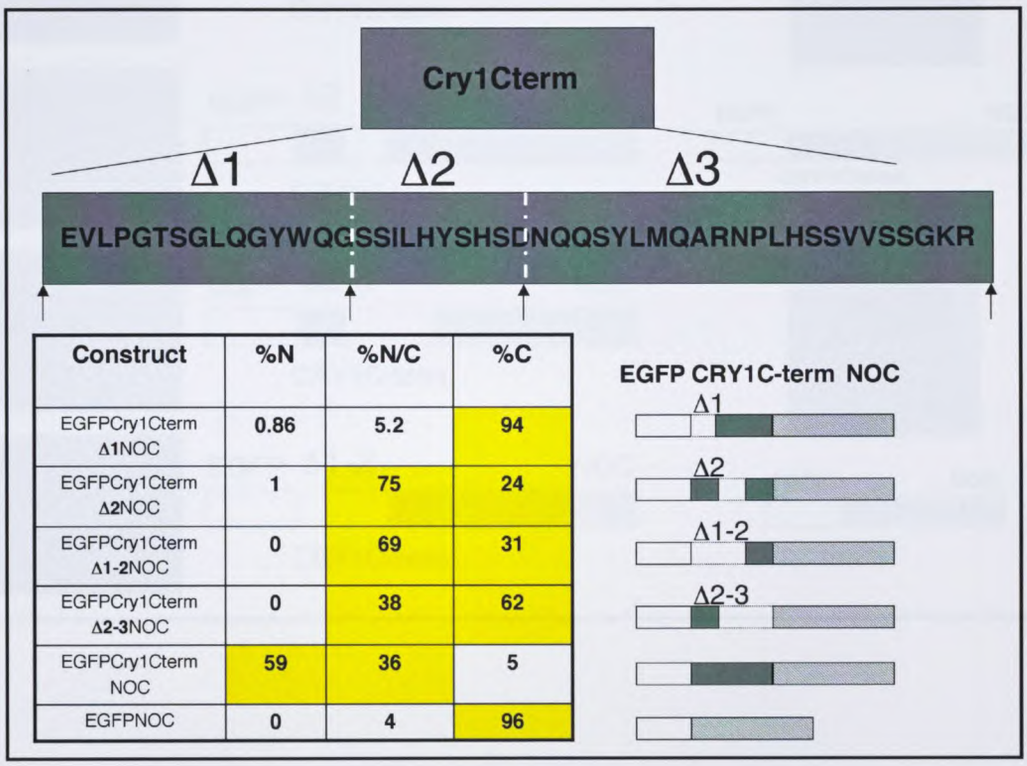
Constructs	%N	% N/C	%C
EGFP Cry1Cterm NOC	60.3	23	16.7
EGFP Cry2bCterm NOC	90.4	9.6	0
EGFP NOC	2.7	5.5	91.8

Figure 4-1: The C-termini of xCry1 and xCry2b function as NLSs

Immunocytochemistry pictures and quantification of Cos-7 cells transfected with the *pEGFPxCry1Cterm-NOC* and *pEGFPxCry2bCterm-NOC* constructs (which contain the sequences encoding the C-terminal regions of xCRY1 (orange) and xCRY2b (blue) between GFP and Nocturnin). Cos-7 cells were seeded to 70% confluency into 6-well plates onto glass cover slips. The cells were then transfected with 1 μg of total DNA. The slides were viewed under an Olympus inverted EPI-fluorescent microscope (IX-70), and the images were taken using Optronics CCD camera (Optronics, Goleta, CA). The slides were randomly numbered and about 200 cells were counted for each construct transfected. N: nuclear, C: cytoplasmic. Green indicates EGFP (fused to xCRY1 and 2b C-terminus and Nocturnin) expression, while blue indicates Hoechst's nuclear stain. Overlay between the two is in light blue (azure).

Figure 4-2: The entire xCry1 C-terminus is required for nuclear localization.

A



Construct	%N	%N/C	%C
EGFPCry1Cterm Δ1NOC	0.86	5.2	94
EGFPCry1Cterm Δ2NOC	1	75	24
EGFPCry1Cterm Δ1-2NOC	0	69	31
EGFPCry1Cterm Δ2-3NOC	0	38	62
EGFPCry1Cterm NOC	59	36	5
EGFPNOC	0	4	96

B

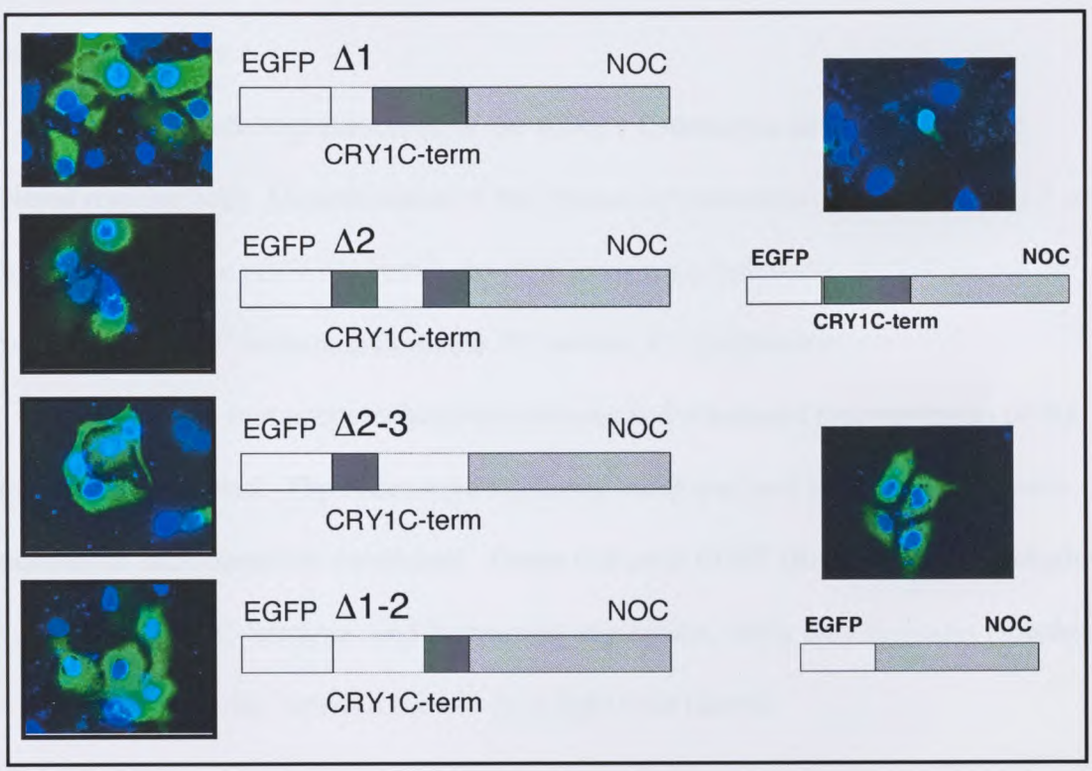

EGFP Δ1
NOC
CRY1C-term
EGFP Δ2
NOC
CRY1C-term
EGFP Δ2-3
NOC
CRY1C-term
EGFP Δ1-2
NOC
CRY1C-term
EGFP
NOC
CRY1C-term
EGFP
NOC

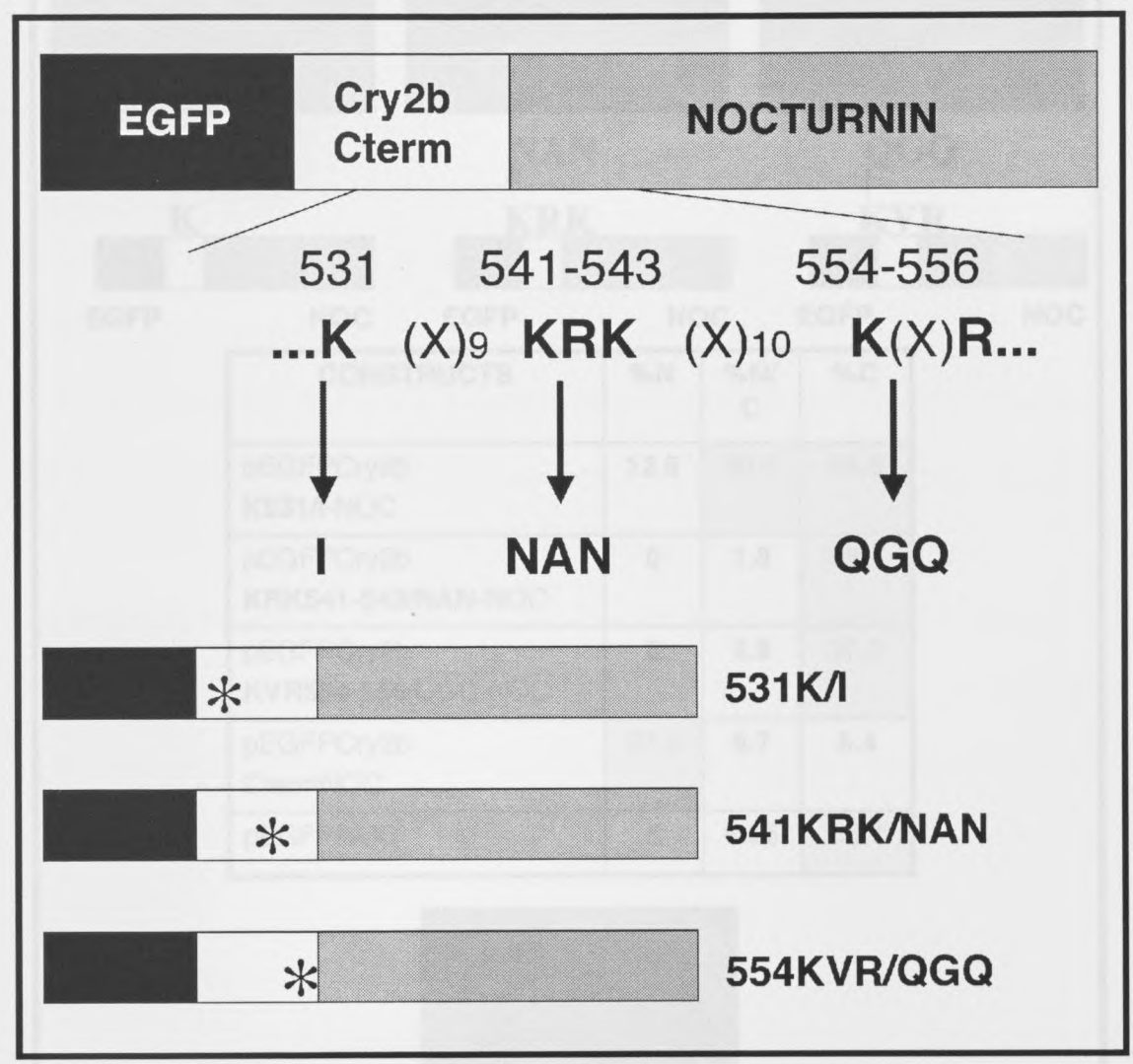
Figure 4-2: The entire xCRY1 C-terminus is required for nuclear localization.

Immunocytochemistry and quantification of Cos-7 cells transfected with *pEGFPxCry1Cterm-NOC* serial deletion constructs. The experiments were carried out as described in Figure 1.

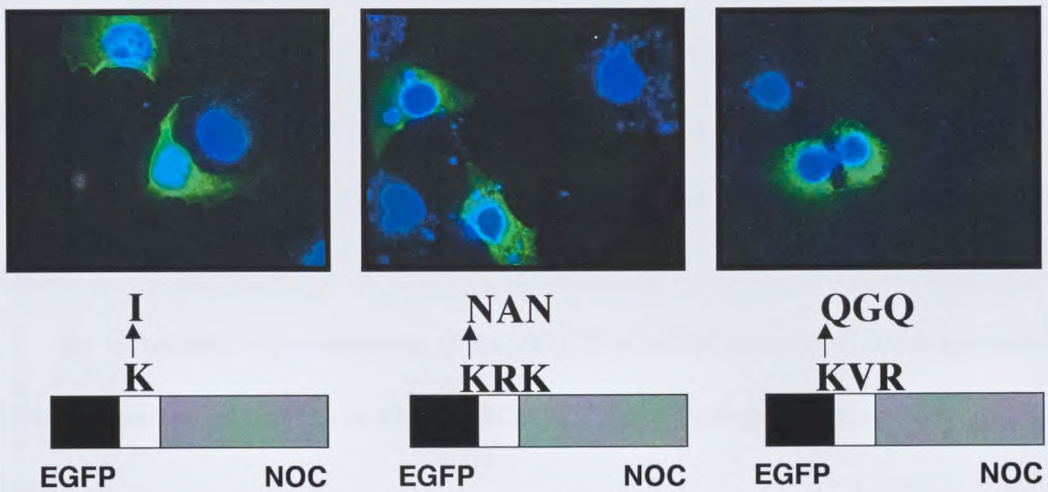
A: Schematic representation of the xCRY1 C-terminus and location of the deleted regions (top). Quantification of the immunocytochemistry pictures of Cos-7 cells transfected with the *pEGFPxCry1Cterm-NOC* constructs (encoding the full-length or truncated xCRY1 C-terminus) (bottom) N: nuclear, C: cytoplasmic.

B: Sample immunocytochemistry pictures and schematic representation of the constructs transfected. The slides were randomly numbered and about 200 cells were counted for each construct transfected. Green indicates EGFP (fused to the full-length or truncated xCRY1 C-terminus and Nocturnin) expression, while blue indicates Hoechst's nuclear stain. Overlay between the two is in light blue (azure).

Figure 4-3: Deletion of the basic residues in the xCry2b C-terminus causes loss of nuclear localization.



B



CONSTRUCTS	%N	%N/C	%C
pEGFPCry2b **K531/I**-NOC	12.5	30.5	56.9
pEGFPCry2b **KRK541-543/NAN**-NOC	0	1.8	98.2
pEGFPCry2b **KVR554-556/QGQ**-NOC	0	2.8	97.2
pEGFPCry2b CtermNOC	87.8	6.7	5.4
pEGFPNOC	5	6.25	88.7

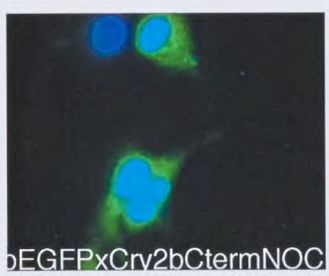


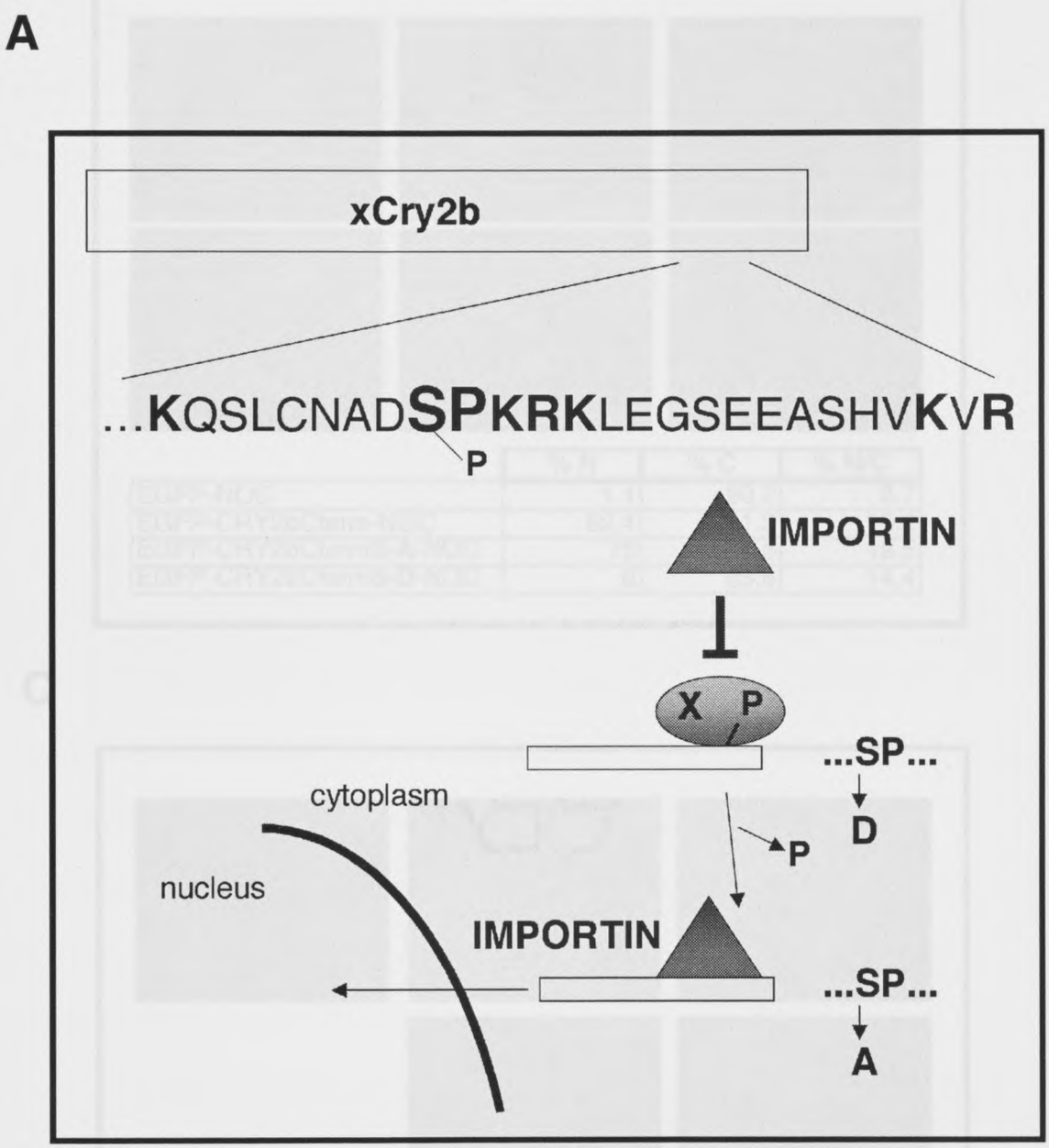
Figure 4-3: Deletion of the basic residues in the xCRY2b C-terminus causes loss of nuclear localization.

Immunocytochemistry and quantification of Cos-7 cells transfected with *pEGFPxCry2bCterm-NOC* constructs where the basic amino acids present in the xCRY2b C-terminus have been mutated. The experiments were carried out as described in Figure 1.

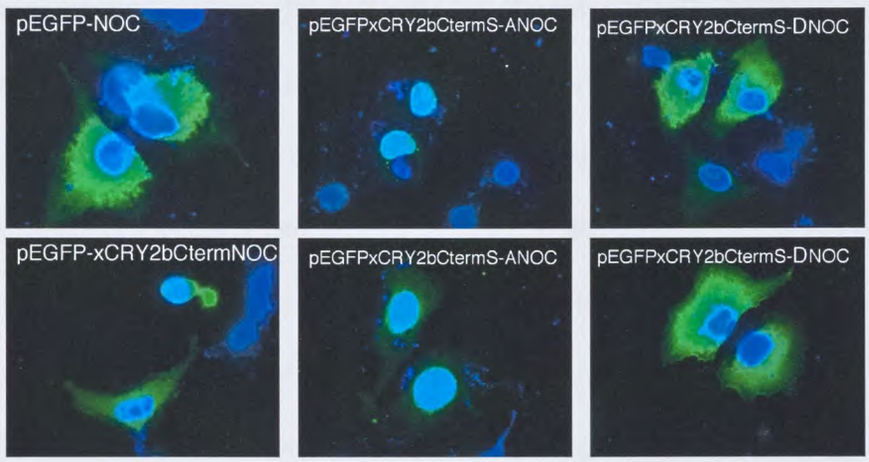
A: Schematic representation of the *pEGFPxCry2bCterm-NOC* construct where the basic amino acids present in the xCRY2b NLS have been mutated: basic residues are in bold.

B: Immunocytochemistry sample pictures of Cos-7 cells transfected with the *pEGFPxCry2b C-term-NOC* wild type and mutant constructs where the basic amino acids present in the xCRY2b NLS have been mutated. The slides were randomly numbered and about 200 cells were counted for each construct transfected. Green indicates EGFP (fused to the wild type or mutated xCRY2b C-terminus and Nocturnin) expression, while blue indicates Hoechst's nuclear stain. Overlay between the two is in light blue (azure).

Figure 4-4: Mutation of S539 to D causes loss of nuclear localization.

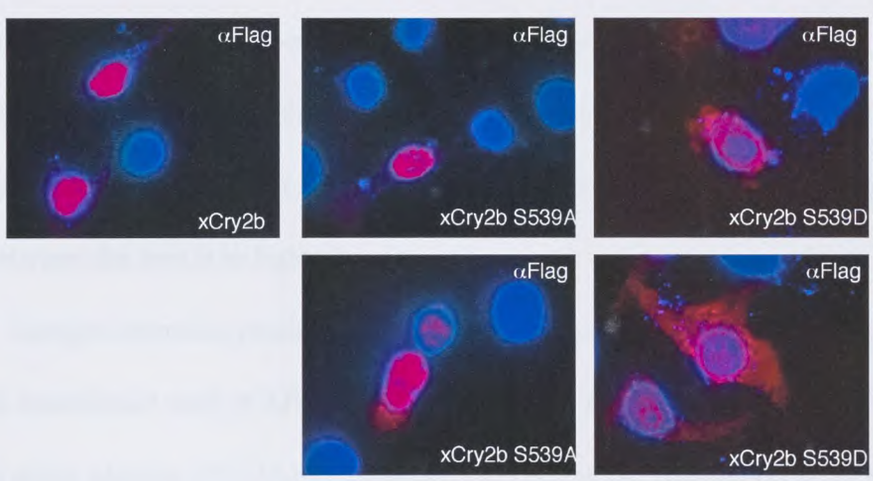


B



	% N	% C	% N/C
EGFP-NOC	1.1	90.2	8.7
EGFP-CRY2bCterm-NOC	82.4	1.3	16.2
EGFP-CRY2bCterm**S-A**-NOC	75	5.5	19.5
EGFP-CRY2bCterm**S-D**-NOC	0	85.6	14.4

C



	%N	%C	%N/C
pCMV-TagxCry2b	93.8	0	6.2
pCMV-TagxCry2bS539A	97.8	0	2.2
pCMV-TagxCry2bS539D	0	10.7	90.3

Figure 4-4: Mutation of S539 to D causes loss of nuclear localization.

Immunocytochemistry of Cos-7 cells transfected with *xCry2bCterm* and full length *xCry2b* constructs where the serine (S539) preceding the xCRY2b NLS has been mutated. The experiments were carried out as described in Figure 1.

A: Schematic of the location of S539 in the xCRY2b C-terminus with respect to the NLS (top). Model of nuclear import (bottom). Normally, importin binds to the NLS in its cargo and translocates it into the nucleus. If the serine preceding the NLS is phosphorylated (or mutated to an aspartate), a competing factor (X) is allowed to bind, preventing importin interaction, and the protein is retained in the cytoplasm.

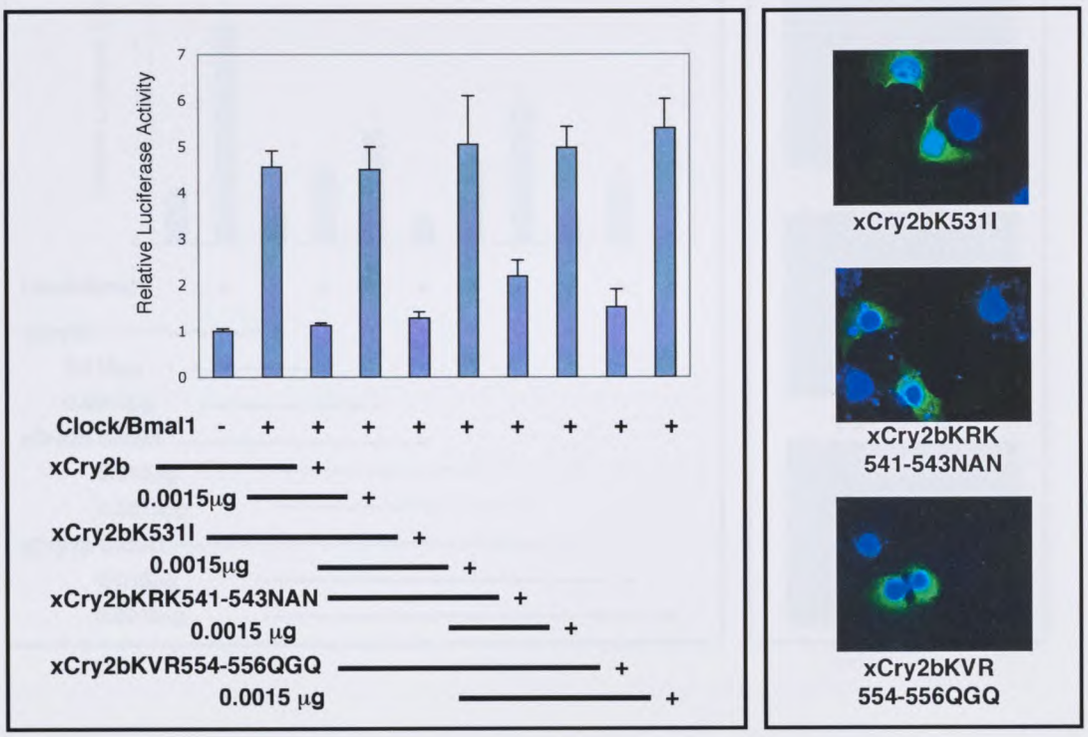
B: Sample immunocytochemistry pictures and quantification of the results of Cos-7 cells transfected with *pEGFPxCry2bCterm**S539A**-NOC* and *pEGFPxCry2bCterm**S539D**-NOC* (S539 was mutated to either alanine (S539A) or aspartate (S539D) in the *xCry2b* C-terminus fused between EGFP and NOC). The slides were randomly numbered and about 200 cells were counted for each construct transfected. N: nuclear; C: cytoplasmic. Green indicates EGFP (fused to the xCRY2b C-terminus and Nocturnin) expression, while blue indicates Hoechst's nuclear stain. Overlay between the two is in light blue (azure).

C: Sample immunocytochemistry pictures and quantification of the results of Cos-7 cells transfected with *pCMVxCry2b**S539A*** and *pCMVxCry2b**S539D*** (S539 was mutated to either alanine (S539A) or aspartate (S539D) in the full-length *xCry2b*). All the *xCry2b* constructs were fused to an N-terminal Flag tag in the pCMV-Tag2b vector. The slides were randomly numbered and about 200 cells were counted for each construct

transfected. N: nuclear; C: cytoplasmic. Proteins are detected with anti-Flag and rhodamine-conjugated antibodies. Anti-Flag staining detecting xCRY2b is shown in red. Nuclear Hoechst's stain is shown in blue. Overlay and co-localization of xCRY2b and Hoechst's stain is shown in purple.

Figure 4-5: xCry2b NLS and S539 mutants display wild-type repressive activity.

A



B

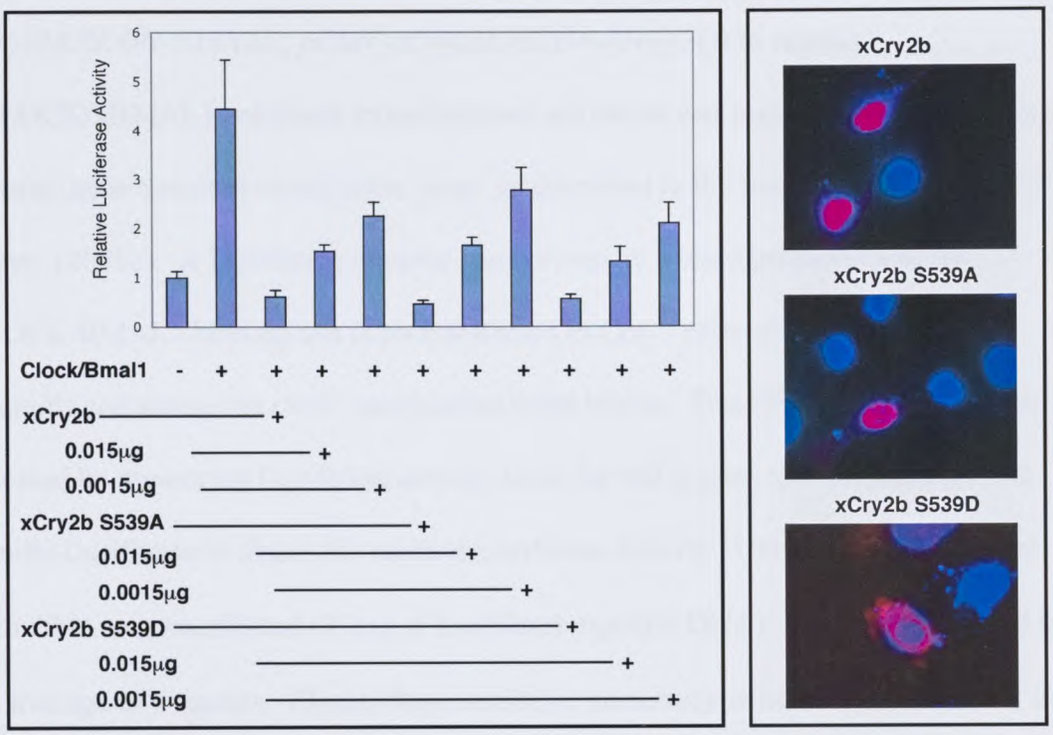

Relative Luciferase Activity
0
1
2
3
4
5
6
Clock/Bmal1 - + + + + + + + + + +
xCry2b +
0.015μg +
0.0015μg +
xCry2b S539A +
0.015μg +
0.0015μg +
xCry2b S539D +
0.015μg +
0.0015μg +
xCry2b
xCry2b S539A
xCry2b S539D

Figure 4-5: xCRY2b NLS and S539 mutants display wild type repressive activity.

A: (Left) Luciferase reporter assay of the xCRY2b NLS mutants. The ability of xCRY2b and of the xCRY2b NLS mutants (*pCMV-xCry2bK531I*, *pCMV-xCry2bKRK541-543NAN*, *pCMV-xCry2bKVR554-556QGQ*) to repress xCLOCK/xBMAL1-mediated transcriptional activation was measured via a Luciferase reporter gene transient transfection assay, as described in the methods and in Zhu and Green, (2001b). A Luciferase reporter gene driven by a basal promoter and the CLOCK/BMAL1 binding site is co-transfected in Cos-7 cells with the expression plasmids containing the clock components listed below. Transcriptional activity was assessed by measuring Luciferase activity from the cell lysates and normalizing it to Renilla Luciferase to obtain the relative Luciferase activity. Unless noted, 0.15μg of each DNA was transfected (0.2μg of Luciferase reporter DNA). Each measurement is the average of 4 repeats. (Right) Representative immunocytochemistry pictures for the relative xCRY2b C-terminus NLS mutants fused to EGFP and Nocturnin. Slides were randomly numbered and 200 cells were counted for each construct transfected. Green denotes EGFP (fused to xCRY2b C-terminus and Nocturnin) expression, while blue indicates Hoechst's nuclear stain. Overlay between the two is in light blue (azure).

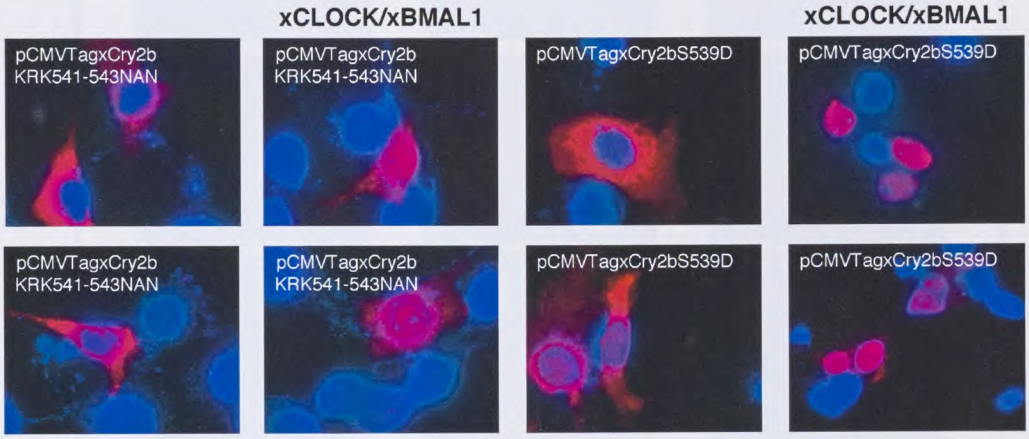
B: (Left) Luciferase reporter assay of the full-length *xCry2b S539* mutants. Luciferase reporter assay was carried out as described above. (Right) Representative immunocytochemistry pictures for each construct. The immunocytochemistry was carried out as described in Figure 4C. Slides were randomly numbered and 200 cells were counted for each construct. Red represents wild-type xCRY2b-Flag or

xCRY2bS539-Flag mutants , Hoechst's nuclear staining is shown in blue and overlay of the two is shown in purple.

A

Figure 4-6: Co-transfection of *xbmal1* re-localizes the xCRY2b mutants to the nucleus.

A



	%N	%N/C	%C
xCRY2bS-D	9.1	51.1	39.8
xCRY2bS-D+C/B	87.6	11.6	0.8
xCRY2bKRK	0	6.9	93.1
xCRY2bKRK+C/B	14.9	83.5	1.6

B

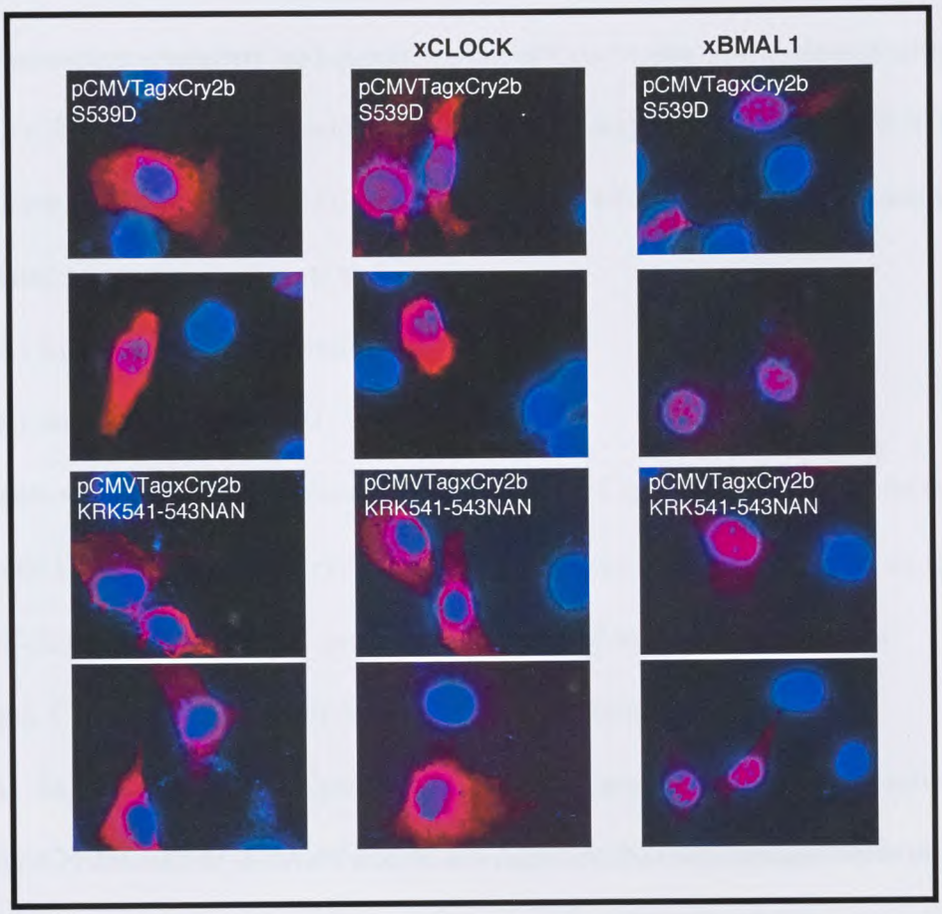

xCLOCK
xBMAL1
pCMVTagxCry2b
S539D
pCMVTagxCry2b
S539D
pCMVTagxCry2b
S539D
pCMVTagxCry2b
KRK541-543NAN
pCMVTagxCry2b
KRK541-543NAN
pCMVTagxCry2b
KRK541-543NAN

Figure 4-6: Co-transfection of *xbmal1* re-localizes the xCRY2b mutants to the nucleus.

Immunocytochemistry and quantification of Cos-7 cells co-transfected with full-length *xCry2b* NLS (where the basic amino acids necessary for the xCRY2b NLS's function have been mutated) or S539 (where the serine affecting the NLS's function has been mutated to aspartate) mutants and:

(A) either both *xClock*/*xbmal1*

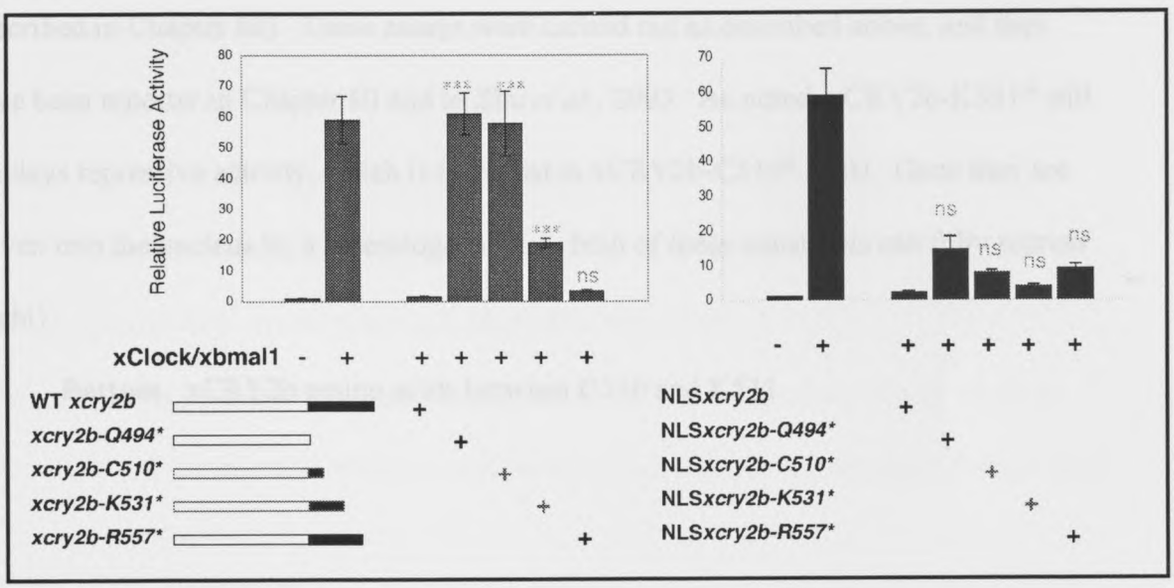
(B) only *xClock* or *xbmal1*

Experiments were carried out as described in Figure 3. Cos-7 cells were transfected with 1 μg of total DNA. All of the *xCry2b* constructs are fused to an N-terminal Flag-tag into the *pCMV-Tag2b* vector. *xClock* and *xbmal1* are cloned into the pVAX vector (Invitrogen, Carlsbad, CA) and driven by the CMV promoter.

A: Sample immunocytochemistry pictures and quantification of the results. *pCMVTagxCry2bKRK541-543NAN* and *pCMVTagxCry2bS539D* mutants were either transfected alone or co-transfected with *xClock* and *xbmal1* in Cos-7 cells. Pictures were taken and cells were counted as described above. N: nuclear; C: cytoplasmic. Red represents xCRY2b-Flag mutants, Hoechst's nuclear stain is shown in blue and overlay of the two is shown in purple.

B: Sample immunocytochemistry pictures. *pCMVTagxCry2bKRK541-543NAN* and *pCMVTagxCry2bS539D* mutants were co-transfected either with *xClock* or *xbmal1* in Cos-7 cells. Pictures were taken as described above. Red represents xCRY2b-Flag mutants expression, Hoechst's nuclear stain is shown in blue and overlay of the two is shown in purple.

Figure 4-7: Repressive activity of xCry2b C-terminal deletion mutants.



510 520 531
CVEDLGGPVTDSSHNPAEAAPK

Figure 4-7: Repressive activity of xCry2b C-terminal deletion mutants.

Top: Luciferase reporter assays of the full-length *xCry2b* truncation mutants (left) and of full-length *xCry2b* truncation mutants with an added heterologous NLS (as described in Chapter III). These assays were carried out as described above, and they have been reporter in Chapter III and in Zhu *et al.*, 2003. As noted, xCRY2b-K531* still displays repressive activity, which is fully lost in xCRY2b-C510* (left). Once they are driven into the nucleus by a heterologous NLS, both of these constructs can fully repress (right).

Bottom: xCRY2b amino acids between C510 and K531.

References

Aitken, A., Howell, S., Jones, D., Madrazo, J., Patel, Y. (1995) 14-3-3 α and δ arethe phosphorylated forms of Raf-activating 14-3-3 β and γ. *J. Biol. Chem.*, **270**: 5706-5709.

Akashi, M., Tsuchiya, Y., Yoshino, T., Nishida, E. (2002) Control of intracellular dynamics of mammalian period proteins by casein kinase Iε (CKIε) and CKIδ in cultured cells. *Molec. and Cell. Biol.*, **22**: 1693-1703.

Busza, A., Emery-Le, M., Rosbash, M., Emery, P. (2004) Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science*, **304**: 1503-1506.

Cashmore, A.R., Jarillo, J.A., Wu, Y.J., Liu, D. (1999) CRYptochromes: blue light receptors for plants and animals. *Science*, **284**: 760-765.

Ceriani, M.F., Darlington, T.K., Staknis, D., Maas, P., Petti, A.A., Weitz, C.J., Kay, S.A. (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science*, **285**: 553-556.

Christophe, D., Christophe-Hobertus, C., Pichon, B. (2000) Nuclear targeting of proteins: how many different signals? *Cell. Signal.*, **12**: 337-341.

Coogan, A.N., Piggins, H.D. (2004) MAP kinases in the mammalian circadian system key regulators of clock function. *J. Neurochem.*, **90**: 769-775.

Dissel, S., Codd, V., Fedic, R., Garner, K.J., Costa, R., Kyriacou, C.P., Rosato, E. (2004 A constitutively active cryptochrome in Drosophila melanogaster. *Nat. Neurosci.*, **7**: 834-840.

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

Fahrenkrog, B., Stoffler, D., Aebi, U. (2001) Nuclear pore complex architecture and functional dynamics. *Curr. Top. Microbiol. Immunol.*, **259**: 95-117.

Görlich, D., Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Develop. Biol.*, **15**: 607-660.

Green, C.B. (2004) Cryptochromes: tail-ored for distinct functions. *Curr. Biol.*, **14**: 847 849.

Hirayama, J., Nakamura, H., Ishikawa, T., Kobayashi, K., Todo, T. (2003) Functional and structural analyses of cryptochrome. *J. Biol. Chem.*, **278**: 35620-35628.

Igarashi, D., Ishida, S., Fukazawa, J., Takahashi, Y. (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell*, **13**: 2483-2497.

Kalderon, D., Roberts, B.L., Richardson, W.D., Smith, A.E. (1984) A short amino acid sequence able to specify nuclear localization. *Cell*, **39**: 499-509.

Kobayashi, K., Kanno, S., Smith, B., van der Horst, G.T.J., Takao, M., Yasui, A. (1998) Characterization of photolyase/blue-light receptor homologs in mouse and human cells. *Nucl. Acid Res.*, **26**: 5086-5092.

Kobayashi, Y., Ishikawa, T., Hirayama, J., Daiyasu, H., Kanai, S., Toh, H., Fukada, I., Tsujimura, T., Terada, N., Kamei, Y., Yuba, S., Iwai, S., Todo, T. (2000) Molecular analysis of zebrafish photolyase/cryptochrome family, two types of cryptochrome present in zebrafish. *Genes to Cells*, **5**: 725-738.

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M. (1999) mCRY1 and mCRY2 are essential

components of the negative limb of the circadian clock feedback loop. *Cell*, **98**: 193-205.

Landford, R.E., Butel, J.S. (1984) Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell*, **37**: 801-813.

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

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., Takahashi, J.S. (2000) Positional synthetic cloning and functional characterization of the mammalian circadian mutation *tau*. *Science*, **288**: 483-491.

Lowrey, P.L., Takahashi, J.S. (2000) Genetics of the mammalian circadian system: photic entrainment, circadian pacemaker mechanism and posttranslational regulation. *Annu. Rev. Genet.*, **34**: 533-562.

Macara, I.G. (2001) Transport into and out of the nucleus. *Microbiology and Molecular Biol. Rev.*, **65**: 570-594.

Mattaj, I.W., Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase. *Annu. Rev. Biochem.*, **67**: 265-306.

Menaker, M. (1969) Biological clocks. *Bioscience*, **19**:681-689.

Miyazaki, K., Mesaki, M., Ishida, N. (2001) Nuclear entry mechanism of rat PER2 (rPER): role of rPER2 in nuclear localization of CRY protein. *Molec. Cell. Biol.*, **21**: 6651-6659.

Muslin A.J., Tanner, J.W., Allen, P.M., Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**: 889-897.

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

Ralph, M.R., Menaker, M. (1988) A mutation of the circadian system in golden hamster. *Science*, **241**: 1225-1227.

Rout, M.P., Aitchison, J.D. (2001) The nuclear pore complex as a transport machine. *J. Biol. Chem.*, **276**: 16593-16596.

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., Costa, R., Kyriacou, C.P. (2001) Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxyl terminus of CRY. *Curr. Biol.* , **11**: 909-917.

Saez L., Young, M.W. (1996) Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. *Neuron*, **17**: 911-920.

Sanada, K., Hayashi, Y., Harada, Y., Okano, T., Fukada, Y. (2000) Role of circadian activation of mitogen activated protein kinase in chick pineal clock oscillation. *J. Neurosci.*, **20**: 986-991.

Sanada, K., Harada, Y., Sakai, M., Todo, T., Fukada, Y. (2004) Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase. *Genes to Cells*, **9**: 697-708.

Sekimoto, T., Fukumoto, M, Yoneda, Y. (2004) 14-3-3 suppresses the nuclear localization of threonine phosphorylated $p27^{Kip1}$. *The EMBO Journal*, **23**: 1934 1942.

Smith, M.R., Greene, W.C. (1992) Characterization of a novel nuclear localization signal in the HTLV-I tax transactivator protein. *Virology*, **187**: 316-320.

Somers, D.E., Devlin, P.F., Kay, S.A. (1998) Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science*, **282**: 1488-1490.

Standiford, D.M., Richter, J.D. (1992) Analysis of a developmentally regulated nuclear localization signal in Xenopus. *J. Cell. Biol.*, **118**: 991-1002.

Suri, V., Qian, Z., Hall, J., Rosbash, M. (1998) Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. *Neuron*, **21**: 225-234.

Takano, A., Shimizu, K., Kani, S., Buijs, R.M., Okada, M., Nagai, K. (2000) Cloning and characterization of rat casein kinase Iε. *FEBS Letters*, **477**: 106-112.

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

Vasu, S.K., Forbes, D.J. (2001) Nuclear pores and nuclear assembly. *Curr. Opinions Cell Biol.*, **13**: 363-375.

Vielhaber, E., Eide, E., Rivers, A., Gao, Z., Virshup, D.M. (2000) Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase Iε. *Molec. Cell. Biol.*, **20**: 4888-4899.

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

Weis, K. (2002) Nucleocytoplasmic transport: cargo trafficking across the border. *Curr. Opinions Cell Biol.*, **14**: 328-355.

Yaffe, M.B., Rittinger, K., Volina, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J., Cantley, L.C. (1997) The structural basis for 14-3-3: phosphopeptide binding specificity. *Cell*, **91**: 961-971.

Yaffe, M.B. (2004) Master of all things phosphorylated. *Biochemical J.*, **379**: 1-2.

Yagita, K., Yamaguchi, S., Tamanini, F., van der Horst, G.T.J., Hoeijmakers, J.H.J., Yasui, A., Loros, J.J., Dunlap, J.C., Okamura, H. (2000) Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes and Dev.*, **14**: 1353-1363.

Yagita, K., Tamanini, F., van der Horst, G.T.J., Okamura, H. (2001). Molecular mechanisms of the biological clock in cultured fibroblasts. *Science*, **292**: 278-281.

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

Yang, H.Q., Wu, Y.J., Tang, R.H., Liu, D., Liu, Y., Cashmore, A.R. (2000) The C-termini of *Arabidopsis* cryptochromes mediate a constitutive light response. *Cell*, **103**: 815-827.

Yang, H.Q., Tang, R.H., Cashmore, A.R. (2001) The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. *Plant Cell*, **13**: 2573 -2587.

Zhu, H., and Green, C.B. (2001) A putative flavin electron transport pathway is differentially utilized in *Xenopus* CRY1 and CRY2. *Curr. Biol.*, **11**: 1945- 1949.

Zhu, H., Conte, F., Green, C.B. (2003) Nuclear localization and transcriptional repression are confined to separable domains in the circadian protein CRYPTOCHROME. *Curr. Biol.*, **13**: 1653-1658.

Chapter V

Conclusion

Summary of My Results

The *Xenopus laevis* retina contains an autonomous circadian clock (Besharse and Iuvone, 1983; Cahill and Besharse, 1991), which regulates several physiological processes such as melatonin production (Besharse and Iuvone, 1983). *Xenopus* is an excellent system for the study of the circadian clock in the retina, and of the circadian molecular loop in general, because *Xenopus* retinas can be cultured *in vitro* (Besharse *et al.*, 1980; Besharse and Iuvone, 1983) and rhythmic melatonin can be monitored over-time (Cahill and Besharse, 1990; Green *et al.*, 1999). In addition, the transgenic technique allows fast generation of tadpoles carrying the desired transgene (Kroll and Amaya, 1996). These powerful techniques have been used successfully in our laboratory for the manipulation of the circadian clock in the retina (Hayasaka *et al.*, 2002). Several *Xenopus* circadian genes have already been cloned in our laboratory, including *xClock* (Zhu *et al.*, 2000), *xper1*, *xper2* (Steenhard and Besharse, 2000) and the *crys* (Zhu and Green, 2001a).

In this dissertation, I present my work on components of the *Xenopus* clock. Here, I present data on the cloning the *Xenopus* clock gene *bmal1*. I have shown that while *xbmal1* is strongly rhythmic in the retina, its mRNA is differentially regulated in the tissues of *Xenopus*. In mouse, CLOCK and BMAL1 form an heterodimer which activates transcription of clock-regulated genes (Hogenesh *et al.*, 1998; Gekakis *et al.*, 1998). I have shown that xBMAL1 binds to xCLOCK and activates transcription of a Luciferase reporter gene driven by CLOCK/BMAL1 binding sites. Transcriptional activation by xBMAL1 is dependent on its functional domains, the basic, HLH and PAS domains.

I have also investigated the function of the C-terminus in the circadian repressors xCRY1 and xCRY2b. In mouse, these proteins repress CLOCK/BMAL1-mediated transcriptional activation (Kume *et al.*, 1999; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999), and they have the same function in *Xenopus* in tissue culture (Zhu and Green, 2001b). In this study I have shown that xCRY1 and xCRY2b contain functional NLSs in their C-termini. These NLSs are differentially regulated, suggesting a possible explanation for the phenotypic difference observed between *mCry1* and *mCry2* knockout mice (van der Horst *et al.*, 1999).

Bmal1 in the *Xenopus* retina and tissues

In order to clone *xbmal1*, I screened a retinal cDNA library and isolated two *bmal1* clones, *xbmal1-a* and *xbmal1-b* (see Chapter II). Even though the two clones are not identical, their DNA sequences are very similar. To investigate if the two cDNAs represent two genes with distinct functions or the same *bmal1* gene duplicated because of *Xenopus* tetraploidy, I compared their mRNA expression. I found no differences in the expression pattern of the two clones. Both are expressed in the photoreceptor layer of the *Xenopus* retina, where they are both rhythmic with an indistinguishable phase, and both are expressed and non-rhythmic in the other non-retinal tissues. Evidence from zebrafish, mouse and rat shows that two *bmal* cDNAs are present in these organisms, *bmal1* and *bmal2* (Cermakian *et al.*, 2000; Oishi *et al.*, 2000; Okano *et al.*, 2001). Nevertheless, their expression patterns in the mouse SCN are different: while *mbmal1* is strongly rhythmic (Oishi *et al.*, 2000), *mbmal2* is constitutively expressed throughout the day (Okano *et al.*, 2001). In addition, the deduced amino acid sequence for xBMAL1-b is

more similar to xBMAL1-a (98.5%) than to mBMAL2 (74%). When the sequences for *xbmal1-a* and *xbmal1-b* are blasted, they both show higher ε-values to *mbmal1*. These results indicate that the *bmal1* cDNAs I cloned from the *Xenopus* retina are probably the result of tetraploidy.

I have found that *xbmal1* is strongly rhythmic in the *Xenopus* retina, peaking at the transition between light and dark. Notably, I could not detect *xbmal1*rhythmicity in the tissues tested (brain, heart and testis). These data demonstrate that either *xbmal1* mRNA is not rhythmic in the *Xenopus* tissues, or that rhythmicity is of much lower amplitude in the tissues versus the retina. This is in contrast to results from mice and rats, where *bmal1* is expressed rhythmically in the tissues tested (liver, heart and kidney of mice; eye, heart, kidney and lung of rats) (Oishi *et al.*, 1998; Oishi *et al.*, 2000). This discrepancy could be due to a regulatory difference between *bmal1* in the retina and tissues of *Xenopus*.

Evidence from the mouse SCN and liver shows that the mCLOCK/mBMAL1 heterodimer drives the expression of the orphan nuclear receptor *Rev-erbα*. Rhythmicity of *mbmal1* is sustained by the direct interaction between REV-ERBα and ROR response elements in the *mbmal1* promoter (Preitner *et al.*, 2002). If the *xbmal1* promoter does contain REV-ERBα response elements and rhythmicity of *bmal1* is regulated via a similar mechanism in the *Xenopus* retina, lack of it in the tissues could be due to different factors binding to the *xbmal1* promoter. On the other hand, the *xbmal1* promoter might not contain ROR response elements and *xbmal1* rhythmicity in the retina could be due to retina-specific rhythmic transcription factors binding specifically to the *bmal1* and not to

the *xClock* promoter, which would also explain the lack of *xClock* rhythmicity in this tissue (Zhu *et al.*, 2000).

Determining the rhythmicity of the clock genes in *Xenopus* is also important to understand the circadian behavior of this animal, which is not well studied. In fact, it is not known if *Xenopus laevis* is a diurnal or nocturnal organism, even though physiological studies suggest that adult *Xenopus* are diurnal. Oxygen consumption in adult *Xenopus* exhibits a diurnal periodicity (Abel *et al.*, 1992). In addition, male *Xenopus* display a courtship song comprised of rapid trills, which we and others have observed as a diurnal occurrence in a laboratory setting (Watson and Kelley, 1992; my unpublished observations). The development of locomotor assays might demonstrate that *Xenopus* is indeed a diurnal organism; if this is the case, existing understanding of its rhythmic gene expression can expand the understating of the circadian molecular differences between diurnal and nocturnal organisms.

Interaction between BMAL1 and CLOCK

Currently, it is not known via which domain CLOCK and BMAL1 interact, since both the HLH and PAS domains have been shown to function as protein-protein interaction domains. Both CLOCK and BMAL1 contain bHLH and PAS domains; in addition, CLOCK contains a Q-rich activation domain (King *et al.*, 1997), which is not present in BMAL1. The xBMAL1 mutants I generated (xBMALΔHLH, xBMAL1ΔPAS-A, xBMAL1ΔPAS-B) are well suited to test this question via co-immunoprecipitation studies. In addition, the phenotype of these mutants can be tested *in vivo* by generating

transgenic *Xenopus* tadpoles; these animals could provide insight on how CLOCK and BMAL1 interact and on how the heterodimer activates transcription.

Comparison between the CLOCK/BMAL1 heterodimer and other bHLH/PAS proteins can also help formulate new hypotheses. The aryl hydrocarbon nuclear translocator (ARNT) protein and the aryl hydrocarbon receptor (AhR) are involved in the protective response against polycyclic and halogenated aromatic hydrocarbons. After the ligand-dependent translocation of AhR to the nucleus, AhR and ARNT heterodimerize and activate xenobiotic metabolic enzymes (Reyes *et al.*, 1992; Hankinson *et al.*, 1995). The response is regulated by the binding of the ligand to AhR and the subsequent translocation of AhR into the nucleus; in contrast to ARNT, which is a constitutively nuclear protein (Hord and Predew, 1994) much as CLOCK, Ahr resides in the cytoplasm bound to two molecules of the 90-kDa heat-shock protein (Perdew, 1988). In the presence of ligand, the dioxin receptor accumulates in the nucleus, where it heterodimerizes with ARNT (Gu *et al.*, 2000). Evidence from the mouse liver and NIH-3T3 fibroblasts shows that while mCLOCK is present in the nucleus at constant levels throughout the circadian cycle, the levels of nuclear mBMAL1 protein vary rhythmically (Lee *et al.*, 2001; Tamaru *et al.*, 2003). The similarities between the two systems indicate that nuclear entry of BMAL1 might be important for the regulation of the circadian loop.

In addition, both nuclear localization and nuclear export (NES) sequences have been identified in the bHLH and PAS domains of AhR (Ikuta *et al.*, 1998; Berg and Pongratz, 2001). AhR contains two NES sequences, which differentially regulate the receptor's nuclear localization. The inactive form of the receptor is actively exported from the nucleus via the NES motif in the PAS domain, while the ligand-activated dioxin

receptor is exported via the NES motif present in the bHLH domain (Berg and Pongratz, 2001). xBMAL1 contains a putative NLS, starting at R37, (**RKRK**) conserved between mouse and *Xenopus* (Figure 2, Chapter II) and two sequences which reveal a substantial similarity to an NES motif ($LX_{2\text{-}3}LX_{2\text{-}3}LXL$; Fornerod *et al.*, 1997). The first one is located between the bHLH and the PAS-A domain (**L**147SDDE**L**KQ**L**I**L**) and the second one is located within the PAS-B motif (**L**367GY**L**PQE**LL**); both sequences are conserved between mouse and *Xenopus* (Figure 2, Chapter II). It is possible that nuclear localization of BMAL1 is regulated via its putative NLS and NES motifs.

Unlike CLOCK (King *et al.*, 1997), the C-terminus of the xBMAL1 protein does not contain a Q-rich region. xBMAL1's C-terminus consists of serine, threonine and proline repeats, many of which are conserved between mouse and *Xenopus* (Figure 2, Chapter II). Of the last 178 amino acids in the xBMAL1 C-terminus, 32 are serines (18%), 17 are prolines (9.6%) and 9 are threonines (5.1%). The C-terminal region of chicken BMAL1 has been show to be phosphorylated by MAPK at S527, T534 and S599 (Sanada *et al.*, 2001). These sites (S525, T531, S539) are all conserved in *Xenopus* BMAL1, raising the possibility that they are phosphorylation targets of xMAPK. This same stretch of amino acids (between R515 and G541) also resembles a proline, glutamic acid, serine and threonine (PEST) region (PESTfind). PEST sequences have been shown to result in rapid intracellular degradation of the proteins containing them (Rogers *et al.*, 1986). In BMAL1, this region is well conserved between *Xenopus*, mouse and zebrafish (Figure 1) (also: Figure 2, Chapter II) and might be involved in degradation, and therefore rhythmicity, of BMAL1 protein.

Therefore, *bmal1* mRNA and protein rhythmicity, nuclear entry and heterodimerization with CLOCK are regulated by transcriptional and post-translational mechanisms. Likely, many of these steps are also modulated by phosphorylation of BMAL1, CLOCK or other co-factors. Phosphorylation is also likely to mediate crosstalk between the circadian mechanisms and other cellular processes; some of these possible pathways are described below.

Regulation of xCRYs subcellular localization

I have shown in Chapter IV that although both xCRYs contains NLSs, they are regulated via different mechanisms. xCRY1 does not contain a defined NLS, while xCRY2b contains a "classical" lysine-rich NLS arranged in a $KX_9KRKX_{10}KXR$ pattern originally identified in the SV40 antigen (Kalderon *et al.*, 1984; Landford and Butel, 1984). For xCRY1, when any portion of the C-terminal region between E550 and R596 is lost, nuclear localization is impaired. This points to the possibility that the whole region functions as a non-canonical NLS or as a protein-binding domain. The basic amino acids present in the xCRY2b NLS's are crucial for its function, and nuclear localization is lost when they are mutated. Most likely, the xCRY2b's NLS is regulated via the importin α/β pathway, which is known to function through the classical lysine-rich nuclear localization signals (reviewed in Görlich and Kutay, 1999; Mattaj and Engleimer, 1998).

I have demonstrated that function of the xCRY2b's NLSs is dependent upon phosphorylation of S539: when this residue is phosphorylated, xCRY2b is retained in the cytoplasm, indicating that phosphorylation induces the binding of a regulatory protein,

which, in turn, inhibits importin binding. One such regulatory protein could belong to the family of 14-3-3 proteins. 14-3-3 proteins, first discovered in 1967 (Moore and Vj, 1967) are some of the most ancient and abundant proteins, and are expressed in all eukaryotic cells. This family of proteins consists of seven isotopes in mammalian cells ($\beta,\varepsilon,\eta,\gamma,\tau,\zeta,\sigma$) (Aitken, 2002) playing critical roles in regulating multiple cellular processes including the maintenance of cell cycle checkpoints, DNA repair, prevention of apoptosis, the onset of cell differentiation and senescence, the coordination of cell adhesion and motility, the orchestration of a sustained response to DNA damage, the control of chromatin remodeling and the defensive response to toxins and environmental stresses (reviewed in Mackintosh, 2004; Wilker and Yaffe, 2004).

Recently, over 200 human binding partners for yeast 14-3-3 were isolated via affinity chromatography. These include the mitotic phosphatases Cdc25B and C, the pro-apoptotic protein BAD, histone deacetylases 4, 5 and 7, the protein kinase Raf, the catalytic subunit of human telomerase (Ponzuelo Rubio *et al*, 2004), arylalkamine/serotonin N-acetyltransferase (AANAT), the enzyme which regulates the synthesis of melatonin (Ganguly *et al.*, 2001) and the Cdk inhibitor $p27^{Kip1}$ (Sekimoto, *et al.*, 2004). Binding to their target proteins results in a variety of effects, spanning from activation, to repression, to regulation of subcellular localization: how this range of effects is achieved by a single family of proteins is unclear, but it is thought that 14-3-3 functions via a modular mechanism, allowing more flexibility (reviewed in Mackintosh, 2004). For example, a 14-3-3 dimer activates phospho-AANAT by restricting the movement of a loop, forcing open the active site of the enzyme into a conformation

which favors substrate binding (Ganguly *et al.*, 2001; Klein *et al.*, 2002). Strikingly, the melatonin biosynthetic pathway displays a number of 14-3-3 targets (Klein, *et al.*, 2003).

14-3- 3 are also associated with dynamic nucleocytoplasmic shuttling. Phosphorylation-dependent binding of 14-3-3 to telomerase promotes its nuclear localization (Seimya *et al.*, 2000), while binding of 14-3-3 to the phosphorylated plant transcription factor *repression of shoot growth* (RSG) promotes its cytoplasmic accumulation (Igarashi, *et al.*, 2001). One proposed general feature of 14-3-3s is that they promote cytoplasmic localization or nuclear export by masking NLSs. $p27^{Kip1}$ is a CDK inhibitor which is translocated into the nucleus by the importin pathway where it regulates cyclin-CDK complexes. Stimulation of the cell cycle by growth factors leads to $p27^{Kip1}$'s phosphorylation and subsequent binding to 14-3-3. Binding of 14-3-3 to the phosphorylated NLS blocks importin binding, which promotes cytoplasmic localization of $p27^{Kip}$, (Sekimoto *et al.*, 2004).

The possibility that 14-3-3 might bind to the phosphoserine in xCRY2b and regulate its NLS by retaining it in the cytoplasm and leading to a delay in the period is very intriguing (Figure2 A, B). Equally intriguing is the fact that xCRY1 does not contain such a motif, possibly indicating that the two proteins are differentially regulated in their nuclear import. In mice, there are prominent functional differences between the two proteins. *mCry1*$^{-/-}$ knockout mice have a free running period faster than 24 hours, while *mCry2*$^{-/-}$ knockouts' free running period is slower than 24 hours. Animals become completely arrhythmic in constant conditions only when both proteins are lost (van der Horst, 1999). This suggests that mCRY1 and mCRY2 are both necessary but that neither one is sufficient for maintenance of a proper rhythm. Furthermore, the two proteins

possibly have antagonistic roles, which help stabilize the period to approximately 24 hours. Since CRY1 and CRY2 are very similar in the core domain, this difference could be achieved via the C-terminus and, more specifically, via the timing of nuclear import: like the forces controlling a pendulum, CRY1 could slow the clock (because, when lost, the period is faster than 24 hours), while CRY2 could speed it up.

Testing of the hypothesis that 14-3-3 regulates xCRY2b's subcellular localization should begin by testing direct binding between these two proteins. Specifically, a xCRY2b peptide encompassing the NLS, S539 and the flanking sequences could be used in co-immunoprecipitation studies; if binding of 14-3-3 is dependent upon phosphorylation of S539, it should be abolished when S539 is mutated to alanine, and constitutive when S539 is either phosphorylated or mutated to aspartate.

If the interaction between 14-3-3 and xCRY2b proves to be true, 14-3-3 could have a profound effect on xCRY2b's subcellular localization *in vivo*, and therefore on the period of the molecular loop. The *tau* mutant hamster displays a shorter period due to a reduction in PER1 phosphorylation and decay (Ralph and Menaker, 1988; Keesler *et al.*, 2000; Vielhaber *et al.*, 2000), which therefore leads to a faster repression of the CLOCK/BMAL1 heterodimer. Retention of xCRY2b in the cytoplasm could introduce a period delay or, possibly, arrhythmicity (Figure2 A, B). This could be tested *in vivo* in tissue culture or in transgenic *Xenopus laevis* (Kroll and Amaya, 1996).

These two approaches, the transgenic technique in *Xenopus* tadpoles and the tissue culture studies, carry advantages and drawbacks. As compared to mice, numerous transgenic *Xenopus* tadpoles are relatively simple to obtain (Kroll and Amaya, 1996). In addition, release of melatonin from the *Xenopus* retina, in adults and tadpoles, has served

as a convenient measurement of the circadian clock (Cahill and Besharse, 1991; Green *et al.*, 1999; Hayasaka *et al.*, 2002). Nevertheless, the REMI technique can damage the genomic DNA of the transgenic sperm or damage the unfertilized embryos, leading to a small number of viable transgenic tadpoles. Moreover, it is not possible to control how many copies of the transgene will be integrated, which leads to variable expression. Many of these setbacks can be resolved by testing the mutants in a tissue culture system.

Immortalized mammalian cell lines have been shown to display circadian rhythms (Basalobre *et al.*, 1998). Mouse embryonic fibroblasts display oscillations that are molecularly comparable to the oscillations in the SCN in temporal expression profiles of the clock genes and in the phase of the mRNAs rhythms (Yagita *et al.*, 2001). Even though rhythms dampen in culture, this phenomenon is now thought to be the result of asynchrony between cells, leading to the assumption that peripheral cells contain self-sustained circadian oscillators (Yoo *et al.*, 2004; Welsh *et al.*, 2004). Recently, bioluminescence Luciferase rhythms have been monitored from rat-1 fibroblasts either acutely transfected with a *mbmal1::luciferase* plasmid or from primary fibroblasts dissociated from $mPer2^{Luciferase\text{-}SV40}$ knockin mice (Welsh *et al.*, 2004).

The xCRY2bS539 mutants could be acutely transfected and tested in the tissue culture system. In contrast to transgenic tadpoles, tissue culture cells carrying the mutant transgene are simple to maintain and easily transfectable. In addition, any effect of the mutant transgene on rhythmicity can be monitored via the Luciferase transgene. This system displays better resolution, allows the possibility for real-time imaging and can be monitored in culture for a much longer duration than the melatonin culture system (Welsh *et al.*, 2004).

Introduction of a mutant form of xCRY2b where S539 has been converted to either alanine (S539A) or aspartate (S539D) could affect the period of the clock via several mechanisms. The overexpressed S539D mutant could bind large amounts of 14-3-3 protein, preventing 14-3-3 from regulating the wild type xCRY2b. Nevertheless, 14-3-3 belongs to a very large family of proteins, many of which might have redundant functions, and it might therefore be a difficult system to overwhelm. Alternatively, expression of the S539A mutant could lead to more severe results. Hypothetically, this mutant has lost the ability to bind to 14-3-3; therefore, if overexpressed, it would be constitutively nuclear. Constitutively nuclear xCRY2b expression might lead to a shortened period phenotype (such as in the *tau* mutant hamster) or to arrhythmicity (Figure 3).

The hypothesis that the S539A mutant could have a dominant negative effect also addresses the concern that either *Xenopus* retinas or tissue culture cells have endogenous CRY2. This limitation could also be handled in the following ways: by reducing the amount of endogenous CRY2 via RNA interference (RNAi), a technique which has been successfully used in tissue culture cells to study the effects of altered *Clock* gene expression on circadian output (Allen *et al.*, 2004), or by using immortalized mouse embryonic fibroblasts (MEFs) from either *mCry2*$^{-/-}$ mice or from *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mice, which could also be acutely transfected with Luciferase reporter genes as described above (Yagita *et al.*, 2001; Welsh *et al.*, 2004).

The possible binding of 14-3-3 to CRY2 describes how the duration of the circadian period can modulated. One of the questions underlying circadian biology is how the organism, starting with the cell, achieves robust and regular oscillations. The

answer is likely not to depend on one protein, but the mechanism I described elucidates one of these steps providing both robustness and regularity to the molecular loop.

Crosstalk between the clock and other signaling pathways: the road ahead

As previously mentioned, phosphorylation is a key step in the regulation of the molecular loop. Both BMAL1 and the CRYs are phosphorylated by CKIε and MAPK (Sanada *et al.*, 2001; Eide *et al.*, 2002; Sanada *et al.*, 2004), and the evidence for kinases regulating the circadian molecular loop is increasing (Tischkau *et al.*, 2004). The MAP kinase signal transduction network comprises an interactive series of protein kinases, which receive, modulate and amplify intra- and extra-cellular signals. The cellular pathways regulated by MAPK include proliferation, development, differentiation and apoptosis. All of the kinases belonging to different MAPK subgroups act by translocating to the nucleus and by activating target transcription factors: this occurs after the kinases themselves have been activated by diverse receptors (reviewed in Dong and Bode, 2003). Many of the signals triggering MAPK cascades involve environmental stresses such as carcinogenic factors and UV irradiation (Cowley *et al.*, 1994; Minden *et al.*, 1994; Kallunki *et al.*, 1994). As mentioned in the introduction, it is possible that the biological clock evolved as a protection mechanism from environmental stresses, providing an ancient link between these pathways (Pittendrigh, 1993; Gehring and Roshbash, 2003). Given the pervasiveness of circadian regulation in biological systems, crosstalk between the circadian and other cellular pathways such as the MAP kinase cascades is likely to be extensive. Animal models are demonstrating the importance of the clock on physiological homeostasis (Rudic *et al.*, 2004); in addition, perturbations of

the circadian clock result not only in disrupted sleep/wake cycles, but also in diseases such as cancer (reviewed in Lowrey and Takahashi, 2004).

Figure 5-1: BMAL1 contains a putative PEST sequence, which is conserved between *Xenopus*, mouse and zebrafish

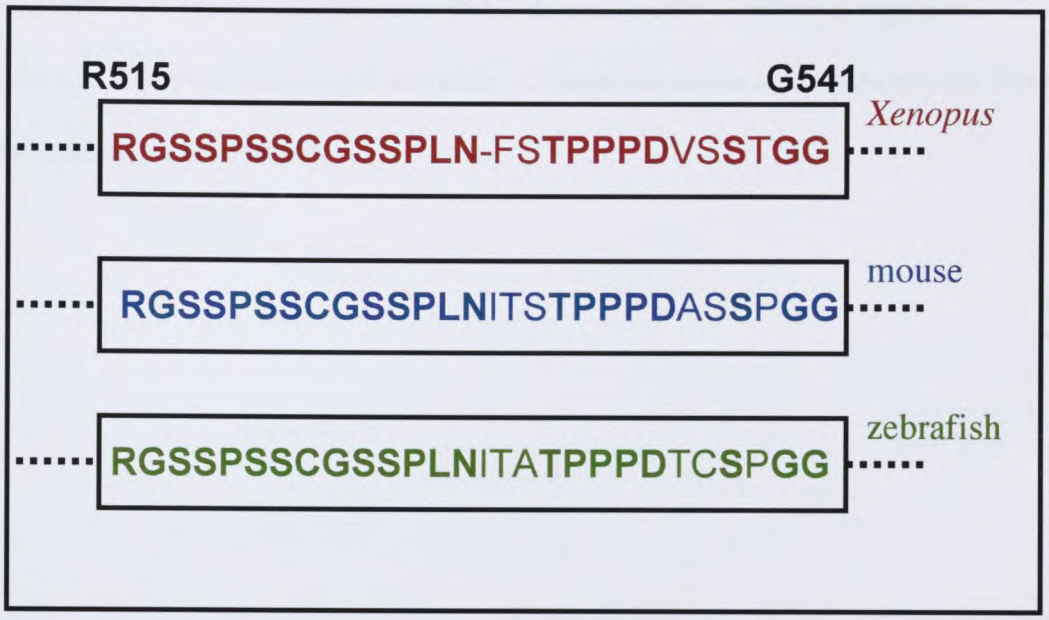


Figure 5-1: BMAL1 contains a putative PEST sequence, which is conserved between *Xenopus*, mouse and zebrafish

Comparison between the putative PEST domain in the C-terminal region of BMAL1 in *Xenopus*, mouse and zebrafish. Conserved amino acids between the three organisms are in bold letters.

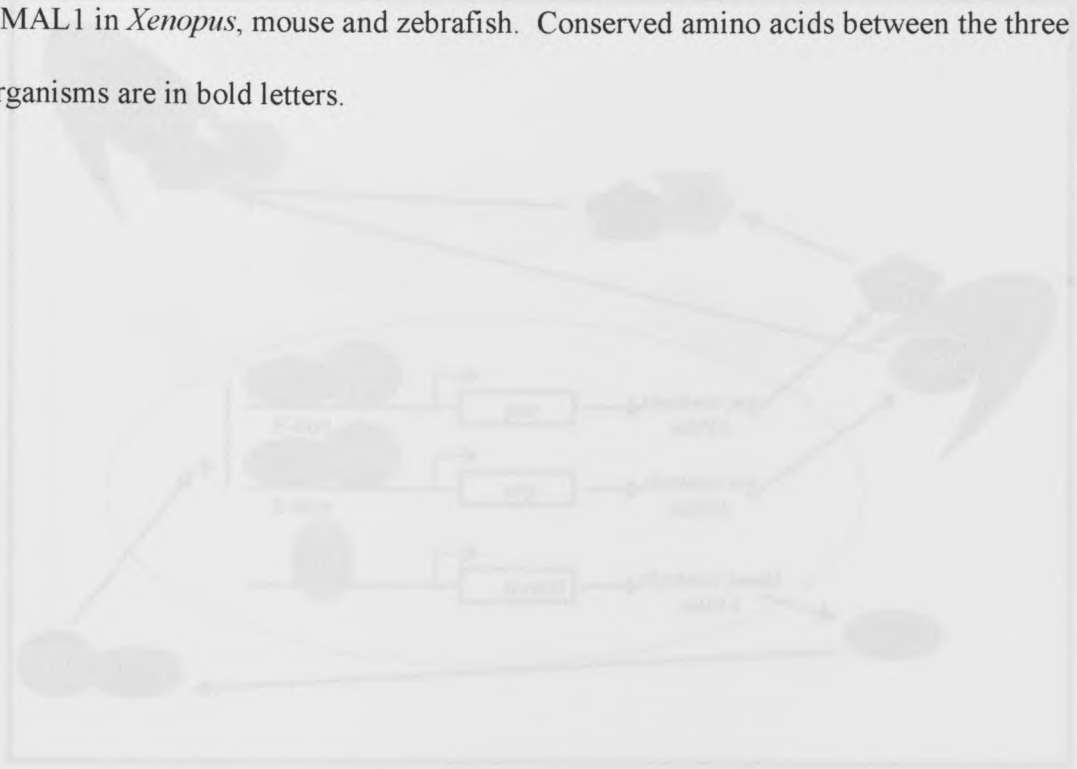
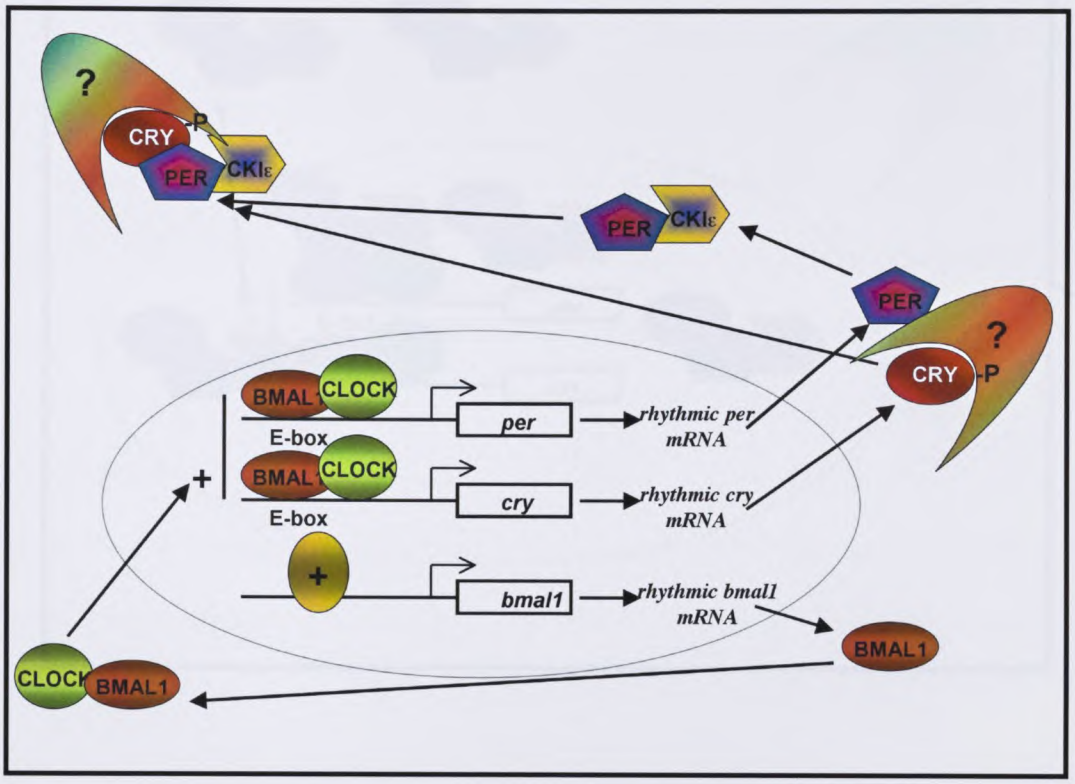


Figure 5-2: Possible regulation of the repressive complex by 14-3-3

A



B

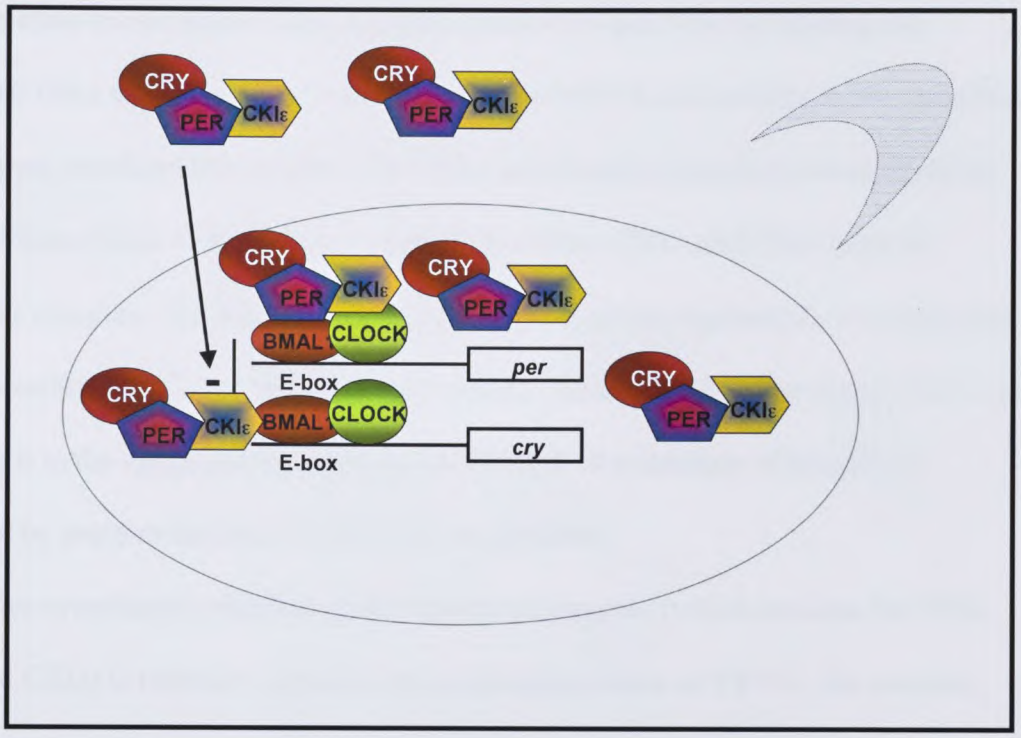

CRY
PER
CKIε
CRY
PER
CKIε
CRY
PER
CKIε
CRY
PER
CKIε
BMAL
CLOCK
E-box
per
CRY
PER
CKIε
CRY
PER
CKIε
BMAL
CLOCK
E-box
cry
CRY
PER
CKIε

Figure 5-2: Possible regulation of the repressive complex by 14-3-3

A: The nuclear CLOCK/BMAL1 heterodimer (the nucleus is defined by the gray oval) activates transcription of the circadian genes *Pers* and *Crys* by binding to a conserved DNA sequence, the E-box. *Per* and *Cry* mRNAs accumulate in the cytoplasm, and are then translated into protein. The PERs are phosphorylated and destabilized by CKIε. When critical PER levels are reached, the PERs, CRYs and CKIε form the repressive complex. Nuclear entry of this complex might be regulated by a cytoplasmic factor, possibly 14-3-3 (red and green half-moon), binding to phosphorylated CRY2 and retaining it in the cytoplasm by masking CRY2 NLS. Rhythmicity of BMAL1 is regulated by positive factors, which bind to its promoter.

B: Once cytoplasmic retention of the repressive complex (which includes the PERs, CRYs and CKIε) is relieved –probably by de-phosphorylation of CRY2-, the complex enters the nucleus and represses CLOCK and BMAL1. Possibly, nuclear entry of the complex is mediated by the importin pathway. Unbound 14-3-3 is represented by the gray half-moon.

Figure 5-3: Hypothetical effect of overexpression of the S539A xCRY2b mutant on the circadian molecular loop

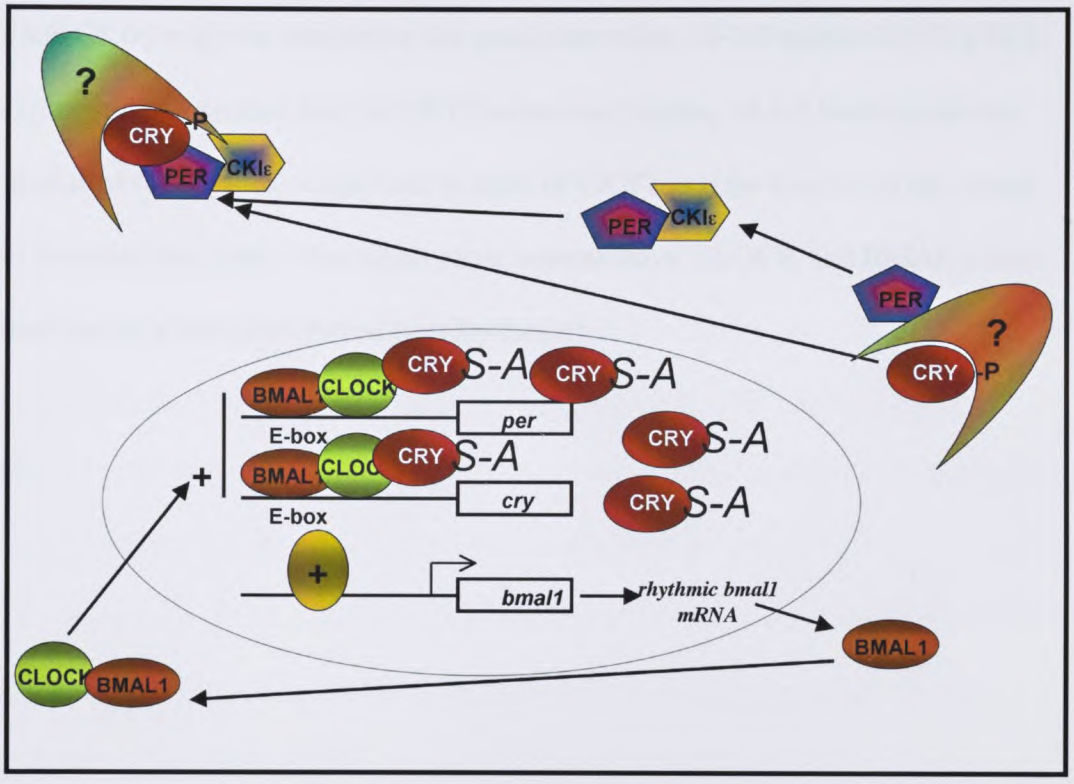


Figure 5-3: Hypothetical effect of overexpression of the S539A xCRY2b mutant on the circadian molecular loop.

As described in Figure 2, the repressive complex (which includes the PERs, CRYs and CKIε) might be retained in the cytoplasm when 14-3-3 masks CRY2's NLS. Overexpression of a mutant form of CRY2 where the binding 14-3-3 binding site has been abolished (CRY2S-A) might lead to entry of CRY2 into the nucleus at the wrong time in the circadian cycle. This might cause repression of CLOCK and BMAL1, and therefore lead to a shortened period or arrhythmicity.

References

Abel, D., Seale, D.B., Boraas, M.E. (1992) Periodicities and transient shifts in anuran (*Xenopus laevis*, *Rana clamitans*) oxygen consumption revealed with flow through respirometry. *Comp. Biochem. Physiol. Comp. Physiol.*, **101**: 423-432.

Aitken, A. (2002) Functional specificity in 14-3-3 isoforms interactions through dimer formation and phosphorylation: chromosome location of mammalian isoforms and variants. *Plant Molec. Biol.*, **50**: 993-1010.

Allen, G.C., Farnell, Y., Bell-Pedersen, D., Cassone, V.M., Earnest, D.J. (2004). Effects of altered Clock gene expression on the pacemaker properties of SCN2.2 cells and oscillatory properties of NIH/3T3 cells. *Neuroscience*, **127**: 989-999.

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

Berg, P., Pongratz, I. (2001). Differential usage of nuclear export sequences regulates intracellular localization of the dioxin (Aryl hydrocarbon) receptor. *J. Biol. Chem.*, **276**: 43231-43238.

Besharse, J.C., Terrill, R.O., and Dunis, D.A. (1980) Light evoked disc shedding by rod photoreceptors in vitro: Relationship to medium bicarbonate concentration. *Invest. Ophthalmol. Vis. Sci.*, **19**:1512-1517.

Besharse, J.C., and Iuvone, P.M. (1983) Circadian clock in *Xenopus* eye controlling retinal serotonin N-acetyltransferase. *Nature*, **305**:133-135.

Cahill, G.M., and Besharse, J.C. (1990). Circadian regulation of melatonin in the retina of *Xenopus laevis*: Limitation by serotonin availability. *J. Neurochem.*, **54**:716-719.

Cahill, G.M., and Besharse, J.C. (1991) Resetting the circadian clock in cultured *Xenopus* eyecups: Regulation of retinal melatonin rhythms by light and D2 dopamine receptors. *J. Neurosci.*, **11**:2959-2971.

Cermakian, N., Whitmore, D., Foulkes, N.S., Sassone-Corsi, P. (2000) Asynchronous oscillations of two zebrafish CLOCK partners reveal differential clock control and function, *Proc. Natl. Acad. Sci.*, **97**: 4339-4344

Cowley, S., Paterson, H., Kemp, P., Marshall, C.J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. *Cell*, **77**: 841-852.

Dong, Z., Bode, A. (2003) Dialogue between ERKs and JNKs: friendly or antagonistic? *Molec. Interventions*, **3**: 306-308.

Eide, J.E., Vielhaber, E.L., Hinz, W.A., Virshup, D.M. (2002). The circadian regulatory protein BMAL1 and Cryptochromes are substrates of Casein kinase Iε. *J. Biol. Chem.*, **19**: 17248-17254.

Fornerod, M., Ohno, M., Yoshida, M., Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**: 1051-1060.

Ganguly, S., Gastel, J.A., Weller, J.L., Schwartz, C., Jaffe, H., Namboodiri, M.A., Coon, S.L., Hickman, A.B., Rollag, M., Obsil, T., et al (2001) Role of a pineal cAMP operated arylalkylamine N-acxetyltransferase/14-3-3 binding switch in melatonin synthesis. *Proc. Natl. Acad. Sci., U.S.A.* , **98**: 8083-8088.

Gehring, W., and Rosbash M. (2003) The coevolution of blue-light photoreception and circadian rhythms. *J. Mol. Evol.*, **57**: S286-289.

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

Green, C.B., Liang, M.-Y., Steenhard, B.M., and Besharse, J.C. (1999) Ontogeny of circadian and light regulation of melatonin release in *Xenopus laevis* embryos. *Brain Res. Dev. Brain Res.*, **117**:109-116.

Görlich, D., Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annual Review in Cell and Developmental Biology*, **15**: 607-660.

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

Hayasaka, N., LaRue, S.I., Green, C.B. (2002) *In vivo* disruption of *Xenopus* CLOCK in the retinal photoreceptor cells abolishes circadian melatonin rhythmicity without affecting its production levels. *J. Neurosci.*, **22**: 1600-1607.

Hankinson, O. (1995) The aryl hydrocarbon receptor complex. *Adv. Enzyme Regul.*, **34**: 159-171.

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

Hord, N.G., Perdew, G.H. (1994). Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: Characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator. *Mol. Pharmacol.*, **46**: 618-626.

Igarashi, D., Ishida, S., Fukazawa, J., Takahashi, Y. (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell*, **13**: 2483-2497.

Ikuta, T., Eguch, H., Tachibana, T., Yoneda, Y., Kawajiri, K. (1998) Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem.*, **273**: 2895-2904.

Kallunki, T., Su, B., Tsigelny, I., Sluss, H.K., Derijard, B., Moore, G., Davis, R., Karin, M. (1994). JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.*, **8**: 2996-3007.

Keesler, G.A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C., Yao, Z. (2000) Phosphorylation and destabilization of human period 1 clock protein by human casein kinase I epsilon. *Neuroreport*, **11**: 951-955.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W., Takahashi, J.S. (1997). Positional cloning of the mouse circadian *Clock* gene. *Cell*, **89**: 641-653.

Klein, D.C., Ganguly, S., Coon, S., Weller, J.L., Obsil, T., Hickman, A., Dyda, F. (2002) 14-3-3 proteins and photoneuroendocrine transduction: role in controlling the daily rhythm in melatonin. *Biochem. Soc. Trans.*, **30**: 365-373.

Klein, D.C., Ganguly, S., Coon, S.L., Shi, Q., Gaildrat, P., Morin, F., Weller, J.L., Obsil, T., Hickman, A., Dyda, F. (2003) 14-3-3 proteins in pineal photoneuroendocrine transduction: how many roles? *J. Neuroendocrinology*, **15**: 370-377.

Kroll, K.L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*, **122**:3173-3183.

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

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

Lowrey, P.L., Takahashi, J.S. (2004) Mammalian circadian biology: elucidating genome wide levels of temporal organization. *Annu. Rev. Genomics Hum. Genet.*, **5**: 407 -441.

Mackintosh, C. (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem. J.*, **381**: 329-342.

Mattaj, I.W., Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase. *Annual Review in Biochemistry*, **67**: 265-306.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R.J., Johnson, G.L., Karin, M. (1994). Differential activation of ERK and JNK mitogen activated protein kinases by Raf-1 and MEKK. *Science*, **266**: 1719-1723.

Moore, B., Vj, P. (1967) *Specific acidic proteins of the nervous system*. In: Fd C editor, Englewood Cliffs: Prentice Hall; p.343-359.

Oishi, K., Sakamoto, K., Okada, T., Nagase, T., Ishida, N. (1998) Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem. Biophys. Res. Commun.*, **253**: 199-203.

Oishi, K., Fukui, H., Ishida, N. (2000) Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and pheripheral tissues. *Biochem. Biophys. Res. Commun.*, **268**: 164-171.

Okano, T., Sasaki, M., Fukada, Y. (2001) Cloning of mouse BMAL2 and its daily expression profile in the suprachiasmatic nucleus: a remarkable acceleration of Bmal2 sequence divergence after Bmal gene duplication. *Neurosci. Lett.*, **300**: 111-114.

Perdew, G.H. (1988) Association of the Ah receptor with the 90-kDa heat-shock protein. *J. Biol. Chem.*, **263**: 13802-13805.

Pittendrigh, C.S. (1993). Temporal organization: reflections of a Darwinian clock watcher. *Annu. Rev. Physiol.*, **55**: 16-54.

Ponzuelo Rubio, M., Geraghty, K.M, Wong, B.H.C., Wood, N.T., Campbell, D.G., Morrice, N., Mackintosh, C. (2004) 14-3-3 affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. *Biochem. J.*, **379**: 395-408.

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

Ralph, M.R., Menaker, M. (1988) A mutation of the circadian system in golden hamster. *Science*, **241**: 1225-1227.

Reyes, H., Reisz-Porszasz, S., Hankinson, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor . *Science*, **256**: 1193-1195.

Rogers, S., Wells, R., Rechsteiner, M. (1986). Amino acid sequences common to rapid degraded proteins: the PEST hypothesis. *Science*, **234**: 364-368.

Rudic, R.D., McNamara, P., Curtis, A.M., Boston, R.C., Panda, S., Hogenesh, J., Fitzgerald, G.A. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PloS. Biol.*, **2**: 377.

Sanada, K., Okano, T., Fukada, Y. (2001). Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *J. Biol. Chem.*, **277**: 267-271.

Sanada, K., Harada, Y., Sakai, M., Todo, T., Fukada, Y. (2004). Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase. *Genes to Cells*, **9**: 697-708.

Seimya, H., Sawada, H., Muramatsu, Y., Shimizu, M., Chko, K., Yamane, K., Tsuruo, T. (2000) Involvement of 14-3-3 proteins in nuclear localization of telomerase. *The EMBO Journal*, **19**: 2652-2661.

Sekimoto, T., Fukumoto, M, Yoneda, Y. (2004) 14-3-3 suppresses the nuclear localization of threonine phosphorylated $p27^{Kip1}$. *The EMBO Journal*, **23**: 1934 1942.

Steenhard, B.M., Besharse, J.C. (2000) Phase shifting and retinal circadian clock: xPer2 mRNA induction by light and dopamine. *J. Neurosci.*, **20**: 8572-8577.

Tamaru, T., Isojima, Y., van der Horst, G.T.J., Takei, K., Nagai, K., Takamatsu, K. (2003). Nucleocytoplasmic shuttling and phosphorylation of BMAL1 are regulated by circadian clock in cultured fibroblasts. *Genes to Cells*, **8**: 973-983.

Tischkau, S.A., Mitchell, J.W., Pace, L.A., Barnes, J.W., Gillette, M.U. (2004). Protein kinase G type II is required for night-to-day progression of the mammalian circadian clock. *Neuron*, **43**: 539-549.

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

van der Spek, P.J. Kobayashi, K., Bootsma, D., Takao, M., Eker, A.P., Yasui, A. (1996) Cloning, tissue expression and mapping of a human photolyase homolog with similarity to plant blue-light photoreceptors. *Genomics*, **37**: 177-182.

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

Vitaterna M.H., Selby C.P., Todo T., Niwa H., Thompson C., Fruechte E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., *et al.* (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci. USA*, 9**6:**12114-12119.

Watson, J.T., Kelley, D.B. (1992) Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate and frequency spectra. *J. Comp. Physiol.*, **171**: 343-350.

Welsh, D.K., Yoo, S.-H., Liu, A.C., Takahashi, J.S., Kay, S.A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.*, **14**: 2289-2295.

Wilker, E., Yaffe, M. (2004) 14-3-3 proteins- a focus on cancer and human disease. *Journal of Molecular and Cellular Cardiology*, **37**: 633-642.

Yagita, K., Tamanini, F., van der Horst, G.T.J., Okamura, H. (2001). Molecular mechanisms of the biological clock in cultured fibroblasts. *Science*, **292**: 278-281.

Yoo, S.-H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.-K., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **101**: 5339-5346.

Zhu, H., LaRue, S., Whiteley, A., Steeved, T.D., Takahashi, J.S., Green, C.B. (2000) The *Xenopus* Clock gene is constitutively expressed in retinal photoreceptors. *Brain Res. Mol. Brain Res.*, **75**: 303-308.

Zhu, H., and Green, C.B. (2001a) Three cryptochromes are rhythmically expressed in *Xenopus laevis* photoreceptors. *Mol. Vis.*, 7: 210-215.

Zhu, H., Green, C.B. (2001b) A putative flavin electron transport pathway is differentially utilized in *Xenopus* CRY1 and CRY2. *Curr. Biol.*, **11**: 1945-1949.