Structural and Biochemical Analysis of Interaction of mCRY2 with mBMAL1

by

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ABSTRACT

Circadian rhythms are intrinsic periodic oscillations of physiological, biochemical and behavioral processes within approximately 24h periodicity in diverse species ranging from bacteria to mammals. CRYPTOCHROME proteins are one of the essential components of the mammalian circadian clock and null mutations of *Cry* leads to severe disruption of normal circadian clock function. Although forward and reverse genetic approaches have successfully unveiled the core and auxiliary circadian clock components in detail, the molecular mechanisms of the transcriptional repression in the circadian clock feedback loop remains elusive.

In this thesis work, evidence will be provided through a reverse genetics approach that interaction of mCRY2 with mPER2 is mediated through evolutionary conserved amino acids on the C-terminus of mCRY2 (R501 and K503) and that interaction is essential for transcriptional repression of both endogenous and exogenous BMAL1:CLOCK heterodimer transcriptional activity. Moreover, it will be shown that mPER2 interaction is not a prerequisite for mCRY2 to bind to other circadian clock components, as ectopically expressed loss-of-function mutant immunoprecipitates with ectopically expressed mBMAL1. Furthermore, the functionality of the loss-of-function protein will be explored in terms of interaction with other core circadian clock proteins and nuclear localization pattern. It will be shown that loss-of-function is solely based on elimination of the interaction and not due to aberrant translocation of the mutant protein, as ectopically expressed tagged proteins can be detected immunocytochemically and also GFP-fusion constructs directly translocate into nucleus. In addition, the mBMAL1 interaction hot spots on mCRY2 are mapped to F171 on the N-terminus and other unresolved residues through another reverse genetics approach.

ÖZET

Sirkadiyen ritimler bakterilerden memelilere kadar geniş bir kapsamdaki canlıların biyokimyasal, fizyolojik ve davranışsal düzenlemesini yaklaşık 24 saatlik periyotta düzenleyen döngülerdir. Kriptokromlar moleküler düzeyde sirkadiyen ritmi oluşturan transkripsiyon geri bildirim döngüsünde büyük öneme sahiptir. Kriptokrom alelleri hasara uğratılmış farelerin sirkadiyen ritimlerinin tamamen yok olması, kriptokromların sirkadiyen ritmlerin oluşması ve korunması için temel unsurlardan biri olduğunu ortaya koymuştur. Şu ana kadar ileri ve geri genetik teknikler kullanılarak sirkadiyen ritim'i değişik seviyelerde düzenleyen proteinler kısmi oranda tespit edilmiştir. Ancak transkripsiyonel mekanizmanın nasıl çalıştığı, BMAL1:CLOCK protein kompleksinin transkripsiyonel aktivitesinin nasıl durdurulduğu, Kriptokrom'un bu aktiviteyi nasıl durdurduğu gibi konular hala belirsizliğini korumaktadır.

Bu çalışmada CRY2 proteininin PER2 ile etkileştiği bölgeler geri genetik yöntemler vasıtasıyla evrimsel olarak korunmuş R501 ve K503 bölgeleri olarak tespit edilmiştir. Bu bölgelerin mutasyona uğratılması PER2 ile etkileşimi ortadan kaldırıp CRY2'nin transkripsiyonel baskılama özelliğinin de yüksek oranda kaybolmasına yol açmıştır. Ayrıca bu bölgelerin mutasyona uğratılması, CRY2'nin BMAL1'e bağlanmasını engellememekte ve hücrenin çekirdeğine lokalize olmasını da etkilememektedir. Ayrıca CRY2'nin BMAL1'e bağlandığı bölge de yine geri genetik yöntemler vasıtasıyla tespit edilmiş olup proteinin N-ucundaki F171 bölgesine ve diğer bazı bölgelere atfedilmiştir.

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NOMENCLATURE

bHLH	basic helip-loop-helix
BMAL1	Brain-muscle-Arndt-like-protein-1
CCG	clock-controlled gene
CK1ε	Casein kinase 1 epsilon
CLOCK	Circadian-locomoter-output-cycles-prote
CRY	Cryptochrome
CYC	Cycle
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FRQ	Frequency
KCl	potassium chloride
LB	Luria-Bertani
LD	light:dark
mM	milimolar
NaCl	sodium chloride
NP-40	Nonidet P-40
NPAS2	Neuronal PAS domain protein 2
PAS	Per-Arnt-Sim
PBS	phosphate buffered saline
PER	Period
PHR	photolyase homology region
REV-ERBα	V-erbα-related protein
RORα	Retinoic acid receptor alpha
rpm	revolutions per minute
SCN	suprachiasmatic nucleus
TBS	Tris-buffered saline
TIM	Timeless
Tris	Tris(hydroxymethyl)aminomethane
WCC	White-Collar complex

Chapter 1

INTRODUCTION

Circadian rhythms are intrinsic periodic oscillations of physiological, biochemical and behavioral processes within approximately 24h periodicity in diverse species ranging from bacteria to mammals. Circadian rhythms are mainly dictated by environmental light-dark cycles which serve as an input for the generation and preservation of the oscillations. The circadian rhythm is ubiquitous in mammals and hierarchically organized into master and peripheral clocks. A master circadian clock is dictated by a small cluster of neurons, called suprachiasmatic nucleus of the hypothalamus. Light stimulus is perceived by the cells lining the inner retina and transmitted to the suprachiasmatic nucleus via neural projections of the retina-hypothalamic tract. Synaptic input to the suprachiasmatic nucleus induces downstream molecular events leading to synchronization of the master circadian pacemaker. Peripheral clocks exist in both neural and non-neural tissues and are synchronized to the master circadian clock by humoral and neural cues.

Forward and reverse genetic approaches have successfully unveiled the molecular determinants of the circadian clock in mammals. There are four proteins that comprise the core clock: CRY, PER, BMAL and CLOCK.[1-3] These proteins have been shown to form a network of transcriptional/translational feedback loops.[4] At the molecular level it is widely accepted that circadian rhythms are generated by an autoregulatory feedback loop that is composed of both positive and negative transcriptional elements. CLOCK and its heterodimeric binding partner BMAL1 comprise the positive arm of the feedback loop by forming an heterodimer through PAS (Per-Arnt-Sim) and bHLH domains and subsequently binding to the regulatory sequences on DNA, called E-box, to activate transcription of target genes including Period(Per) and Cryptochrome(Cry) which comprise the negative arm of the feedback

loop. Increase in the concentration and the activity of the negative elements result in robust repression of the CLOCK:BMAL1 heterodimer mediated transcription . This repression is progressively cleared through degradation of the negative components by ubiquitin-proteasome pathway, thereby creating an oscillation of approximately 24h. [4]

Previous research reports indicate that cryptochromes are essential for generating the circadian rhythms. Although each of the mammalian paralogs of the cryptochrome, Cry1 and Cry2, are redundant, in that targeted disruption of each one of the alleles individually results in a shift in the circadian clock, a double mutant bearing disruption of both alleles is arrhythmic. [5, 6]Complementary to these reverse genetic studies, studies encompassing the use of mPer1 or AVP-luciferase transfected fibroblasts showed that both Cry1 and Cry2 results in a potent repression of BMAL1:CLOCK transcriptional activity. [7]Altogether these data point out the eminent role of cryptochromes in circadian clock function.

Several studies have scrutinized the functional motifs and structural domains of cryptochromes with unique functions that account for the observed functionalities of cryptochrome. Domains dictating the binding to other core circadian clock components and repression of the transcriptional activity of the BMAL1:CLOCK heterodimer have been mapped to separate regions. The region that show high homology to the bacterial photolyase on Cry2 has been shown to sufficiently repress the BMAL1:CLOCK transcriptional activity. Unlike the other three proteins of the core circadian clock which interact through their PAS or bHLH domains, Cryptochromes do not bear any PAS or bHLH domains. It has been shown that C-terminus of Cry2 dictates heterodimerization with Per2. However the domain on CRY2 that mediates binding to BMAL1 has not been characterized. In this thesis work, the molecular nature of the interactions of mCRY2 with mPER2 and mBMAL1 will be elaborated. The region of mCRY2 that dictates physical interaction with mPER2 is assessed in the molecular scale and the functional significance of this interaction is examined through transcriptional and localization assays. Moreover it will be shown that the C-terminus of mCRY2 is not required for binding to mBMAL1 and this physical interaction is mediated by

evolutionarily conserved amino acids (Phe-171 and other unresolved residues) on the mCRY2 by making use of mammalian two hybrid system complemented with a random mutagenesis screen in the scope of this thesis work.

In Chapter 2, detailed background of the circadian rhythms, molecular architecture of circadian clocks in different organisms and the functional significance of each core circadian clock components are presented. Moreover contemporary research on the interactions of core circadian clock proteins will be discussed.

In Chapter 3, detailed explanation of the materials and methods used in generation of the random mutant allele library, screening of the mutant alleles for loss of function by mammalian two hybrid technique, biochemical validation of the interactions through co-immunoprecipitation, assessment of subcellular localization by GFP-fusion constructs and immunocytochemistry, homology modeling of mCRY2 will be presented.

In Chapter 4, the results obtained through the experimental work are included.

In Chapter 5, discussion of the results presented in this thesis work is included.

Chapter 2

LITERATURE REVIEW

This chapter includes the summary of the detailed review of the literature about the general features of circadian clock, mammalian circadian organization and the genetic control of the circadian clock machinery.

2.1. Overview of Circadian Rhythms

Environment persists as an abiotic selective pressure on organisms with both temporal and spatial cues. The Darwinian (survival of the fittest) theory dictates that organisms which exhibit a higher level of adaptation to exploit the environmental conditions, dominates the genetic population by means of survival. Temporal variations in the environment occur due to daily and seasonal variations of the position of earth with respect to the sun. Therefore periodic variations in the sunlight dictate a necessity of a periodicity for environmental adaptation. [8] Thus, organisms have evolved rhythmic mechanisms to respond and adapt to this rhythmic abiotic pressure exerted by the sunlight. These daily rhythmic adaptations are called circadian rhythms, derived from latin *circa* (about) and *dies* (day).

Circadian rhythms are intrinsic periodic oscillations of physiological, biochemical and behavioral processes within approximately 24h periodicity. The discovery of the circadian rhythms dates back to 18th century with Jean Jacques Ortous de Marian's observations of mimosa, a widely known heliotrope plant. He found that when the mimosa is incubated complete darkness, the rhythmic opening and closing of the leaves continues even if there is no external light dark cycle. He concluded that mimosa plants under external temporal isolation exhibit normal rhythmic behavior in the absence of external sunlight.[9] This observation led to the basics of current

understanding of the circadian rhythms. These endogenous rhythms can be observed in the isolation from the external temporal cues and endogenous period is approximately 24h. The deviation of the endogenous rhythm from the external temporal rhythm of 24h is due to the absence of zeitgeber (time giver) under the temporal isolation. The ability to respond to environmental light-dark cycles is the common feature of all circadian systems.[8] These endogenous rhythms are discovered in very diverse species like unicellular organisms[10-12], cyanobacteria[13], fungi[14], fruit fly[15], and rodents[16, 17].

Circadian rhythms are mainly dictated by environmental light-dark cycles which serve as an input for the generation and preservation of the oscillations. Abundance of light at the dawn and the scarcity of the light at the dusk create a phase for adjusting the clock in which there is a differential response to the changing light intensity. This differential response is called a phase response curve.[18] Phase response curve is independent of taxa or phototransduction pathways.[19]

One other important hallmark of the circadian rhythm is temperature compensation. It is a basic rule in biochemistry that the rates of biochemical reactions are drastically affected by the varying temperature. However, interestingly, circadian machinery is unaffected by varying temperature. [1] This feature common to all circadian systems provide a basis for stability in organismal adaptation.

Even though this feature implies independence of circadian systems from external factors, circadian clock is modulated by environmental factors such as change in light intensity, nutrient availability and social pressure to be able to provide adaptive advantage. [20] This is also a common feature of all circadian systems and generally called “resetting of the circadian clock”. For a circadian clock machinery to be useful in environmental adaptation, it should be entrained or reset by the periodic stimuli from various environmental factors. The environmental factors are collectively called zeitgeber (time giver) and they modulate the internal clock by convergent mechanisms.

Circadian clock in multicellular organisms is a cell-autonomous feature, such that every single cell has a different circadian oscillator, which is not necessarily

connected to a master circadian oscillator network. In vertebrates the circadian clock is hierarchically organized such that there are peripheral clocks under the control of a master circadian clock, which is entrained by the environmental stimuli (zeitgeber) and entrains the other peripheral clocks.[19] The organization of the circadian systems can be explained in three interconnected parts in which the input component perceives the zeitgebers and entrain the central oscillator which then generates the rhythmic output as a response to the stimuli.

2.2. Molecular architecture of the circadian clocks

Circadian clocks are well conserved among the six kingdoms, due to a selective pressure of the rhythmic changes in environmental stimuli. Therefore it should be expected that not all the molecular elements, but the molecular architecture, itself, should be well conserved among species. The basis of the molecular architecture is a transcriptional-translational negative feedback loop, which oscillates with the periodic changes in the zeitgebers. This oscillation arises from an autoregulatory negative feedback loop that includes at least one clock protein that has transcriptional activation or repression capability. This protein either directs or inhibits its own transcription directly or indirectly via other accessory proteins. In higher eukaryotes, the molecular architecture of the circadian clock is binary.

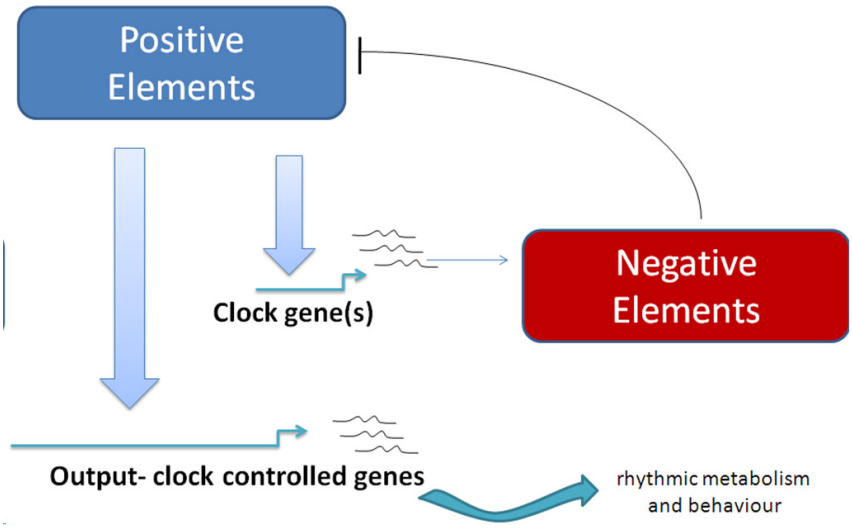


Figure 2.1- Basic molecular architecture of the circadian clock, adapted from [14]

At the molecular level, circadian clock in higher eukaryotes is composed of both positive and negative elements. Positive elements are transcriptional activators that induce transcription of negative elements and various output genes. Negative elements inhibit the transcriptional activity of the positive elements (negative feedback), thereby creating a molecular oscillator with rhythmic period. [21]

Originally, this negative feedback loop was comprehended as a chemical equilibrium, in which the peak concentrations of the negative transcriptional elements induce transcriptional repression. However experimental evidence indicates that not the gradual increase in the concentration of the negative transcriptional elements at both protein and RNA levels, but rather rate limiting steps such as post-translational modifications on the negative elements determine the precise timing of the circadian clock. [22]The roles of the kinases CKIε, CKIδ and GSK3β have been shown to regulate both the positive and the negative transcriptional elements by means of protein stability, subcellular localization and interaction.[23-26] Interestingly the core clock protein CRY impairs kinase activity of these regulators, which in turn leads to reduction of phosphorylated BMAL1 and BMAL1:CLOCK transcriptional activity.[27]

2.3 Circadian clocks in prokaryotes, simple eukaryotes and invertebrates

Since the observation of the circadian system in mimosa plant [2], circadian clocks have been characterized and assessed in further detail in simple unicellular organisms such as *Synecoccus elongatus*, fungus *Neurospora crassa*, plant model organism *Arabidopsis thaliana* and the fruit fly *Drosophila melanogaster* as well as in mammals. The negative feedback loop is well conserved in generation and maintenance of circadian rhythms among these organisms. Apart from cyanobacteria, the rhythm is an interplay between the negative and the positive transcriptional elements. In the following section, a brief summary of the circadian clocks in each organism will be presented.

2.3.1 Cyanobacterial clock

Before the discovery of the circadian clock in prokaryotes, it was thought that the circadian clock machinery was limited to the eukaryotic organisms and arose at the later stages of evolution. Strikingly, elegant observations of the photosynthetic freshwater cyanobacteria led to identification of the circadian clock machinery in prokaryotes for the first time.[28] As ancient cyanobacteria evolved approximately 3.5 billion year ago, this finding suggests that the circadian regulation is more common and conserved throughout evolution.[29] The core circadian oscillator in cyanobacteria consists of three proteins namely KaiA, KaiB and KaiC. [30] The cyanobacterial clock is unique, such that it does not involve transcriptional feedback like other organisms. A similar model for the cyanobacteria was also put forward as the KaiA induces transcription from the *kaiBC* operon and the KaiC represses its own transcription.[31] However, it was later shown that inhibitors of neither transcription nor translation abolished circadian rhythmicity of the cyanobacteria.[32] The same research group showed that the phosphorylation was the core of the cyanobacterial clock. It was shown previously that the KaiC protein had both autophosphorylation and autodephosphorylation activity. They showed that the KaiA increases the autophosphorylation activity of KaiC and KaiB impedes the effect of KaiA and interplay of these proteins in KaiC phoshporylation creates an oscillation. Strikingly, they also showed that these proteins can sustain rhythmic oscillations of KaiC phosphorylation in vitro.[33] These findings led to proposal of a new model for the circadian clock in cyanobacteria.[34] According to this model, KaiA binds to KaiC and prompts KaiC phosphorylation. KaiB binds to the phosphorylated KaiC and dephosphorylates KaiC. After dephosphorylation KaiB dissociates from the complex and the phosphorylation/dephosphorylation loop starts again, which creates a rhythmic oscillation to sustain the core circadian clock. The unorthodox cyanobacterial clock regulates many of the cellular processes such as photosynthesis, uptake of amino acids, cell cycle and carbohydrate synthesis.[29]

2.3.2. *Neurospora crassa* clock

The fungus *Neurospora* is the simplest organism to have the orthodox circadian clock machinery composed of a transcriptional-translational feedback loop. The positive limb of the circadian clock is a transcriptional activator complex called White Collar Complex (WCC), which is composed of two PAS domain containing proteins WC-1 and WC2. [35] WCC activates transcription of clock control genes that regulate metabolism and reproduction and also *frq* (frequency) gene, which comprises the negative limb of the circadian clock to repress the WCC transcriptional activity.[19] The originality of the *Neurospora* clock is that WCC complex harbors a blue-light photoreceptor, WC-1, which senses the light and activates downstream events.[36]

2.3.3 *Arabidopsis thaliana* circadian clock

Plants, as widely known photosynthetic organisms, depend on light more than other organisms. Therefore circadian rhythms are expected to have a more vital role for the plants, as light serves as an input for the circadian systems. As expected, circadian rhythms in plants control a wide variety of physiological and biochemical events, such as stomata opening, flowering time, localization of the chloroplasts, growth of the hypocotyls, germination and emission of odor.[37] The components of the circadian clock have been characterized in detail in the plant model organism *Arabidopsis thaliana* extensively. Circadian regulation of RNA and protein expression, controlled protein degradation, ion influx and protein phosphorylation have been characterized at the molecular level.[31] Forward genetics have identified a set of genes that regulate the circadian clock of *Arabidopsis* in a transcriptional- translational feedback loop. In the positive limb of the transcriptional feedback loop ELF4 and TOC1 play crucial roles. Neither of these two proteins contains a DNA binding domain, which implies the necessity of accessory proteins to facilitate activation of transcription. Recent findings indicate the role of a MYB domain containing protein complex called PCL1/LUX in the ELF4 and TOC1 dependent activation of transcription. Therefore TOC1, ELF4 and PCL1/LUX protein activate transcription of the proteins that constitute the negative

limb of the transcriptional feedback loop, namely CCA1 and LHY. [38] Interestingly post-translational mechanisms play a crucial role in the negative loop such that phosphorylation of CCA1 by CK2 initiates the negative limb of the transcriptional feedback loop. Moreover plant cryptochromes are unique that they do not participate in the negative limb of the transcriptional feedback loop, but together with ZTL and PHY they act as photoreceptors to transmit the input from the light to the core circadian clock components.[31]

2.3.4 *Drosophila melanogaster* circadian clock

Fruit fly has shown to be very useful in circadian clock research. Research with the goal of identifying the essential molecular components of the circadian clock has been initiated in *Drosophila* and resulted with the cloning of first circadian clock genes *period* and *timeless* from the mutants with perturbed circadian rhythms.[2, 39, 40] Research shows that the mammalian core circadian clock mechanisms are highly homologous to Drosophila in terms of physiology and molecular components except that there is an auxiliary loop in Drosophila that sustains the core transcriptional loop. However, as a nearly universal feature of the circadian clocks, Drosophila circadian clock is also a unified network of negative and positive transcriptional loops.

Drosophila is a peculiar organism for circadian clock research, since the physiological regulation is much more simple, but highly homologous to the mammalian counterpart. Circadian regulatory regions in the brain have been identified by immunohistochemistry, after cloning of *period* and *timeless* genes. Results indicated that clock regulatory neurons were localized in two separate anatomical regions in the brain as judged by extensive immunostaining. [41, 42] One group of neurons were located in the dorsal side of the brain, called dorsal neurons, and the other cluster of neurons were localized to the left side of the brain, called lateral neurons. A small subgroup of the lateral neurons localized to a more ventral position and called ventrolateral neurons, play a central role in Drosophila clock since they are the major

pacemaker in Drosophila.[43, 44] Moreover recent research indicates that other clock neurons also act as accessory pacemakers in Drosophila.[45]

The positive limb of transcriptional loop is constituted by two transcription factors called CLOCK and CYCLE, which contain a bHLH DNA binding motifs and PAS domains.[20] These proteins activate rhythmic transcription of target genes including PERIOD and TIMELESS via binding to an enhancer motif called E-box (CACGTG). PERIOD and TIMELESS participate in the negative limb of transcriptional feedback loop. PERIOD associates with TIMELESS through their PAS domains and PERIOD:TIMELESS protein complex repress CLOCK:CYCLE transcriptional activity through binding to CLOCK:CYCLE complex and impeding its binding to DNA rather than dissociating the CLOCK:CYCLE complex [46], thereby inhibit their own transcription thus completing the feedback loop. Post-translational mechanisms are also essential for the self-sustained ~24h circadian rhythm. Post-translational modifications fine-tune the Drosophila molecular circadian clock, DOUBLETIME (mammalian CKIε homolog) phosphorylates PERIOD rhytmically and modulates its subcellular localization and proteasomal degradation. [47, 48] Other components of the core circadian clock are also subject to post-translational modifications, such as TIM[49] and CLOCK[50].

Another fascinating feature of the Drosophila circadian clock is that it has additional secondary feedback loops that sustain the core transcriptional loop, which increases the complexity of the molecular architecture of the circadian clock. The first identified secondary loop revolves around the transcription of *clock.* [51]In the core loop, CLOCK:CYCLE induces rhythmic transcription of target genes including *vrille* and *pdp1*. PDP1 activates transcription by binding to an enhancer element in the *clk* promoter (also present in the *cry* promoter) whereas VRI competes with PDP1 in binding to the same promoter region to repress the transcription. [52] Yet there exists another secondary transcription feedback loop in Drosophila circadian clock. A recently identified bHLH containing transcription factor has repressive capacity and expression of this gene is controlled by the CLOCK:CYCLE protein complex at the mRNA level.

This protein can directly bind to the E-box enhancer elements and compete with CLOCK:CYCLE in binding to these regions, therefore results in potent repression of CLOCK:CYCLE transcriptional activity and in turn shuts down its own transcription.

One important distinction of the Drosophila circadian system is the role of CRY proteins in the circadian clock mechanism. As the mammalian cryptochromes play a central role in the core circadian clock mechanism, Drosophila cryptochrome is a deep brain photoreceptor (DNs) to facilitate the proteasomal degradation of TIM protein. [45] Moreover Drosophila *cry* is not essential for the maintenance of the physiological rhythms, yet it can repress the CLOCK:CYCLE transcriptional activity *in vitro* suggesting its role in the peripheral clocks. [53] This aspect renders Drosophila cryptochrome more similar to the mammalian cryptochromes in terms of function.

2.3.5 Mammalian circadian clock

Mammalian circadian clock has significant evolutionary resemblance compared to lower eukaryotic species. The difference does not lie in the molecular architecture of the circadian clock, yet the physiological organization of the clock and molecular control of the circadian clock is different with respect to up to date research.

Physiological control of the circadian system is regulated by the highly specialized cellular structures in terms of input component, pacemaker and the ouput components.

Mammalian circadian clock has an approximately ~24hr period (24.2 hours in humans and 23.5 hours in mice) and it is entrained mainly by sunlight. Sunlight, as the input component of the circadian system, is perceived by the photoreceptive cells in retina. Retina is essential for normal circadian functions as removal of both eyes disrupts locomotor activity and body temperature rhythms. [54] Retina is composed of layers of distinct cells, photoreceptive cells, bipolar cells, amacrine cells and retinal ganglion cells are localized through the coronal axis. Altogether these cells engage in photoreceptive and visual functions of the retina. The retinal ganglion cells are photoreceptors that transmit the light signal into circadian system. [55]Axons of the

ganglion cells form the optic nerve of the eye and extend projections into brain via four different tracts. [56] One of these tracts, the retinohypothalamic tract directs these projections mainly to the suprachiasmatic nucleus in the brain. Suprachiasmatic nucleus (SCN) is a pea-like structure that is localized anterior of the hypothalamus over the optic chiasm. Surgical lesions to the SCN disrupts the rhythmic behavior and transplantation of new SCN restores circadian function.[57] Neurons in the SCN have circadian rhythms in neural firing rate and they sustain this rhythmicity *in vitro*.[58] Moreover immortalized SCN neurons also can restore the abolished circadian rhythms in SCN-lesioned mice.[59]

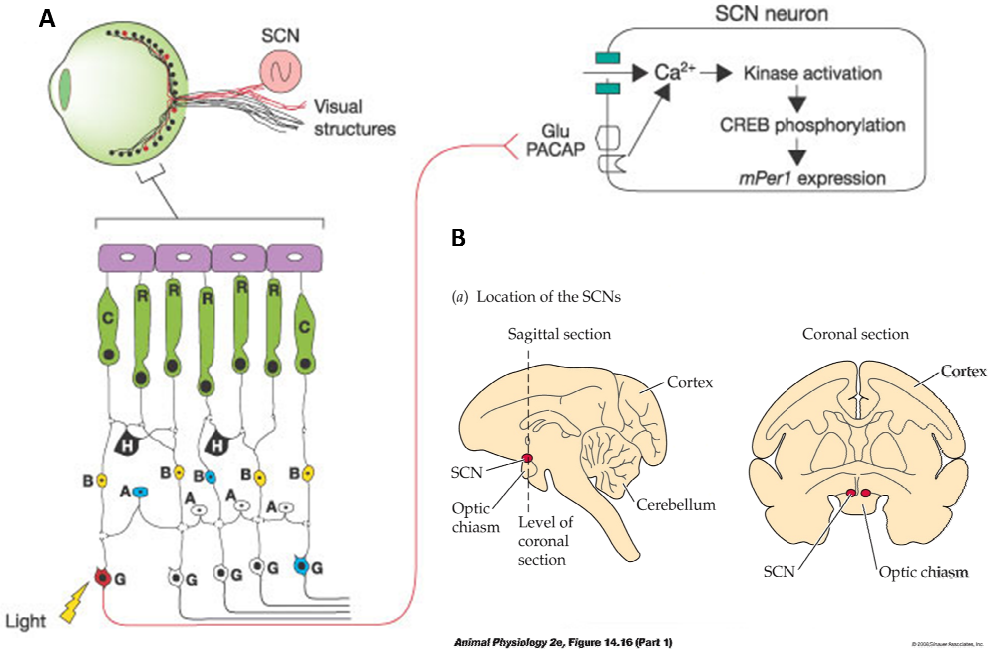


Figure 2.2- Physiological organization of the circadian clock. **(A)** Light input is perceived by the retinal ganglion cells to be transmitted to the neurons of the suprachiasmatic nucleus **(B)** to initiate and engage in downstream events. Figure (**A**) is taken from [60] and Figure **(B)** is taken from [61]

These lines of evidences indicate that SCN is indeed the master circadian pacemaker in mammals. Therefore light input is transmitted to SCN and SCN resets the clocks in other parts of the body (peripheral clocks) to regulate physiological,

biochemical and behavioral events. SCN has the identical molecular clockwork of the peripheral clocks; however SCN receives constant input from the photoreceptive cells and entrained by light to entrain the peripheral clocks. [60]

Molecular architecture of the clock is based on the universal transcriptional feedback loop. Up to date there is one main and one secondary feedback loop concerning the mammalian circadian system. The main loop is very similar to the Drosophila in terms of molecular components. Drosophila homologs of *period* and *clock* are present in the mammalian circadian systems with the same transcriptional activation (CLOCK) and repression (PER) capabilities. The molecular distinction of the mammalian clock from the Drosophila clock is the interaction partners of the CLC and PERIOD are replaced with BMAL1 and CRYPTOCHROME respectively. These four proteins are referred as the core circadian clock components in mammalia. The main transcriptional feedback loop revolves around the transcriptional activity of the BMAL1:CLOCK complex. This complex binds to E-box enhancer elements in DNA and activates transcription of *Cry* and *Per* genes and their paralogs in addition to other clock controlled genes. CRYPTOCHROME and PERIOD proteins then form a complex comprising the negative limb of the feedback loop by repressing BMAL1: CLOCK transcriptional activity through an unknown mechanism. [4, 62] There exists a secondary feedback loop to modulate *Bmal1* expression. Two mammalian specific proteins called *Rev-erbα* and *RORα* are direct transcriptional targets of the BMAL1:CLOCK transcriptional complex and they repress *Bmal1* transcription by binding to *Bmal1* promoter region.[4] There are other important proteins which regulate the core circadian clock proteins through transcriptional, translational and post-translational modifications in order to modulate their stability, activity, localization and interaction. [4, 21]

Mammalian core circadian clock revolves around four proteins. These proteins include BMAL1, CLOCK, PERIOD paralogs (PER1, PER2 and PER3) and CRY paralogs (CRY1 and CRY2). These proteins comprise the negative and positive limbs of the transcriptional feedback loop that forms the infrastructure of the core circadian

clock. In the next section, structural and functional aspects of these proteins relevant to the circadian clock will be discussed in detail.

2.3.5.1 *Clock*

Clock is the first mammalian circadian clock gene to be identified through a forward genetics screen.[17] *Clock* gene encodes a 855 amino acids long, basic helix-loop-helix motif containing protein that act as transcriptional activator when bound to its interaction partner BMAL1. [63] CLOCK contains a PAS domain, which is named after Period-Arnt-Sim proteins. PAS domains are common to other clock proteins except CRY paralogs. CLOCK contains a glutamine rich C-terminus extension, which is thought to mediate the transcriptional activity of CLOCK. CLOCK has been shown to interact with BMAL1 and PERIOD, but there are contradictory reports for interaction with CRY. [64, 65]

An interesting feature of the CLOCK protein is that the absence of rhythmicity in both protein and mRNA levels throughout the circadian period. [64] This implies that the rhythmic expression of BMAL1 is the main determinant in setting the rhythms for the positive limb of the circadian clock.

Moreover the disruption of the *Clock* allele does alter, but does not abolish the circadian rhythms in mice.[66] This implies that *Clock* gene product is redundant and not essential for generation of circadian rhythms in mammals. The inconsistency between these two studies on *Clock* gene disruption possibly arises from a dominant-negative effect of CLOCKΔ19 which competes in binding to other protein factors. It has been suggested that this redundancy occurs since NPAS2 protein also mediates similar functions as CLOCK[67]

CLOCK also has a chromatin remodeling capability, which results from the histone acetyltransferase activity that is mediated through C-terminus of CLOCK. [68] Histone remodeling activity of the CLOCK has been shown to be an essential component for transcriptional activity, which was long thought to have a role in the circadian clock mechanism.

2.3.5.2 *Bmal1*

BMAL1 (also MOP3) is also a bHLH-PAS domain containing protein like its interaction partner CLOCK. *Bmal1* is identified and cloned as a highly abundantly expressed and alternatively spliced gene in the brain and muscle. [3] Though nonexistent in primitive clocks, *Bmal1* has very high structural and sequence homology to Drosophila (CYCLE). *Bmal1* was identified through a yeast two hybrid screen to find the interaction partners of the CLOCK. [3, 69] Unlike its interaction partner, CLOCK, *Bmal1* oscillates at the protein and mRNA levels.[70] As the total CLOCK protein levels remain constant and BMAL1 protein levels oscillate, it is evident that BMAL1 is an essential for the positive limb of the feedback loop. Evidence from the studies with *Bmal1* knockout mice indicates that BMAL1 is an essential component of the circadian clock since *Bmal1* null mouse displays abolished rhythmicity at the molecular and behavioral levels. [71] Moreover *Bmal1* null mouse has a variety of other physiological aberrations, indicating that BMAL1 is not only an essential component of the circadian clock, but also an important mediator of physiological events. [72]

BMAL1 also has a nuclear translocator activity, which has dire importance for core circadian clock function. BMAL1 has a rhythmic expression and forms a complex with CLOCK in the cytosol. Upon formation of a stable complex BMAL1:CLOCK complex is translocated into nucleus. Despite the nearly constant levels of CLOCK in the cell, nuclear CLOCK oscillates with the circadian rhythm via BMAL1 dependent translocation. [73]

2.3.5.3 *Period*

Period was first identified in Drosophila from a forward genetics screen. The mammalian homolog of the *period* was identified and cloned. [74] Subsequently the other mammalian paralogs of *period* were cloned and conventionally named *per1*, *per2* and *per3*. [75-79] These orthologs are all homologous to Drosophila *period* gene and they have rhythmic expression patterns in phase with the circadian rhythm in suprachiasmatic nucleus and retina. [80] Expression of *period* is not confined to the

master circadian pacemaker or other regions of the brain, but it is also expressed in peripheral tissues such as heart, liver, lung and testis suggesting its eminent role in the circadian clock function. [76] Spatial expression patterns of these three orthologs overlap extensively with exceptions of some tissues.[78] In general these genes are co-expressed in the tissues confirmed by co-localization studies.[79] This suggests that these gene products might function through interaction with each other. Such homodimerization phenomenon is observed in Drosophila and disruption of this homodimer results in significant perturbations in the rhythmicity at the molecular and behavioral level. [81]

PERIOD protein has a variety of disctinct structural domains. It has a bHLH domain in the N-terminus, followed by two repetitive PAS domains and an additional PAS-like domain. PERIOD orthologs contain two PAS domains which are also common in BMAL1 and CLOCK. Protein-protein interactions are known to be mediated through the PAS domains between the core circadian clock proteins. These two PAS domains, PAS A and PAS B, are localized as tandem repeats in the N-terminus of PERIOD.[1] Elimination of one of these PAS domains (PAS B) in m*Per2* results in arrhythmicity in constant darkness in mice[82], indicating the eminent function of the PERIOD in circadian clock function. PAS domains also mediate homodimerization and heterodimerization of PERIOD paralogs in mammals.[83] These dimerizations do not affect the function of these proteins, but alter the nucleocytoplasmic shuttling patterns of these proteins. mPER3, which contains a functional nuclear localization signal, heterodimerizes with mPER1 and mPER2 and subsequently facilitates the nuclear translocation of these proteins.[84] In the carboxy terminus of PERIOD, there is a coiled coil region which renders functional significance as this region mediates the interaction with CRY and E4BP4.[85, 86]

In the transcriptional feedback loop of the core circadian clock system, PERIOD associates with CRY paralogs, which then interact to form a protein complex to create the negative limb of the feedback loop. [1] Mice with a null allele of m*Per1* or m*Per2* has a shorter circadian rhythm, and m*Per2* knockout mice displays a total disruption of

circadian rhythmicity.[82, 87] On the contrary, mice with a null allele of m*Per3* display a slightly shortened circadian period with nearly normal circadian function.[60] Mice carrying double null alleles of m*Per3* with either m*Per1* or m*Per2* have a circadian phenotype that is indistinguishable from the m*Per1* or m*Per2* knockout phenotype.[88] These findings suggest that the differential role of PERIOD paralogs in the core circadian clock and m*Per2* is the essential component in the negative limb of core feedback loop.

Mammalian PER has repressive activity by itself, though a more potent repression is imposed on the BMAL1:CLOCK heterodimer by mammalian CRY paralogs. [7, 89] Though a component of the transcriptional feedback loop, PERIOD paralogs do not have DNA binding activity.

2.3.5.4 *Cryptochrome*

Cryptochromes were first identified in plants as putative blue light photoreceptors and thought to have photolyase activity as they possess high sequence homology to the bacterial photolyase. [90] Mammalian *cryptochromes* genes were later identified through analysis of a human EST cDNA library and subsequently cloned. [91] A second human paralog was also identified. Surprisingly, none of the human CRY proteins displayed photolyase activity.[92, 93] DNA photolyase is present in prokaryotes and some eukaryotic species that has blue light dependent DNA repair activity.[94] Sequence analysis suggests that the mammalian *cryptochromes* display a higher similarity to 6-4 DNA photolyase than bacterial DNA photolyase, suggesting a differential evolution mechanism of *cryptochromes*.

CRY proteins, despite not possessing DNA photolyase activity, were thought to be photoreceptors as they are colocalized to the retinal ganglion cells together with mPER1. Moreover mice carrying a null allele of *cry1* or *cry2* are rhythmic and they do not display highly altered circadian rhythm output, whereas mice carrying both null alleles of *Cry1* and *Cry2* are arrhythmic. [5] However it was later shown that light pulses can induce m*Per1* expression in *Cry1/Cry2* double knockout mice.[6]

Mammalian CRY proteins are structurally unorthodox circadian clock proteins, since they do not contain any PAS domains, which dictate the protein protein interactions between other circadian clock proteins. Although they do not contain PAS domains, mammalian CRY proteins interact with core circadian clock proteins PERIOD (PERIOD1 and PERIOD2), CLOCK and BMAL1.[95] They repress transcriptional activity of BMAL1:CLOCK complex stronger than PERIOD proteins through an unknown mechanism.[7]

Structurally mammalian CRY proteins contain a highly homologous region that resembles significantly to 6-4 DNA photolyase and followed by a unstructured carboxy terminus extension, which mediate important functions such as interaction with PER2 and nuclear localization.[96]

CHAPTER 3

MATERIALS AND METHODS

3.1 Construction of Mutant Library

PCR based mutagenesis was employed in order to create a mutant DNA library. PCR reaction mixture contained 10 pmole of primers, dNTP mixture (1mM dTTP, 1mM dCTP, 1mM dGTP and 0.2mM dATP) 0.5 mM $MnCl_2$, varying concentrations of $MgCl_2$ and 25 ng of plasmid DNA template and 1µL of homemade *Taq* polymerase.

PCR products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and resolved in 0.8% agarose gel. The specific bands corresponding to the PCR products were excised and PCR products were extracted using gel extraction kit (Qiagen,CA).

Gel purified PCR products were digested with EcoRV (Fermentas) and NotI (Fermentas) for 3 hours at 37ºC and subsequently ligated to EcoRV and NotI digested, alkaline phosphatase treated pACT vector using T4 DNA ligase (Fermentas). Ligation products were transformed into chemical competent *E.coli* DH5α strain by heat shock transformation method. Transformation reactions were spread onto LB (Luria Broth)-agar petri dishes supplemented with 100µg/ml ampicillin. Transformants were inoculated into LB medium supplemented with 100µg/ml ampicillin and incubated overnight at 37ºC. Plasmid DNA was extracted using an ion exchange column (Axygen) according to manufacturer's instructions. The efficiency of mutagenesis was verified by DNA sequencing.

3.2 Cell culture

HEK293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's Medium with high glucose (Gibco) supplemented with 10% fetal bovine serum

(HyClone), 2mM L-glutamine (HyClone), 100U/ml penicillin and 100µg/ml streptomycin (Gibco). Cells were maintained under a humidified 5% CO_2 atmosphere at 37 ºC.

3.3 Immunocytochemistry

HEK293T cells were grown on coverslips and cultured in DMEM supplemented with 10%FBS. Cells were seeded at $2x10^5$ density 20h prior to transfection and transfected with pACT-Cry2-HA and pACT-Cry2-HA by Fugene HD (Roche, IN). Transfected cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.3% Triton-X. Permeabilized coverslips were blocked in 10% normal goat serum for 1 hour and incubated with monoclonal mouse anti-HA antibody (1:500) (Santa Cruz) for 1 hour. After incubation with goat anti-mouse (H+L) FITC conjugated antibody (1:200) (Invitrogen) for 1 hour, nuclei were stained with DAPI. Coverslips are mounted onto glass slides and fluorescent images are generated by Zeiss Axioplan 2 fluorescent microscope. 200 blind counts of transfected cells were performed to generate the statistical data.

3.4 Nuclear Localization Studies

NIH3T3 cells were grown on coverslips and cultured in DMEM+10%FBS. Cells were seeded at $1x10^5$ density at 6-well plates 24h prior to transfection. Both wild type and mutant Cry2 genes were cloned into pEGFP-N1 (Clontech, CA) and constructs were transfected by Fugene HD (Roche, IN) transfection reagent according to the manufacturer's instructions. The transfected cells were fixed with 4% paraformaldehyde for 10 minutes and washed with PBS. Fixed cells were subsequently stained with DAPI. Fluorescent images were generated by confocal microscopy (Leica). Localizations of the proteins were assessed by microscope and 200 blind counts of transfected cells were performed to generate the statistical data.

3.5 Co-immunoprecipitation

HEK293T cells were seeded at $2.0x10^6$ density in 100 mm plates 24h prior to transfection. Cells were transfected with relevant expression plasmids using calcium phosphate co-precipitation technique. 48h post transfection, cells were lysed in lysis buffer containing 50 mM Tris-HCl(pH=7.5), 0.5% NP-40 and 150 mM NaCl for 20 min on ice and sonicated once. Lysates were clarified by centrifugation at 14000 rpm for 10 minutes at 4ºC.

Clarified lysates were mixed with 20 µL of anti-FLAG M2 agarose beads (Sigma) and rotated overnight to generate antibody:antigen complexes. After three subsequent washes with ice cold lysis buffer, resin is precipitated by centrifugation at 14000 rpm for 1 min at 4ºC. Resin bound proteins were eluted in 2X laemmli sample buffer and resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis.

3.6 Mammalian two-hybrid assays

HEK293T cells were seeded into 24-well cluster plates (TPP) with 10^5 cells/mL concentration 24h prior to transfection. Cells were transfected with equimolar ratios of pACT-m*Cry2*, pBIND-m*Bmal1* and pG5-*luc* plasmids at a total DNA amount of 500 ng/well. For transcriptional assays, $2.5x10^5$ cells were seeded into 6-well dishes and transfected with 200 ng of pGL3-m*Per1* promoter construct and 250 ng of the m*Cry2* variants.

At 48 h post-transfection, cells were lysed with passive lysis buffer (25 mM Tris-phosphate (pH=7.8), 1% *Oxytoxynol-9*, 2mM CDTA, 2mM DTT, 0.1% BSA and 10% glycerol) and both the firefly luciferase and *Renilla* luciferase enzyme activities were measured from the same cell lysate sample using the Dual-Luciferase Reporter Assay System, according to the manufacturer's instructions (Promega). Luciferase activities were measured in a Fluoroskan Ascent FL luminometer (Thermo Scientific). Luciferase values are background subtracted and normalized to *Renilla* luciferase activity.

3.7 Western blot

Total lysates or immunoprecipitates were separated by 10% SDS-PAGE and transferred onto polyvinyldifluoride membranes. Membranes were blocked with 5% BSA (Amresco) in Tris buffered saline supplemented with 0.1% Tween 20 to alleviate nonspecific binding. Blocked membranes were incubated with mouse anti-Hemagglutinin (1:250) (Santa Cruz Biotechnology,CA) or mouse anti-FLAG (1:1000) (Sigma) for 1 hour at room temperature. After three subsequent 10 minute washes with TBS+ 0.1% Tween 20, the membranes were incubated with alkaline phosphatase conjugated goat anti-mouse IgG(1:10000)(Santa Cruz Biotechnology,CA). Finally blots were washed thrice with TBS+ 0.1% Tween 20 and developed using NBT-BCIP detection system (Roche, IN)

3.8 Site-directed mutagenesis of m*Cry2*

F171L substitutions were carried out using following primers:

5'-CCTTACCTACAAGCGCTTACAGGCCCTCATCAGCCGC-3'
5'-GCGGCTGATGAGGGCCTGTAAGCGCTTGTAGGTAAGG-3'

A plasmid based site directed mutagenesis method was employed using 10µM of each mutagenic primers, 10 ng of plasmid template, 200 µM of dNTP mixture and 2.5U DreamTaq DNA polymerase (Fermentas) in 50µL of PCR mixture. PCR reactions were cycled on the thermal cycler as follows: initial denaturation at 95°C for 2 min; 18 cycles at 95°C for 30 s, 55°C for 30 s and 68°C for 13 min; final extension at 68°C for 7 min. The template was digested with 10 U of *Dpn*I (Fermentas), and incubated at 37°C for 1.5 h. Five microliters of the final sample was used to transform chemically-competent *E.coli* cells (DH5α). Mutagenesis was confirmed by DNA sequencing.

3.9 Homology modeling of mCRY2 and other bioinformatics analysis

A three-dimensional model of mCRY2 is generated using homology modeling based on the existing crystal structure of Drosophila 6-4 DNA photolyase (PDB ID: 3cvv). In order to generate this model, two sequences were aligned using CLUSTALW, and resulting alignment were used to build the 3D structure using DeepView. The generated structure was energy minimized in AMBER force field for 40 ps to eliminate the high energy structures with side chain clashes. Quality of the structure was assessed by the public ProSA[97] quality assessment server. The images of the structure were generated using PyMOL.

Alignments were generated using CLUSTALW to analyze the evolutionarily conserved regions on mCRY2.

CHAPTER 4

RESULTS

4.1 Structural modeling of mCRY2

Although there are preexisting models of mammalian CRY proteins based on the *Escherichia coli* CPD DNA photolyase or cyanobacterial photolyases, mammalian CRY proteins have nearly 50% sequence homology to the 6-4 DNA photolyase group of proteins than CPD DNA photolyases of lower species which are around 30% homologous to mammalian CRY proteins. Therefore, a three-dimensional model was generated using a X-ray resolved crystal structure of Drosophila 6-4 DNA photolyase [98] in order to have a better molecular model. The obtained model shows that the mCRY2 protein has an α/β domain in the N-terminus, followed by a long interdomain loop structure and a long helical domain that extends towards the carboxy-terminus of the protein. (**Figure 4.1.1**) Although the model is energy minimized using AMBER force field, there are still some side chain clashes and geometrical constraints in the structure that arise mainly from the loop structures contained within the protein. However assessment of the global protein structure by using ProSA, indicates that the overall quality of the structure is comparable to a crystal structure with 2.5Å resolution.

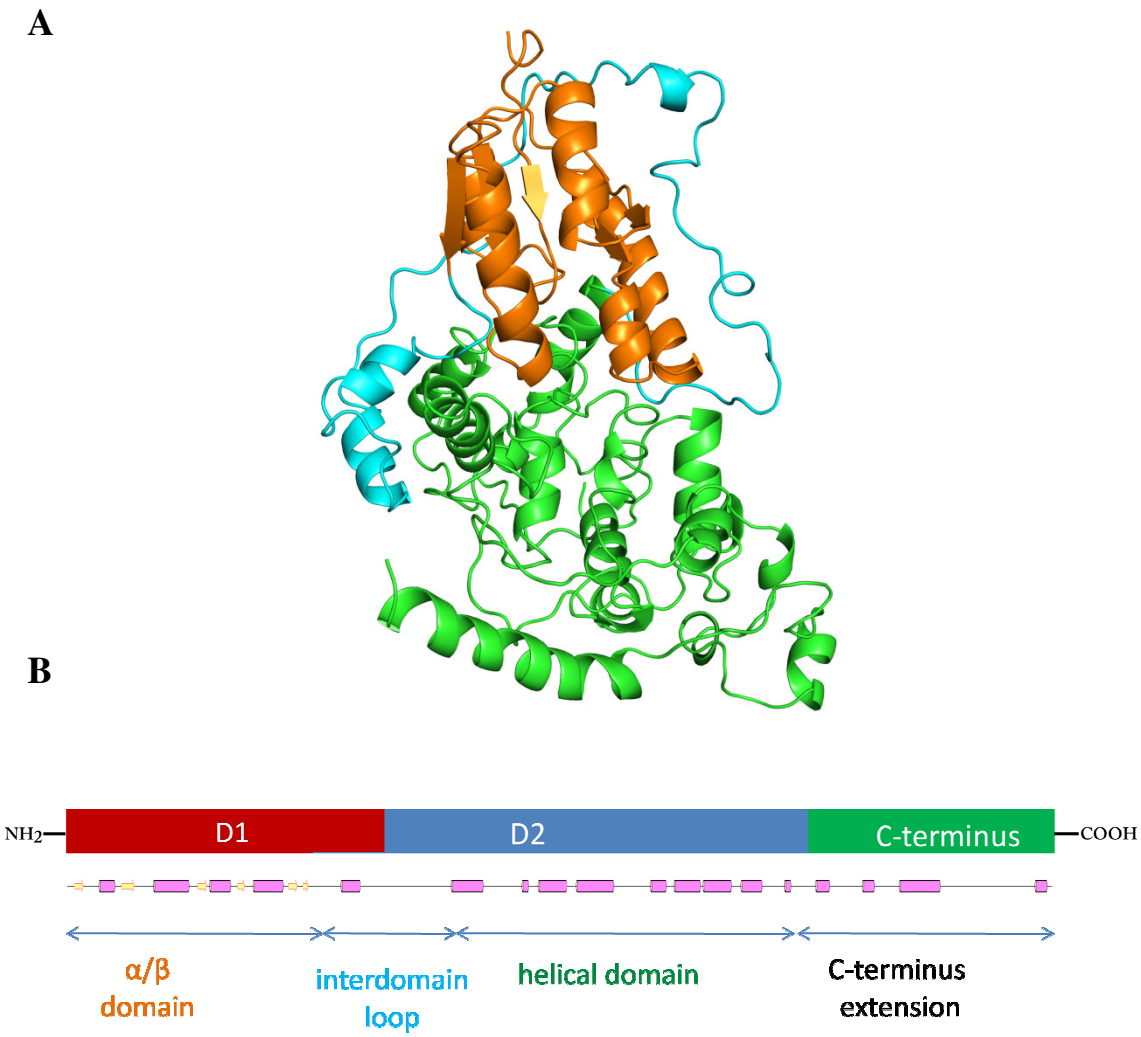
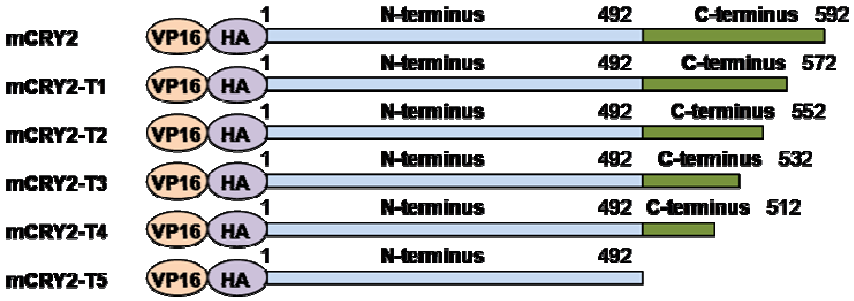


Figure 4.1.1- A three dimensional model of the mCRY2 based on the existing Drosophila 6-4 photolyase crystal structure. The evident motifs are shown on the structure with different colors. (**A**) Secondary structures are plotted along the amino head to carboxy tail axis. (**B**)

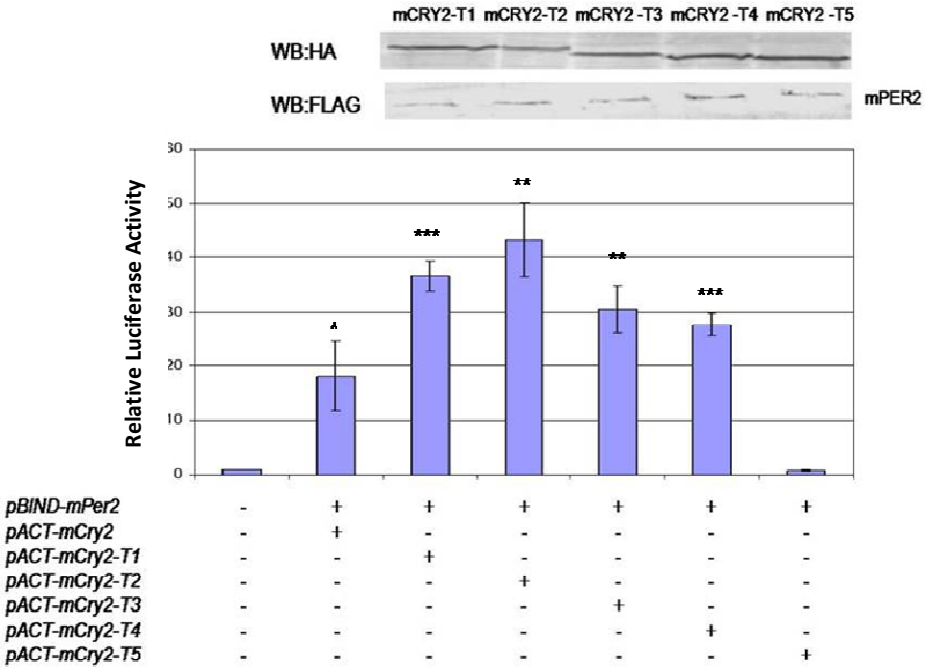
4.2 Interaction between mCRY2 and mPER2

Previous research reports indicated that C-terminus of mCRY2 mediates the interaction with mPER2. [96] In order to assess the mCRY2 and mPER2 interaction in molecular detail and to determine the amino acids on the mCRY2 molecular surface that mediate binding to mPER2 , sequential C-terminus deletion constructs complemented with a degenerate oligonucleotide based PCR was combined in the mammalian two-hybrid system. Results indicated that the region between residues 493-512 encompassed the amino acids that mediate mCRY2-mPER2 interaction (**Figure 4.2.1-A**) and the residues R501 and K503 constitute the interaction surface (Mutant bearing the mutations on these residues is referred as mCRY2-Mut). (**Figure 4.2.1-B**) These residues are then assessed on the three dimensional model (**Figure 4.2.1-C**) and

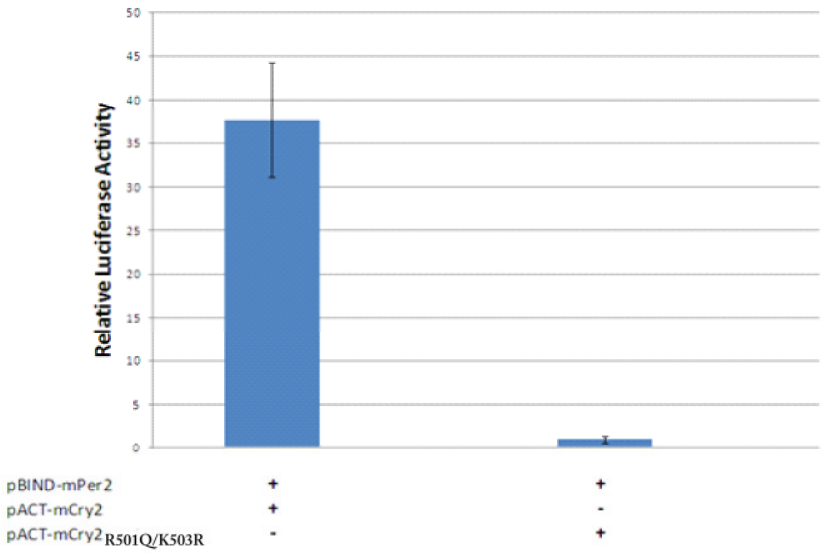
A



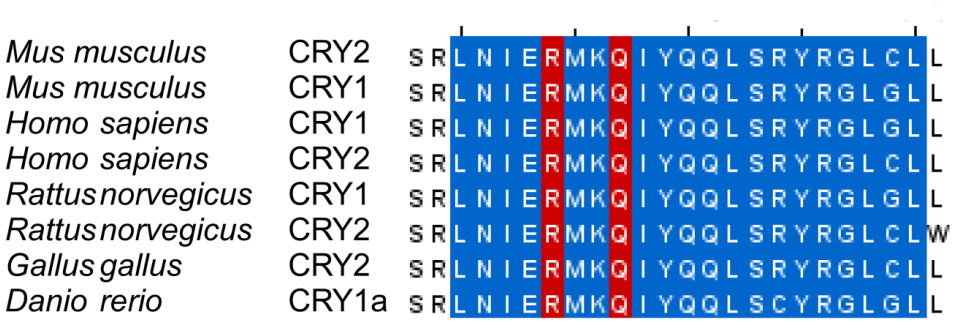
B



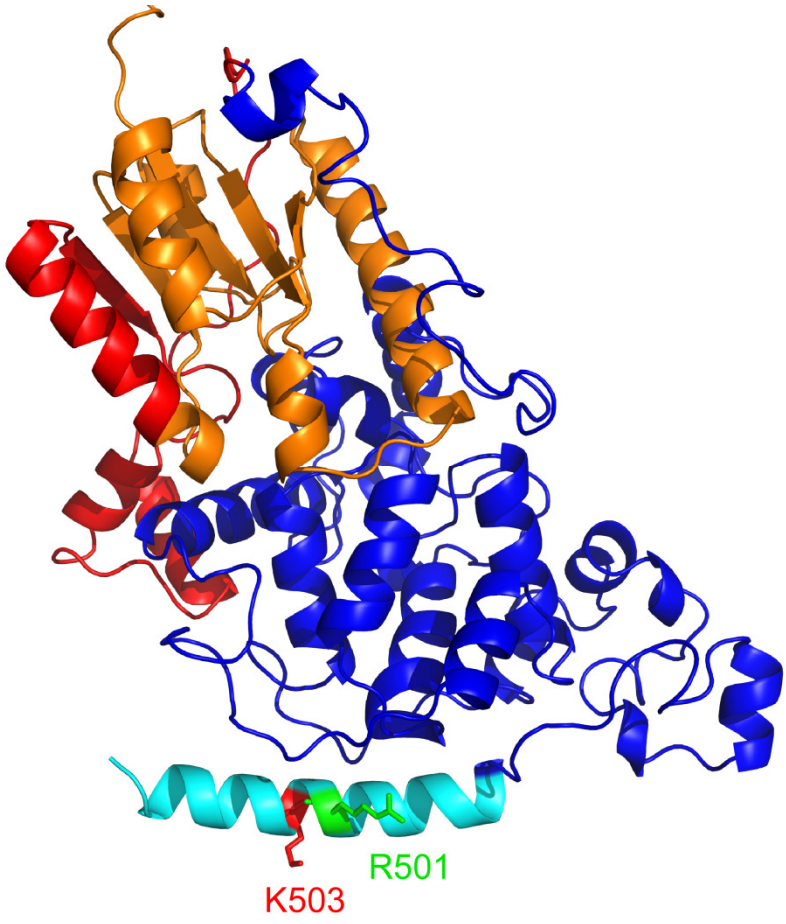
C


Relative Luciferase Activity
50
45
40
35
30
25
20
15
10
5
0
pBIND-mPer2 + +
pACT-mCry2 + -
pACT-mCry2R501Q/K503R - +

D


Mus musculus CRY2 SRLNIERMKQIYQQLSRYRGLCLL
Mus musculus CRY1 SRLNIERMKQIYQQLSRYRGLGLL
Homo sapiens CRY1 SRLNIERMKQIYQQLSRYRGLGLL
Homo sapiens CRY2 SRLNIERMKQIYQQLSRYRGLCLL
Rattus norvegicus CRY1 SRLNIERMKQIYQQLSRYRGLGLL
Rattus norvegicus CRY2 SRLNIERMKQIYQQLSRYRGLCLW
Gallus gallus CRY2 SRLNIERMKQIYQQLSRYRGLCLL
Danio rerio CRY1a SRLNIERMKQIYQQLSCYRGLGLL

E


R501
K503

F

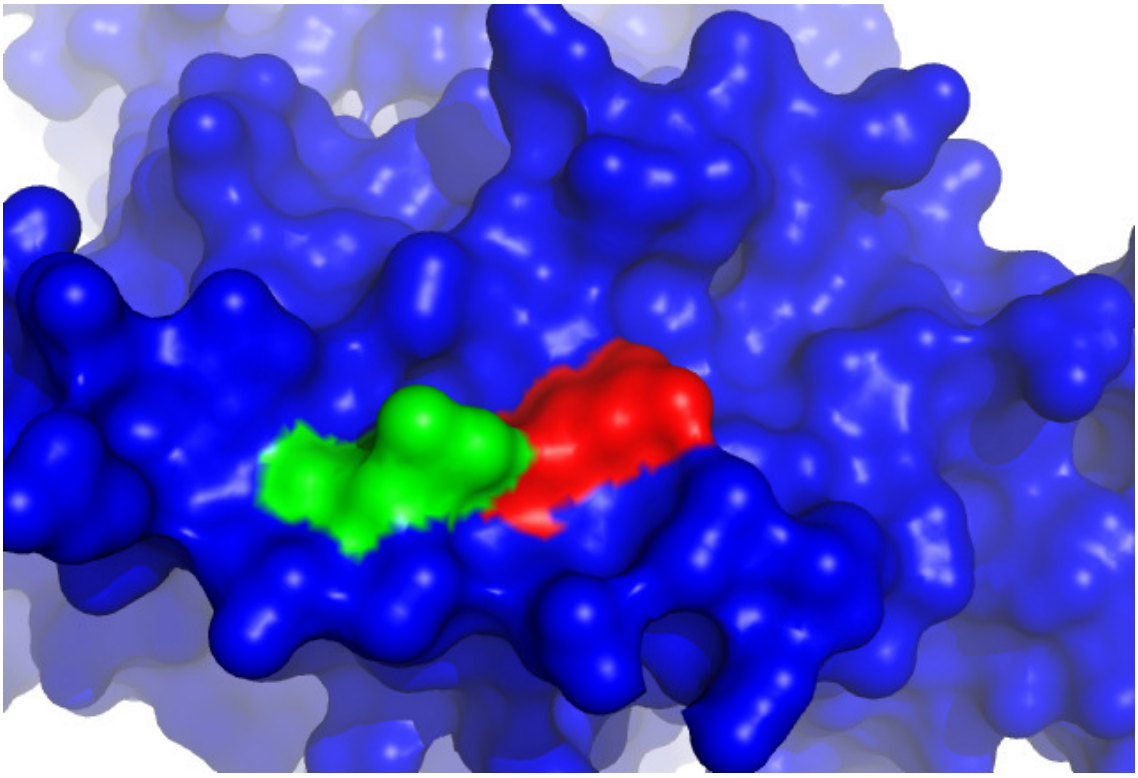


Figure 4.2.1- Analysis of the role of C-terminus in the interaction between mCRY2 and mPER2. Sequential C-terminus deletion constructs of m*Cry2* and full length m*Per*2 were employed in the mammalian two hybrid assay. These constructs were transfected in conjunction with a reporter plasmid encoding a firefly luciferase enzyme driven by five consecutive GAL4 promoter elements. (**B**) Interaction of wild type and R501Q/K503R substituted m*Cry2* with m*Per2* is assessed by the mammalian two hybrid method (**C**) as in (**B**). Standard errors of three independent measurements are represented in the bar graphs in (**B**) and (**C**). The mutated residues are highly conserved, localized onto a C-terminal α-helix and exposed onto the surface. (**D,E,F**) R501 is highlighted in green color whereas K503 is highlighted in red color.

it was observed that these residues are localized onto an α-helical element on the C-terminus of mCRY2 and exposed on the molecular surface.

4.3 Cellular localization and the repressive capability of mutant mCRY2

The transcriptional feedback loop lies at the heart of the core circadian clock mechanism. Circadian clock proteins have distinct nuclear localization and nucleocytoplasmic shuttling patterns. Mouse CRY2 protein has been shown to have a C-terminal nuclear localization sequence and can directly translocate into nuclei without other factors. Moreover mCRY2 facilitates the nuclear translocation of mPER2

to form the negative feedback complex. [7, 83] Therefore the perturbation of interaction by mutating these two residues might affect the nuclear localization pattern of the mCRY2 and influence the interaction in an indirect manner. In order to assess the localization of mCRY2, the gene is cloned into GFP-fusion cassettes and transfected into NIH3T3 murine fibroblast cell line. The results clearly show that there is no disruption of the nuclear localization of mCRY2 as judged by the assessment of subcellular GFP localization using confocal microscopy. (**Figure 4.3.1-B**)

Moreover the disruption of the repression is verified by a slightly modified transcriptional assay described in [7]. (**Figure 4.3.1-A**) In this transcriptional assay, the reporter construct harboring GAL4 promoters was used in lieu of an E-box promoter fused reporter construct. These results indicate that two amino acids, R501 and K503, disrupt the mPER2 interaction and result in subsequent loss of repressive capability without altering the nuclear localization pattern of mCRY2 protein.

Moreover the localization of the mutant and wild type proteins are confirmed by employing different constructs and different cell line immunocytochemistry with monoclonal antibody raised against the HA tags. Also transcriptional activities of the mutant and wild type proteins are investigated with the same constructs to ensure that the mutations do not affect the nuclear localization patterns and perturb the transcriptional activity of mBMAL1:mCLOCK complex. In this transcriptional assay, the repressive capability of mCRY2 proteins of the endogenous BMAL1:CLOCK activity is analyzed by employing an E-box promoter fused reporter cassette. Results clearly confirmed the prior nuclear localization assay as there were no perturbations in the nuclear localization patterns of mutant and wild type proteins as determined by the subcellular localization of the FITC fluorescence. (**Figure 4.3.2-B**) Moreover the mutant protein was also defective in repression of the endogenous BMAL1:CLOCK transcriptional activity. (**Figure 4.3.2-A**)

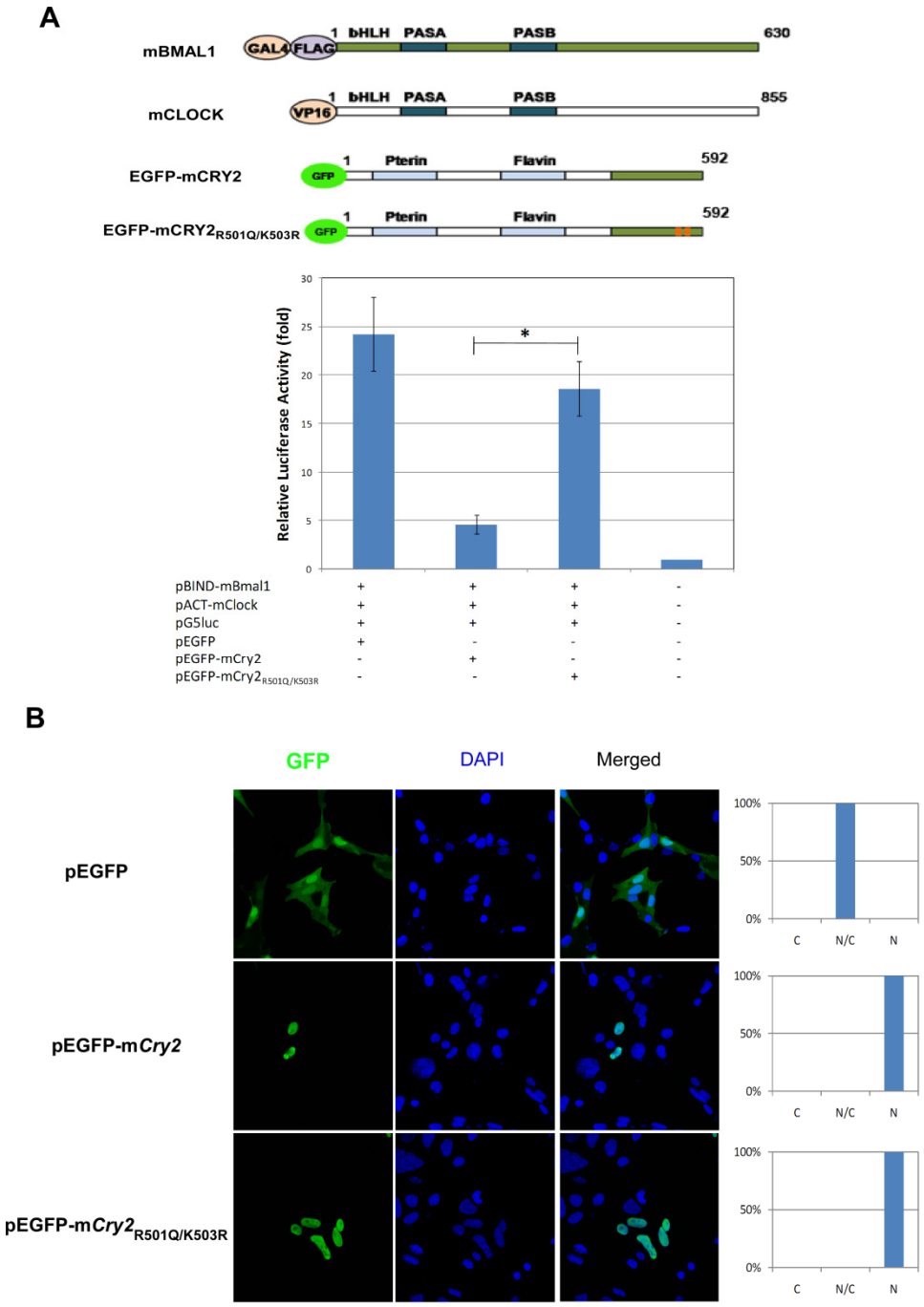


Figure 4.3.1- In order to assess the effect of the R501Q/K503R mutations on nuclear localization of mCRY2 GFP-fusion constructs were employed and wild type and mutant constructs were transfected into NIH3T3 cell line. The bar plots indicate the quantitative assessment of localization after 200 blind counts.(**B**) Transcriptional repression capability of wild type and mutant mCRY2 is investigated using a reporter based transcriptional assay with the constructs depicted in top panel. Standard errors of three independent measurements are included in the bar graph. (**A**)

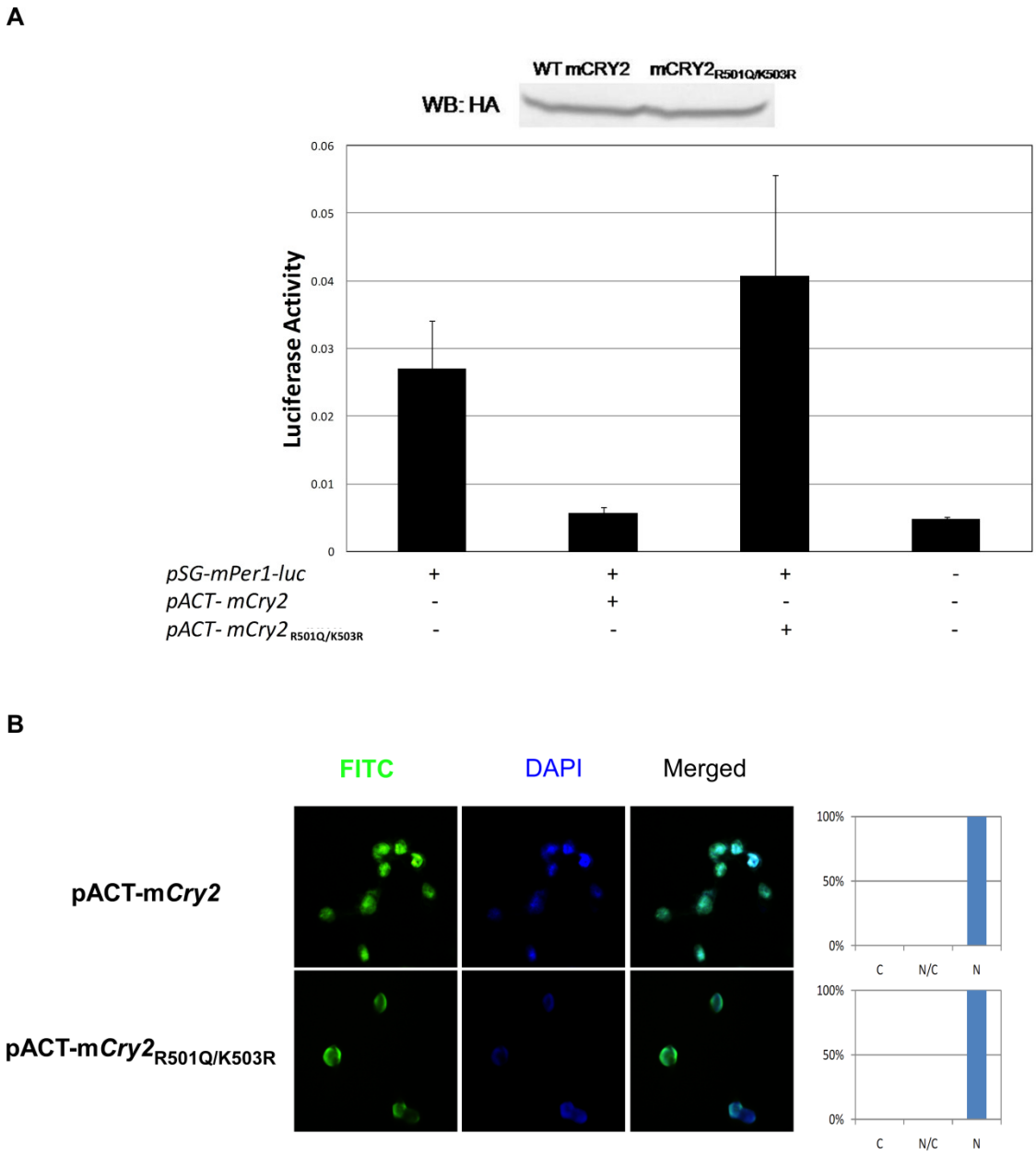


Figure 4.3.2- In order to examine the effect of the mutant protein's transcriptional inhibition activity on endogenous BMAL1:CLOCK transcriptional activity, HEK293T cells were transfected with the wild type and mutant constructs in addition to a E-box promoter fused luciferase reporter construct. (**A**) Immunocytochemical analysis of the subcellular localization of the mutant and wild type proteins in the HEK293T cell line transfected with either wild type (pACT-C2F) or R501Q/K503R mutant constructs.

4.4 Interaction of mCRY2 with mBMAL1

In order to assess whether interaction of mCRY2 with mBMAL1 is mediated through mPER2 or through direct interaction of the two proteins, sequential C-terminal deletion constructs of m*Cry2* and m*Bmal1* are employed in the mammalian two hybrid assay. These constructs were transfected into HEK293T cell line together with a reporter plasmid encoding firefly luciferase enzyme expressed through five GAL4 binding promoter elements (pG5-*luc*).

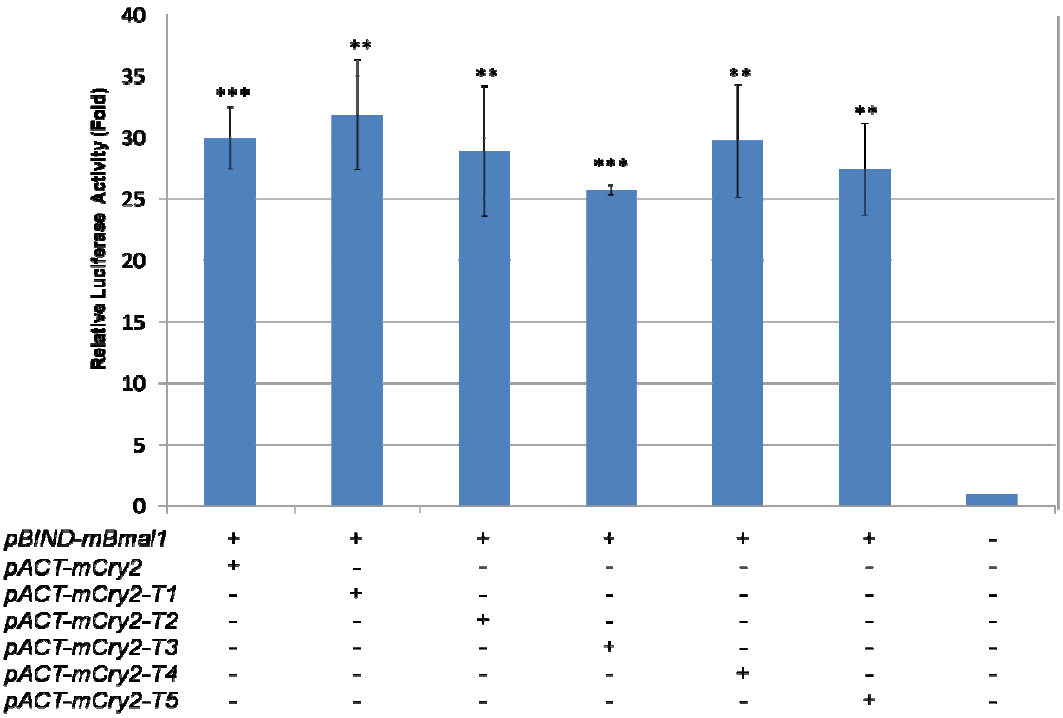


Figure 4.4-1- Analysis of interaction between mCRY2 and mBMAL1 employing mammalian two hybrid method. Sequential truncation constructs of the C-terminus of m*Cry2* (T1-T5) with 20 amino acid amplitudes are transfected together with the pBIND-m*Bmal1* expression and pG5-*luc* reporter constructs into HEK293T cells. Luciferase activity is background subtracted and normalized to Renilla luciferase activity encoded by the pBIND plasmid. Standard errors of three independent measurements are included in the bar graphs.

The results indicate that the C-terminus of mCRY2 is not essential for interaction with mBMAL1. (**Figure 4.4-1**) This is an implication of direct interaction of mCRY2 with mBMAL1 and eliminates the possibility of a scaffold function of mPER2 between these two proteins.

The amino acid residues on mCRY2 that determine the interaction with mPER2 were determined in the previous chapters. Therefore I set out to determine whether the mPER2 interaction is essential for mBMAL1 and mCRY2 interaction using a biochemical assay. Ectopically expressed wild type and mutant mCRY2 proteins are assessed in the capability of interaction with ectopically expressed Flag-tagged mBMAL1 protein using co-immunoprecipitation technique.

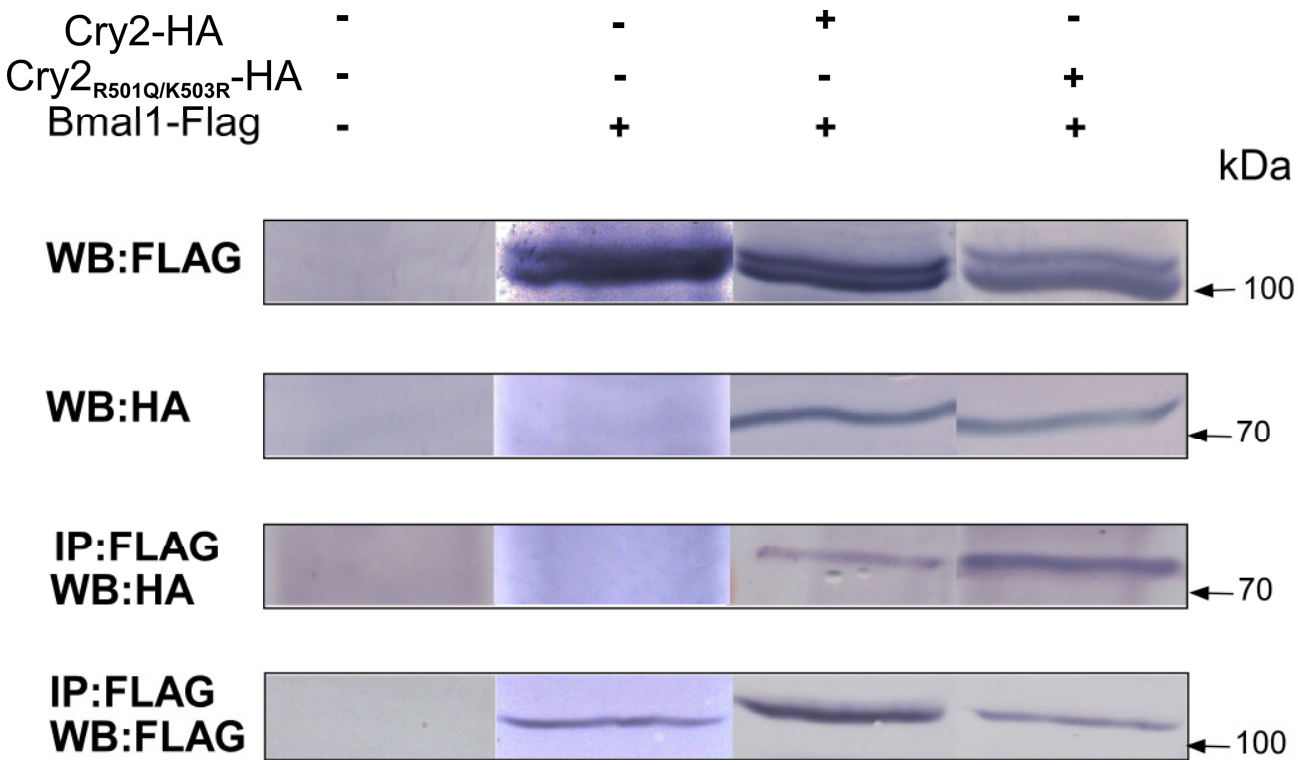


Figure 4.4-2- Analysis of interaction between wild type and mutant mCRY2 and mBMAL1 using co-immunoprecipitation technique. HEK293T cells were transfected with mutant and wild type m*Cry2* expression constructs (pACT-m*Cry2*) in conjunction with m*Bmal1* expression construct (pBIND-m*Bmal1*) into HEK293T cells. Cellular lysates were immunoprecipitated with anti-FLAG agarose resin and immunoprecipitates were subsequently resolved with SDS-PAGE electrophoresis and immunoblotted with HA antisera.

Results indicated that both wild type and mutant mCRY2 proteins were coimmunoprecipitated with mBMAL1 (**Figure 4.4-2**), suggesting that mPER2 interaction is not essential in binding of mCRY2 to mBMAL1. This result indirectly implicates that the C-terminus of mCRY2, which mediates interaction with mPER2, is not also essential in mBMAL1 binding which confirms the results depicted in **Figure 4.4-1**.

4.5 Random mutagenesis of the N-terminus of m*Cry2* (m*Cry2*-D1)

Previous results point out a direct interaction between mCRY2 and mBMAL1. However domains or residues dictating this interaction are not determined in the molecular level. As the contribution of the C-terminus to this interaction was determined and found to be nonessential, we thought that the N-terminus of mCRY2 might mediate this interaction. Therefore the first 200 amino acids of m*Cry2* is subjected to detailed analysis in mammalian two hybrid screen explained in the previous sections. The N-terminus of mCRY2 interacts with mBMAL1 comparably to the full length mCRY2 as assessed by the mammalian two hybrid assay. Therefore a global random mutagenesis strategy was employed using the error prone PCR method in order to determine the binding surface on N-terminus of mCRY2 (referred to mCRY2-D1). To achieve desired level of mutations, the mutagenic PCR reactions were optimized with a gradient magnesium curve. Mutagenesis is saturated with increasing level of magnesium and polymerization of DNA fragments is inhibited at further elevated magnesium concentrations. m*Cry2*-D1 fragment is amplified through this mutagenic PCR reaction, digested with EcoRV and NotI restriction enzymes and subsequently cloned into EcoRV-NotI double digested, dephosphorylated pACT plasmid vector to encode mCRY2-D1 fused to VP16 transactivation domain for further use in the mammalian two hybrid based screening. Results show that the PCR reactions require a magnesium concentration of 4 mM and are inhibited at elevated magnesium concentrations. (**Figure 4.5.1-A**) The ligation products were transformed into chemically competent bacterial cells and plasmids were isolated by ion exchange

columns. The efficiency of cloning was determined by PCR using primers targeting the pACT plasmid. (**Figure 4.5.1-B**) Sequencing of random clones indicate that the mutagenesis is achieved at around 0.66% frequency per 600 nucleotides, which enables further screening of residue based interactions without altering the global structure of the expressed protein. Mutagenized m*Cry2*-D1 encoding plasmid vectors are employed in the mammalian two hybrid system in conjunction with the m*Bmal1* encoding plasmid vector and also the firefly luciferase reporter construct. 150 mutant constructs were screened for the loss of interaction and the three of the verified loss of function mutants were sequenced in order to determine the binding region. The two of the loss of function mutants encoded a C-terminus truncated protein through nonsense mutation of the codon, whereas one of the mutants expressed complete mutagenized D1 construct. The combinatorial logic indicates that the C-terminus of D1 is important as it is absent in two clones that encode a loss of function protein and the last clone has only one mutation in this region through a missense mutation resulting in substitution of phenylalanine to leucine. (**Figure 4.5.1-C**)

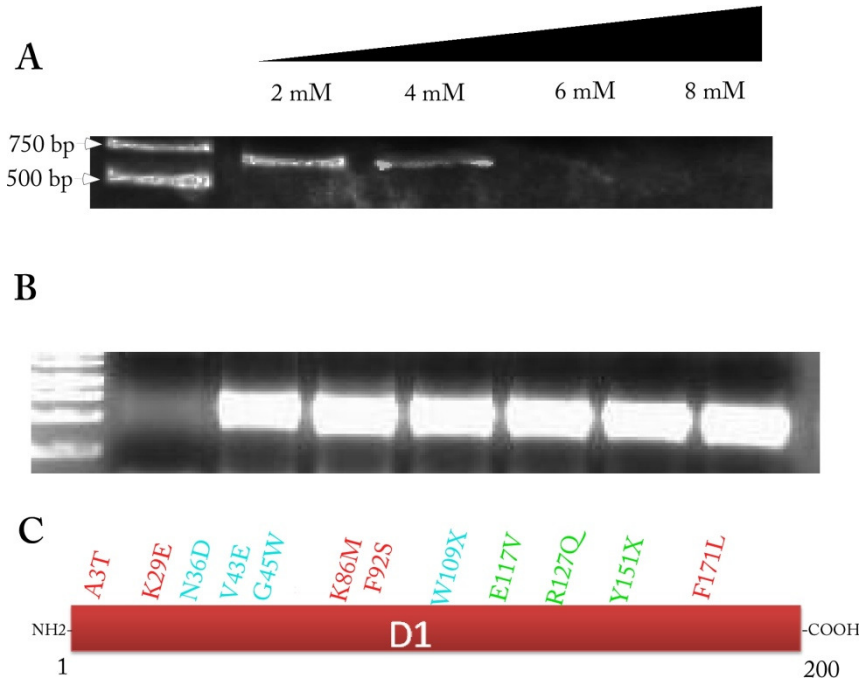
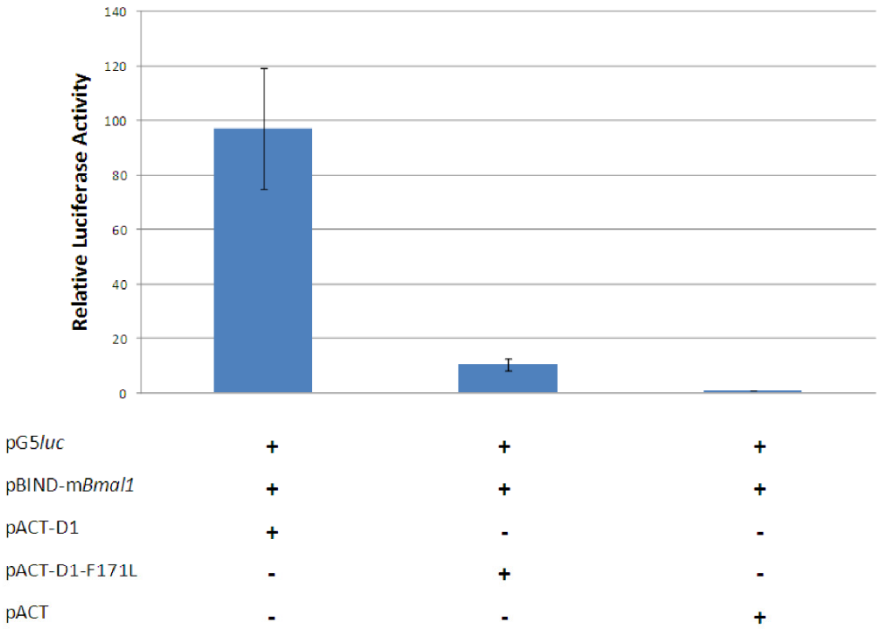
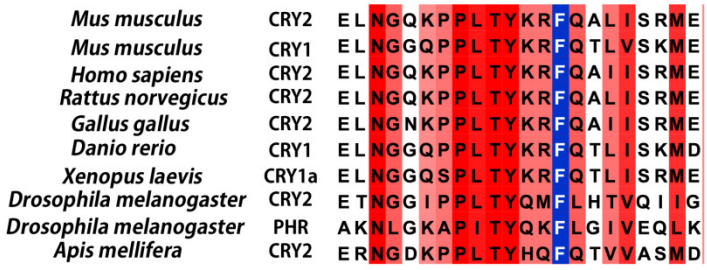


Figure 4.5.1- Titration of the mutagenic PCR reactions with a magnesium curve (**A**) and verification of the cloning efficiency by colony PCR method (**B**) The loss of function mutants are depicted on representative mCRY2-D1. The individual colors represent distinct loss of function clones.(**C**)

A



B



C

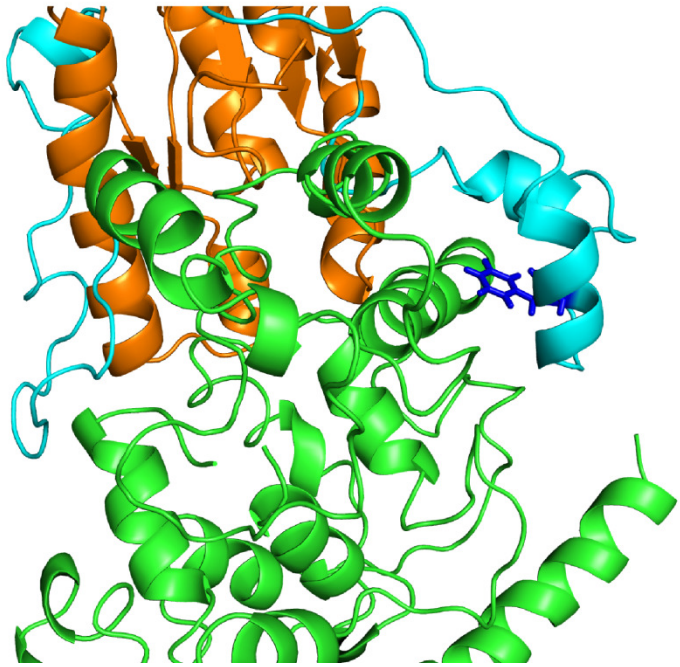


Figure 4.5.2- Mammalian two hybrid analysis of the interaction between wild type and mutant mCRY2-D1 between mBMAL1. Standard errors are included on bar graphs to represent three independent measurements.(**A**) This residue is highly conserved in invertebrate and vertebrate cryptochromes. (**B**) Structural mapping of the F171 residue (highlighted in blue sticks) on the in *silico* model. (**C**)

Site directed mutagenesis of this residue, results in obliterated binding of mCRY2-D1 to mBMAL1 as assessed by the mammalian two hybrid method. This residue is highly conserved in vertebrate and invertebrate cryptochromes and lies in the interdomain loop region on the three dimensional structure. The F171 residue is interestingly on the surface aligned with DNA with respect to the Drosophila photolyase structure. (**Figure 4.5.2)** However interaction is not fully eliminated as the mutant has still weak binding affinity towards BMAL1.

4.6 Random mutagenesis of the central domain of m*Cry2* (m*Cry2*-D2)

Mutagenesis of D2 was performed using the same magnesium concentration as D1 (4mM). However the mutagenesis was not saturated, since the sequencing results of two random clones indicated very low or no mutations of the D2 template. Therefore the magnesium concentration was raised to 8mM which yielded a considerable mutation rate depending on sequencing of two random clones. Screening procedure for loss of function mutants were carried out by the mammalian two hybrid method using the same protocol in D1 screening.

There were five loss-of-function mutants in the screen constructed with 120 individual clones and the loss of function is confirmed by the repetitive mammalian two hybrid assays. (**Figure 4.6.1**)

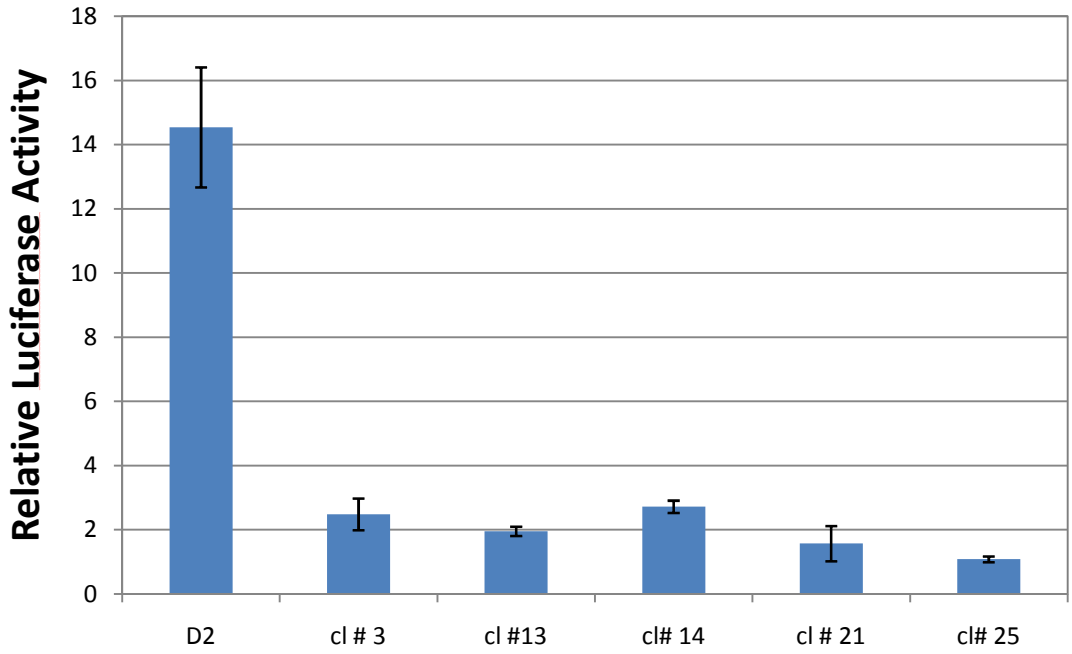


Figure 4.6.1- Analysis of the interaction between the loss of function mutants and mBMAL1 using mammalian two hybrid method. Standard errors are included in the error bars to represent the deviation between three independent measurements.

Unlike D1 screen, the loss-of-function mutants for D2 did not yield any truncation mutants, which prompted a difficulty for selecting the hot spots. However, some of the mutations on the N-terminus were repetitive indicating a functional significance of these residues in the mBMAL1 interaction. (Figure **4.6.2**) Although there are not any consensus mutations between the clones, the clones exhibited significant loss of BMAL1 interaction. The focal mutations on the N-terminus includes mostly proline substitutions (S212P,L216P and L236P) and these are mapped onto different plane than the surface constituted by F171. However there are other mutations on different loss-of-function clones on L308P and W311R. These residues are in the vicinity of the plane constituted by F171L. However the clones still have weak binding affinity towards BMAL1, indicating that the BMAL1 binding surface is constituted by different amino acids probably with a combination of the mutated residues. The focal

mutations on N-terminus imply the binding surface on D2 is constructed by these amino acids, whereas the distal mutations indicate a probable disturbance on the previously identified surface on D1.

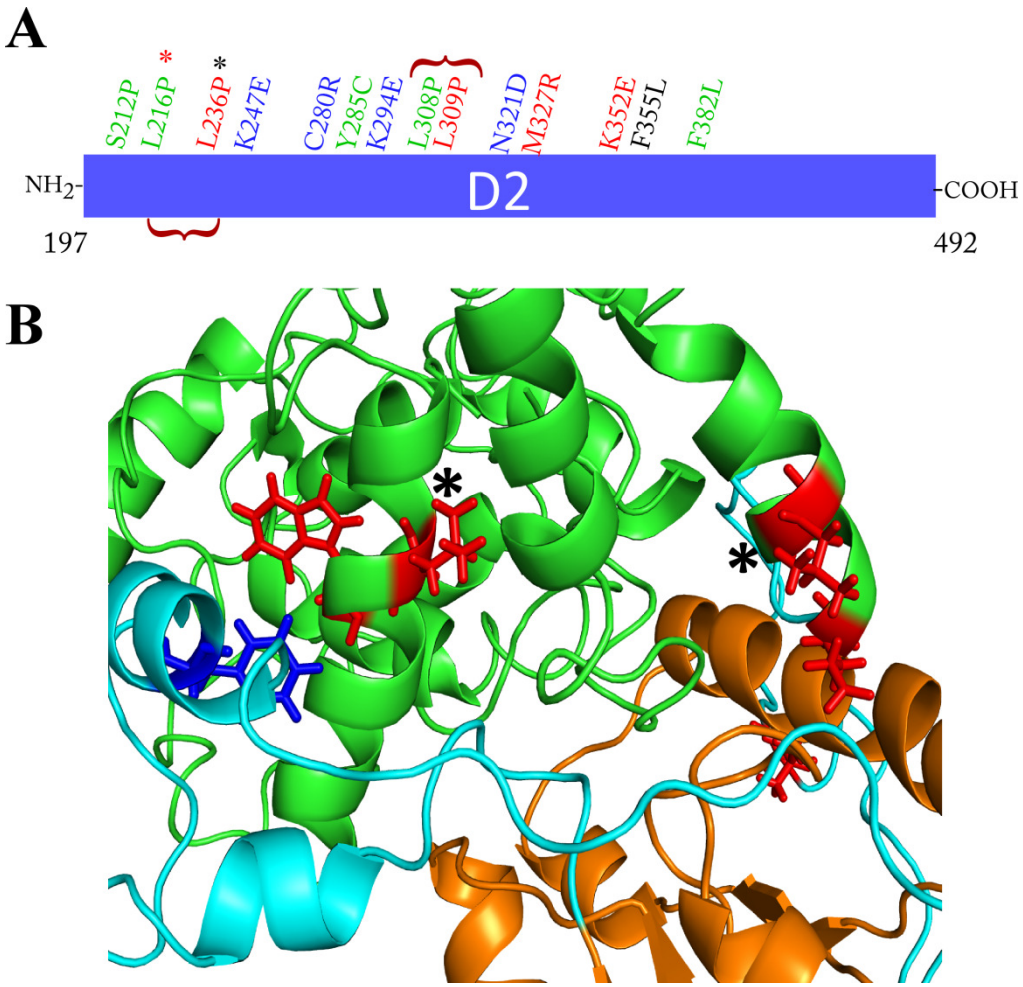


Figure 4.6.2- Pictorial representation of the D2 loss-of-function mutations. Different clones are indicated with different colours. Focal mutations on the N-terminus of D2 are also highlighted.(**A**) The D2 loss-of-functions are mapped onto the three dimensional CRY2 model and highlighted in red color whereas F171 residue is depicted as blue. Asterisks indicate the repetitive mutations.

Chapter 5

DISCUSSION

In this thesis work, the molecular mechanism of the interaction of circadian clock proteins mCRY2, mPER2 and mBMAL1 are assessed using different reverse genetic approaches and the functional analysis of these interactions on the transcriptional feedback loop is conducted.

Previous work for assessing functional domains on the cryptochromes, indicated that the C-terminus of CRY proteins mediate the protein's repressive function in *Xenopus* circadian system.[99] Another recent study maps the interaction hot spot of CRY2 to G354 which abrogates PER2 binding and decreases BMAL1 binding. Moreover it is claimed that the deletion of C-terminus domain does not alter the transcriptional repression capability of CRY1.[100] Opposingly, there are other reports indicating that deletion of the C-terminal portion does not affect the repressive function, but alter the nuclear accumulation of the CRY1.[96] Therefore in the first part of this study, the effect of C-terminus on mPER2 interaction is assessed using sequential C-terminus deletion constructs in order to unravel the function of mCRY2 C-terminus in the core circadian clock mechanism. It was observed that deletion of 100 amino acids of the C-terminus disrupts mPER2 interaction in cellular and biochemical assays. Furthermore mPER2 interaction hot spots are mapped to the evolutionarily conserved R501 and K503 residues located on an α-helix on the C-terminus.

The central role in the negative limb of the transcriptional feedback loop is attributed to CRY proteins, which have prevailing repressive function than PER proteins as overexpression of mPER2 in the fibroblast cell line does not yield significant repression of BMAL1:CLOCK transcriptional activity whereas overexpression of mCRY2 yields dramatic repression of BMAL1:CLOCK transcriptional activity. [7] In contrast, recent studies indicate that PER proteins have a more repressive function than CRY[101], and CRY proteins only facilitate the nuclear

translocation of PER proteins. Therefore we hypothesized that the CRY:PER complex but neither of these proteins alone, mediate the transcriptional repression. In order to explore this hypothesis, we mutated the mPER2 interaction hot spots on mCRY2 and eliminated the interaction between these two proteins. By using m*Per1*-luciferase reporter construct, the transcriptional repression activity of the mutant protein on the endogenous BMAL1:CLOCK transcriptional activity is observed to be dramatically reduced. This disruption is confirmed in the transcriptional assay defined by [7], and it was also observed that the mutant mCRY2 cannot induce transcriptional repression. C-terminus region of mCRY2 is shown to regulate the nuclear localization of both mCRY2 itself and mPER2 as a modulatory mechanism to control circadian timing.[99] Therefore the loss-of-transcriptional activity and also mPER2 interaction might be due to aberrant nuclear localization of the protein, since the regulatory functions of C-terminus and the nuclear localization signal harbored in the proximity of the interaction hot spots.[96] Furhermore it is also claimed that the nuclear accumulation of the CRY:PER complex through deletion of the endogenous NLS in these two proteins and claims that the NLS in these two proteins are essential for translocating the CRY:PER complex into nucleus. [83]However, the C-terminus deletion of the mutant allele results in a more severe loss of transcriptional repression activity.[96] In order to determine whether the loss of transcriptional repression activity is due to loss of interaction or aberrant nuclear localization of the mutant protein, we assessed the nuclear localization of the mutant (R501 and K503) by means of GFP-fusions and immunocytochemistry. It was observed that the mutations at the residues R501 and K503 do not alter nuclear localization pattern of mCRY2. These findings support our initial hypothesis that interaction of mCRY2 with mPER2 is essential for the repression of BMAL1:CLOCK activity.

Our results support a direct C-terminus mediated interaction of CRY2 with PER2 which in turn determine the transcriptional repression activity. Also it was previously shown that CRY2 exerts a more potent repression than PER2 on the BMAL1:CLOCK transcriptional activity. Our results indicate that the transcriptional

repression activity of CRY2:PER2 is not exerted by either CRY2 or PER2 individually but rather the transcriptional repression activity is exerted by the CRY:PER2 complex together. These findings agree with the other studies which suggest that CRY2 alone is not sufficient for transcriptional repression of BMAL1:CLOCK activity.[102] Moreover it is shown that conserved residues on PER2 determine the CRY1 binding surface and the PER2 is essential for CRY specific repression.[103] The results presented in [102] deviate from our findings that CRY2 can directly bind the BMAL1:CLOCK complex. Their results indicate that PER2 acts as a scaffold between BMAL1 and CRY2 and essential for their interaction. This deviation between the results might arise from the difference between the two experimental approach as our system is in vitro whereas the experiments in [102] are performed *in vivo* with the knockout mice. Therefore in our system the endogenous PER paralogs (preferably PER1) might favor the association between the CRY2 and BMAL1 proteins, although the interaction between the ectopically expressed CRY2 and PER2 is disrupted.

Moreover the disruption of PER2 interaction through mutagenesis of R501 and K503 residues did not alter the nuclear localization pattern of CRY2 indicating that PER2 interaction is not essential for CRY2 nuclear translocation. However the disruption of transcriptional inhibition might arise from aberrant PER2 translocation as PER2 nuclear translocation is mainly dependent on CRY2 interaction.[103]

Opposing to our findings, another recent study maps the interaction hot spot of CRY2 to G354 which abrogates PER2 binding and decreases BMAL1 binding.[100] Moreover it is claimed that the deletion of C-terminus domain does not alter the transcriptional repression capability of CRY1.[95] However, the Cterminus deletion of the mutant allele results in a more severe loss of transcriptional repression activity. Therefore this implicitly indicates that the C-terminus have an unattributed function in the transcriptional feedback loop and the loss-of-function mutant can be attributed to abrogation of other regulatory roles of CRY2 such as loss of protein stabilization and loss of post-translational modifications rather than direct loss of interaction with other circadian clock proteins as the domains of interaction has been mapped to C-terminus

with PER2 in *Xenopus*. [94] It is also reported that the activity of CRY proteins are modulated by post-translational modifications in C-terminus, which in turn regulate the circadian clock function.[104, 105] Therefore the contradiction between these results might be due to the modulatory functions of C-terminus, C-terminus either affects interaction with mPER2 or it modulates some prerequisite modifications.

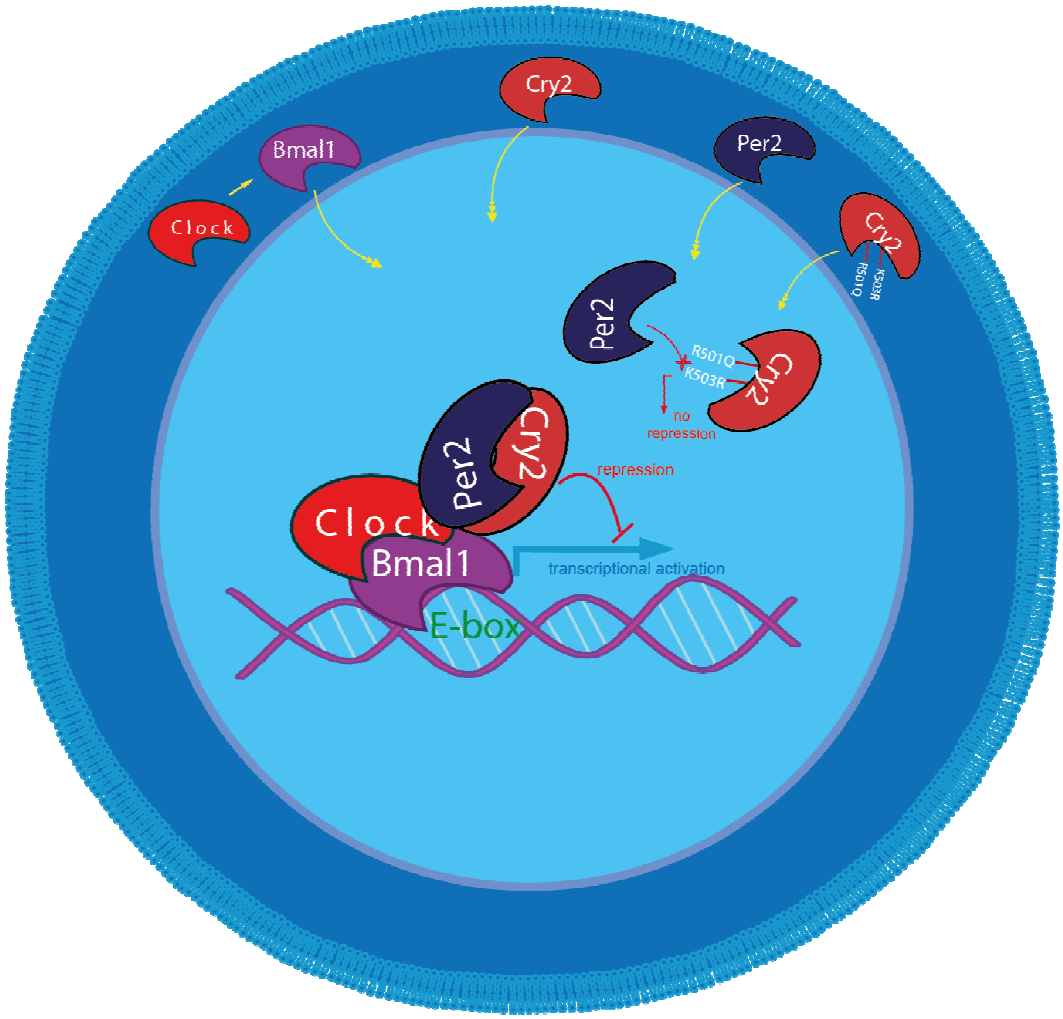
Furthermore our study is extended on elucidation of BMAL1 interaction hot spots of CRY2. Through C-terminal deletion mutants it was observed that the BMAL1 interaction capability of CRY2 has not been altered suggesting that the interaction with BMAL1 and PER2 is mediated through separate domains on CRY2 in agreement with our previous results. Previous research reports indicate that CRY2 interacts with BMAL1 through interdomain loop structure in zebrafish.[106] In this study we have shown that the first 200 amino acid portion (D1) interacts with BMAL1 comparably with the full length protein through mammalian two hybrid assay as well as the succeeding protein domain extending till the C-terminus extension. The hot spot of interaction on D1 is mapped to F171 amino acid residue. This residue is located in the interdomain loop of the CRY2 through analysis of the *in silico* three dimensional model. Moreover this residue is on the surface that is aligned with the DNA in *Drosophila* photolyase. This might imply that the CRY2 interacts with the DNA bound BMAL1 to induce transcriptional repression. However it was previously shown that CRY2 does not alter the binding affinity of BMAL1:CLOCK heterodimer to DNA thereby suggesting a different mechanism for repression. As the CRYPTOCHROME family of proteins evolved from the bacterial photolyase, it is highly likely for these proteins to exert DNA binding activity without showing DNA repair activity, but in turn these proteins might compete with the BMAL1:CLOCK complex to bind the E-box sequences.

Furthermore the loss-of-function mutations on the D2 are mainly clustered along the N-terminus. This also implies that the BMAL1 binding domain of CRY2 is mainly dictated by the interdomain loop structure formed by the C-terminus of D1 and N-terminus of D2. Therefore mammalian two hybrid studies with constructs encompassing

only this region should be implemented to deduce if the interdomain loop structure is solely responsible for mediating the interaction between CRY2 and BMAL1.

Moreover the mutations on D2 mostly include a leucine to proline substitution which induces turns in the structure that distorts not only the local structure but also the global structure of the protein. Therefore the loss-of-function mutations could be due masking of the binding surface due to disruptive alterations through proline substitution. Therefore the contribution of proline mutations should be investigated with preferably alanine mutations that do not have disruptive effect on the secondary structures of the protein. Moreover the high incidence of the proline substitutions implicitly indicates that the D2 binding surface is also substituted by several residues that lie apart from each other in the primary structure. Hence this question should further be addressed by a degenerate oligonucleotide based reverse genetics approach to identify residues on the interdomain loop structure that have direct effect on interaction with BMAL1. Furthermore the contribution of BMAL1 interaction to CRY2 specific repression should be elucidated to conclude a possible explanation for the mechanistic details of the CRY2's role in the circadian clock.

A



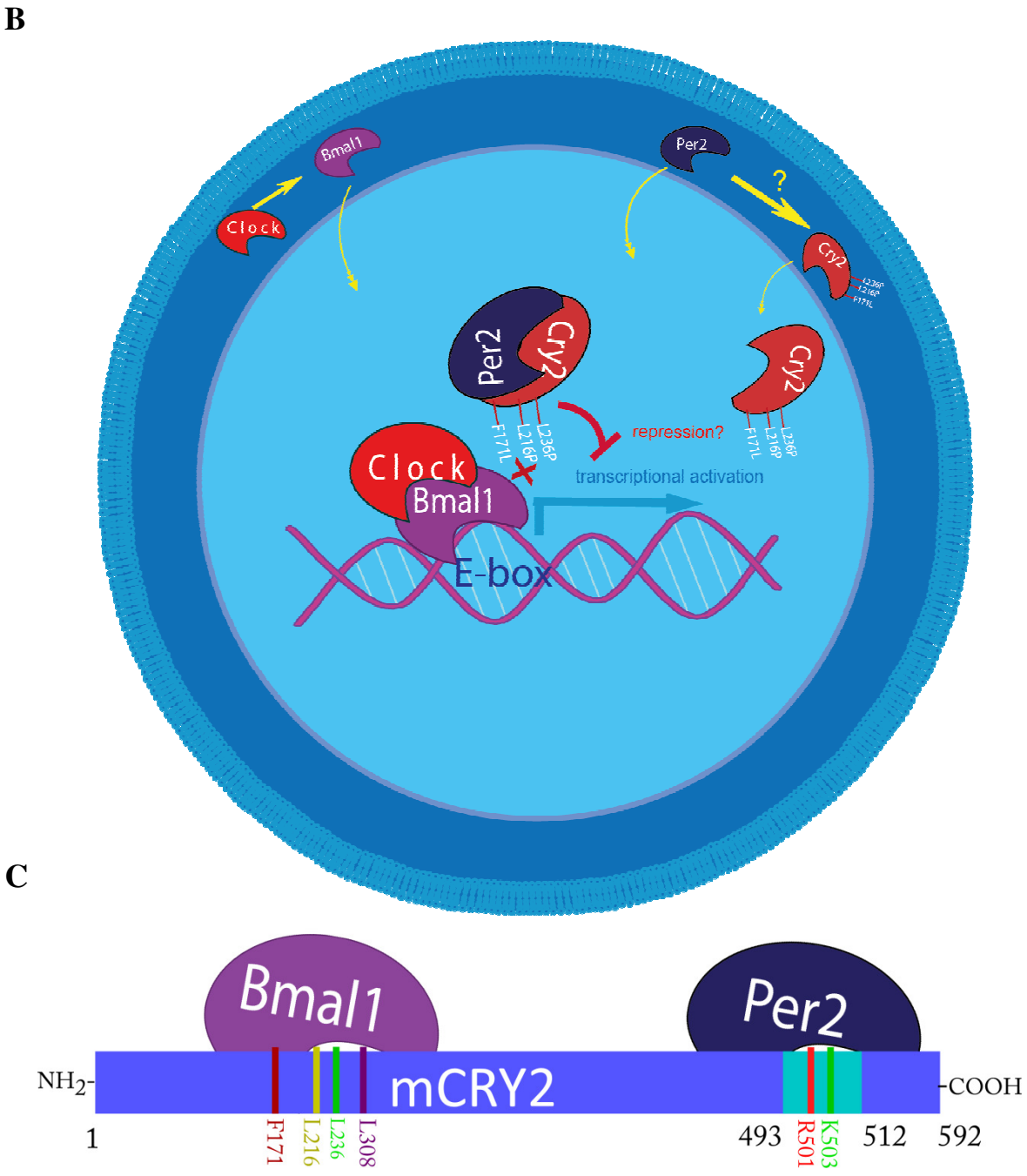


Figure 5.1- Summary of the loss-of function mutations that alter interaction patterns in the core circadian clock mechanism. Evolutionarily conserved amino acids on the C-terminus of CRY2 (R501 and K503) dictate PER2 binding and in turn mediate the CRY2 specific repression of the BMAL1:CLOCK transcriptional activity.(**A**) BMAL1 binding surface on CRY2 is complex, but altered due to F171,L216 and L236 mutations. However the role of BMAL1 interaction on the CRY2 specific repression remains elusive. (**B**) General overview of the regions on mCRY2 that mediate mPER2 and mBMAL1 interaction presented in this thesis work **(C)**

APPENDIX

Transient transfection of HEK293T cells using calcium phosphate co-precipitation

1. Split cells the night before to give ~60% confluence at the day of transfection. The culture should not be overconfluent or subconfluent as this will lead to dramatic decrease in transfection efficiency and very low protein expression respectively.

2. For 10 cm plate, add 10µg DNA to ddH_2O (250 µl total) in 1.5-ml sterile tube, then add 250 µl 0.5M $CaCl_2$ dropwise into diluted DNA. Briefly vortex the solution and incubate 10 minutes at room temperature.

3. Aliquot 500 µL of 2X HBS (274 mM of NaCl, 10 mM KCl, 1.5mM Na_2HPO_4, 50 mM HEPES) for each plate to be transfected in the meantime. Add the $CaCl_2$-DNA solution dropwise into 2XHBS while vortexing slowly (setting node 2).

4. Incubate for 20 minutes and add the precipitate onto the cells dropwise. Very fine, dust-like precipitate should be visible after 4 hours. If the precipitate is visible just after adding the mixture onto cells, the transfection efficiency will be very low.

5. After 16h change the medium and supplement fresh DMEM+10%FBS to keep the cells in healthy state. HEK293T cell line is tolerant to elevated calcium levels, but the growth rate will decrease if the cells are kept in old medium.

6. Harvest cells, 48h after transfection and assay for protein expression.

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