

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**FLEXIBILITY OF C-TERMINUS OF CRYPTOCHROME PROTEIN FOR
PHOTORECEPTOR FUNCTION**

BİHTER MURATOĞLU
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
2018

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**FOTORESEPTÖR FONKSİYONU İÇİN KRİPTOKROM
PROTEİNİ'NİN KARBOKSİ (C) UCUNUN ESNEKLİĞİ**

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MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. NURİ ÖZTÜRK

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2018

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SUMMARY

Cryptochrome (CRY) proteins function as a photoreceptor or a component in the core circadian clock. Light exposure opens the C-terminus of *Drosophila* CRY (dCRY), which is normally bent towards the FAD-binding domain. This opening makes the masked surface of the FAD-binding domain accessible to JETLAG (JET) and TIMELESS (TIM) proteins for binding. The binding of these proteins to dCRY starts a signaling cascade resulting in the degradation of TIM and CRY. TIM degradation resets the clock while CRY degradation gates the resetting of the clock by disrupting the photoreceptor function.

The C-terminus of dCRY is important for photoreception because its deletion generates a constitutively active form causing arrhythmicity in flies. To determine the flexibility of the C-terminus, we replaced the C-terminus of dCRY with C-termini of Type1, 2, and 4 CRYs from different organisms. Then we expressed these chimeric dCRY variants in fruit fly cell line S2R+. In this cell line, expressed dCRY can be degraded by the proteasome pathway following light exposure and this degradation is followed by immunoblot technique. We measured the light activated degradation of chimera.

We found that the replacement of C-terminus of dCRY with the C-termini from very distant CRYs, even from Type2 CRYs, can support the light-dependent proteolysis of dCRY. We also found that the mutation of an arginine residue in the C-terminus of dCRY, which is conserved in all types of CRYs, does not interfere with the light-dependent proteolysis of dCRY. These findings suggest that C-terminus is very flexible for the light-dependent function(s).

Keywords: Cryptochrome, photoreceptor, structural flexibility, circadian clock.

ÖZET

Kriptokrom (CRY) proteinleri, sirkadiyen saatte fotoreseptör ya da çekirdek osilatör mekanizmasının bir parçası olarak işlev görür. *Drosophila* CRY (dCRY) proteini ışığa mazur kalınca, normalde FAD-bağlama alanına doğru kıvrılarak protein gövdesine yapışık olarak kapalı olan C-terminal ucu gevşeyerek açılır. FAD-bağlanma alanının C-terminalce maskelenmiş yüzeylerinin açılmasıyla JETLAG (JET) ve TIMELESS (TIM) proteinlerine bağlanmasını sağlar. Bu proteinlerin dCRY'ye bağlanması bir sinyal kaskadı başlatır. TIM degradasyonu saati sıfırlarken, CRY bozulması ise fotoreseptörün ortadan kaldırılmasıyla saatin sıfırlanmasını sağlar.

dCRY'nin C-terminali fotoreseptör fonksiyonu için önemlidir. Çünkü C-terminalin silinmesi, sineklerde aritmiye neden olan konstitütif aktif bir form oluşturur. dCRY'nin C-terminalinin esnekliğini belirlemek için, dCRY'nin C-terminalini farklı organizmalardan Tip1, 2 ve Tip 4 CRY'leriyle değiştirdik. Daha sonra meyve sinek hücre hattı S2R+'daki bu rekombinant kimerik dCRY varyantlarını eksprese ettik. Bu hücre hattında, eksprese edilen dCRY, ışığa maruz kalmanın ardından proteazom yolağı tarafından degrade edilebilir ve bu rekombinant dCRY üzerindeki etiketlere karşı antikolar kullanılarak immüno blot tekniği ile takip edilir. Kimeraların ışıkla aktive olan degradasyonunu ölçtük.

Uzak akraba CRY'lerin C-terminallerinin, Tip2 CRY'ler de bile dCRY'nin ışık-bağımlı proteolizi destekleyebildiğini gösterdik. CRY'nin tiplerinde korunmuş olan Arjinin amino asidinin mutasyonunun bile dCRY'nin ışık-bağımlı proteolizini engellemediğini de bulduk. Bu bulgular C-terminalin ışık-bağımlı fonksiyon(ları) için çok esnek olduğunu göstermektedir.

Anahtar kelimeler: Kriptokrom, fotoreseptör, yapısal esneklik, sirkadiyen saat.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
c.	: chimeric
C-	: Karboksi
mW/cm ²	: Miliwatt per centimeter squared
μg	: Microgram
μl	: Microliter
APS	: Amonium persulfate
β-Gal	: β-Galactosidase
BLUF	: Blue-Light Using FAD domain
BRWD3	: Bromodomain and WD repeat domain containing 3
CRY	: Cryptochrome
CRYΔC	: DeltaC Cryptochrome
dCRY	: <i>Drosophila</i> Cryptochrome
DpCRY1	: <i>Danaus plexippus</i> Cryptochrome 1
DpCRY2	: <i>Danaus plexippus</i> Cryptochrome 2
FAD	: Flavin adenine dinucleotide gel electrophoresis
HsCRY2	: <i>Homo sapiens</i> Cryptochrome 2
JET	: JETLAG
LOV	: A Light-oxygen-voltage-sensing domain
LB	: Luria bertani
PCR	: Polymerase chain reaction
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide
TcCRY2	: <i>Tribolium castaneum</i> Cryptochrome 2
TIM	: Timeless
TTFL	: Transcription-Translation Feedback Loop
ZfCRY4	: <i>Zebrafish</i> Cryptochrome 4

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1. INTRODUCTION

The subject of this thesis is understanding the flexibility of photosensitivity of circadian clock in C-terminus of animal Type 1, Type 2 and Type4 CRYs in Type 1 *Drosophila melanogaster* CRY that are important for photoreception.

The C-terminus in all animals is different from each other with regards to length and sequence. The C-terminus of Type 4 ZfCRY4 (*Danio rerio*-zebra fish) and Type 2 TcCRY2 (*Tribolium castaneum*-red flour beetle) are required for circadian core oscillator protein whereas Type 1 DpCry1 (*Danaus plexippus*-monarch butterfly) and Type 1 dCRY (*Drosophila melanogaster*-fruit fly) are required for circadian photoreceptor protein.

1.1. The Purpose, Contribution and Content of Thesis

We aimed to show the flexibility of the C-terminus of dCRY. Therefore, we will replace the C-terminus of dCRY with C-termini of Type 1, 2, and Type 4 CRYs from different organisms. Then we will express these recombinant chimeric dCRY variants in fruit fly cell line S2R+. In this cell line, expressed dCRY can be degraded by the proteasome pathway following light exposure and this degradation will follow by immunoblot technique using antibodies against tags on recombinant dCRY.

We expect to show that the replacement of C-termini from other species can support chimeric dCRY degradation and it has the great flexibility of dCRY and even a mutation of the arginine residue which is conserved in all types of CRYs does not affect dCRY function.

Chapter 2 gives detailed information about circadian clock mechanism and animal cryptochromes. Chapter 3-4 explain all experimental procedures and materials. Chapter 5 presents the results. The thesis is finalized in chapter 6 by giving the discussion part.

2. LITERATURE REVIEW

2.1. Circadian Clock

Circadian rhythms are daily oscillations in biological events as a manifestation of an internal clock oscillation with a periodicity of about 24 hours. The circadian is derived from the Latin words; *circa* and *dies* that means *about a day*. Organisms can be active during night (nocturnal), during the day (diurnal) or in the twilight of dawn and dusk (crepuscular) depending on their circadian clock.

In animals, the circadian clock regulates many physiological events such as body temperature, sleep duration, hormone levels and blood pressure. In mammals, the circadian clock is generated by a transcription-translation feedback loop (TTFL) consisting of a positive and negative limb. While CLOCK and BMAL1 transcription factors make the positive limb of the TTFL, Period (PER) and Cryptochromes (CRYs) are circadian clock-regulated genes and their products form the negative limb of the TTFL. PER1, PER2, CRY1, and CRY2 which are translated products of *PER* and *CRY* genes translocate into the nucleus where they inhibit CLOCK-BMAL1 transcriptional activity. The feedback loop generates a molecular oscillation in the expression of circadian clock-regulated genes (CCGs) through the regulation of BMAL1-CLOCK transcriptional activity. In this way, the synchronous oscillation of the cellular molecular clocks in cells generates the circadian clocks of tissues and organs [1]. In fruit fly *Drosophila*, circadian clock is also generated by a TTFL as in mammalian circadian clock, however this time there is some difference at the molecular components.

Drosophila TTFL involves CLOCK (CLK) and CYCLE (CYC) transcription factors in the positive limb; PER (PER) and TIMELESS (TIM) in the negative limb. CLOCK (CLK) and CYCLE (CYC) generate a heterodimeric complex and induces the transcription of CCGs including PER and TIM. During the night, the translated products of PER and TIM genes accumulate and form a heterodimer. Newly formed PER/TIM complex then translocates into the nucleus in order to inhibit further CLK–CYC transcriptional activity. Moreover, PER and TIM are consistently modified by phosphorylation during

2.1.1. Cryptochromes

The term “cryptochrome” originates from the term from Greek “cryptic”, hidden and “chrome” pigment word [6]. In research with plants, it was found out that Cryptochrome (CRY) proteins are responsible for growth and development and their structurally relevant photolyase proteins are involved in light dependent DNA repair of UV-induced dimers[7],[8]. After discovery of two proteins with no DNA repair activities in humans, they were shown that they are either blue-light photoreceptors such as in *Drosophila* or a molecular regulators of the core circadian clock such as in humans [9].

Cryptochromes (CRYs) are members of a family of flavoproteins called Cryptochrome-Photolyase Family (CPF). These proteins use flavin adenine dinucleotide (FAD) as cofactors and some members might have a second chromophore mostly in the form of pterin derived methenyltetrahydrofolate (MTHF). This second chromophore is called photoantenna, and absorbs the light and gives this energy to FAD in order to increase reaction efficiency by fluorescent resonance energy transfer (FRET) mechanism [10],[11].

CRYs have also been identified in bacteria, archaea, plants and animals. Animal cryptochromes also are classified as photosensory or clock proteins [12] or Type 1, 2 and 4 [12]. In animals and plants, Cryptochrome play a role as signaling molecules, which regulate several biological responses. Many scientists also suggest that CRYs may be magnetoreceptors in migratory birds [7],[13],[12]. However, the role of candidate CRYs in bacteria is not known.

In the very basic way, a newly identified protein can be called CRY if it doesn't have light-dependent DNA repair activity [14]. Strong UV light caused a selective pressure in the ocean's life and early metazoans abstained from irradiation by dipping into the oceans during the day. The development of blue-light photoreception is not accidental in an aquatic ecosystem as only blue-light can diffuse into depths in water. This has provided an optimal environment for the detailing of blue-light photoreception and ancestral relationship with circadian rhythm [12]. Therefore, blue-light from the sun has provided the driving force for the co-evolution of these blue-light photoreceptors and circadian clocks.

2.1.2. Animal Cryptochromes

A detailed description of animal cryptochromes classification which are used in this thesis are presented below.

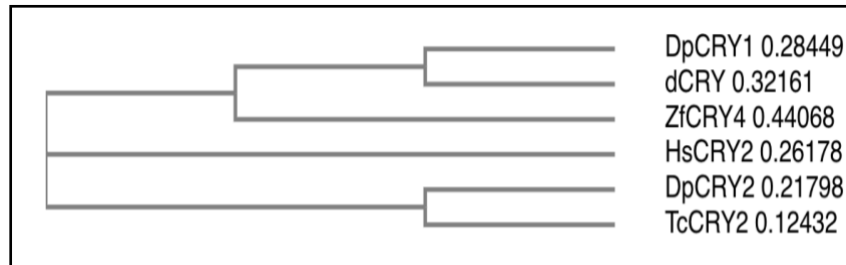


Figure 2.2: Phylogeny of the study's animal Cryptochromes.

Branch lengths are shown Cladogram. Alignment is performed using Multiple Sequence Alignment (MSA) Tool which has MUSCLE, Phylogenetic tree is created by EMBL-EBI Simple Phylogenetic tool using Neighbor joining methods. DpCRY1, *Danaus plexippus* (monarch butterfly) CRY1; c.CRY, *Drosophila* CRY; ZfCRY4, *Zebrafish* CRY4; HsCRY2, *Homo sapiens* (human) CRY2; DpCRY2, *Danaus plexippus* (monarch butterfly) CRY2; TcCRY2, *Tribolium castanum* (red flour beetle) CRY2.

2.1.2.1. Type 1 and Type 2 Cryptochromes

Firstly, animal cryptochromes were classified as Type 1 and 2. However, it was later discovered that some insects have a Type2 CRY either alone or together with a Type1 CRY. Whereas Type1 CRYs act as a photoreceptor for resetting of the circadian clock, Type2 CRYs function in the core clock instead of the photoreception [15].

- *Drosophila melanogaster* (fruit fly) CRY

Three types of photosensory flavoproteins in organisms are identified which are the photolyase/CRY family, the LOV domain proteins like phototropin, and the BLUF domain proteins like the photoactivated adenylyl cyclase. While photolyase realizes catalysis via light-induced cyclic electron transfer, LOV and BLUF domain proteins start photosignaling via a light-induced conformational change. In *Drosophila*, dCRY is the only CRY protein and the main photoreceptor protein for the resetting of the circadian clock .

It is now widely accepted that blue-light induces an important conformational change in the C-terminus of dCRY and this structural change allows binding of E3 ligases BRWD3 and JET onto dCRY. Light-induced conformation change also induces the binding of dCRY to TIM protein. Therefore, in the light-activated photoreceptor complex, JET ubiquitylated TIM which leads to the degradation of TIM and the resetting of the circadian clock by removing PER-TIM dependent inhibitory effect on CLK-CYC activity. After TIM degradation, dCRY is also degraded following BRWD3 mediated ubiquitylation and this desensitizes the photoreceptor by removing dCRY from the system. In this way, the circadian clock resetting is gated by dCRY (Fig. 2.3 is modified from [5]).

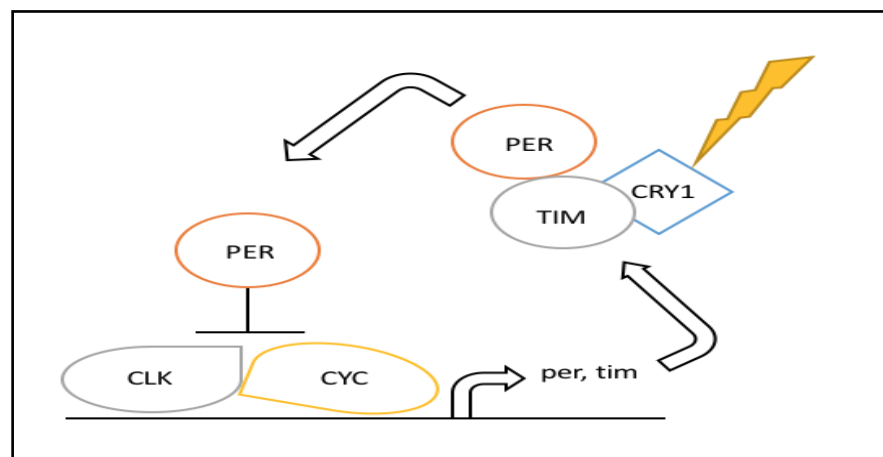


Figure 2.3: Clockwork mechanism of the *Drosophila melanogaster*.

- *Danaus plexippus* (monarch butterfly) CRY1 and CRY2

DpCRY1 and DpCRY2 have been identified in *Danaus plexippus* (monarch butterfly). Homology search has revealed that DpCRY1 shared high sequence homology with dCRY, however, DpCRY2 shared high sequence homology with mammalian cryptochromes [16].

Sancar and colleagues assessed DpCRY1 protein *in vitro* for photoreduction and *in vivo* for photosignaling as measured by light-induced DpCRY1 degradation [17] and Hsu *et al.* tested the relative *in vivo* photosensitive and transcriptional repressor activities of these proteins with expression in *Drosophila* Schneider 2 cells. DpCRY1 supports light-induced photoreceptor activity and DpCRY2 plays a role as transcriptional regulation of the core clock in a similar way to mammalian

cryptochromes. With the aforementioned studies, DpCRY1 belongs to photosensitive animal cryptochromes and is also classified as a *Drosophila*-like [16] or insect-like [18] animal cryptochrome (Fig. 2.4 is modified from [5]).

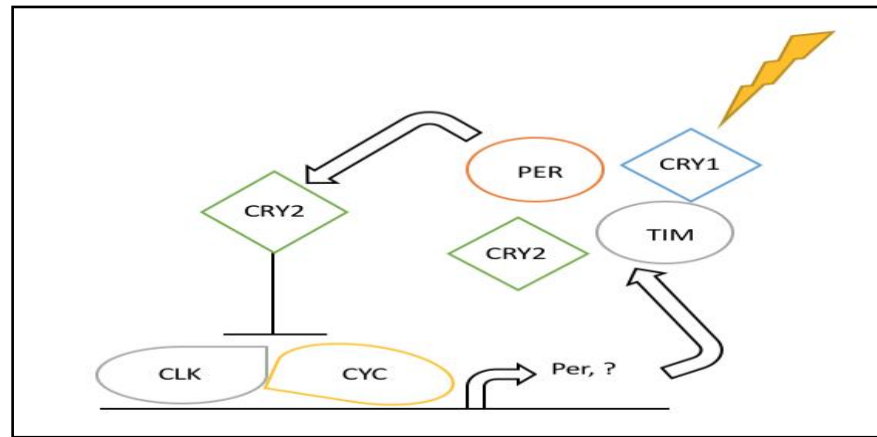


Figure 2.4: Clockwork mechanism of the *Danaus plexippus*.

- *Homo sapiens* (human) CRY2

Human CRY2 (HsCRY2) and CRY1 function in the core circadian clock as classical mammalian circadian clock [19]. Even though, it was shown that human CRY2 is involved in the magnetoreception, a light-dependent photoreception for the HsCRY2 could not be shown at the molecular level (Fig. 2.5 is modified from [1]).

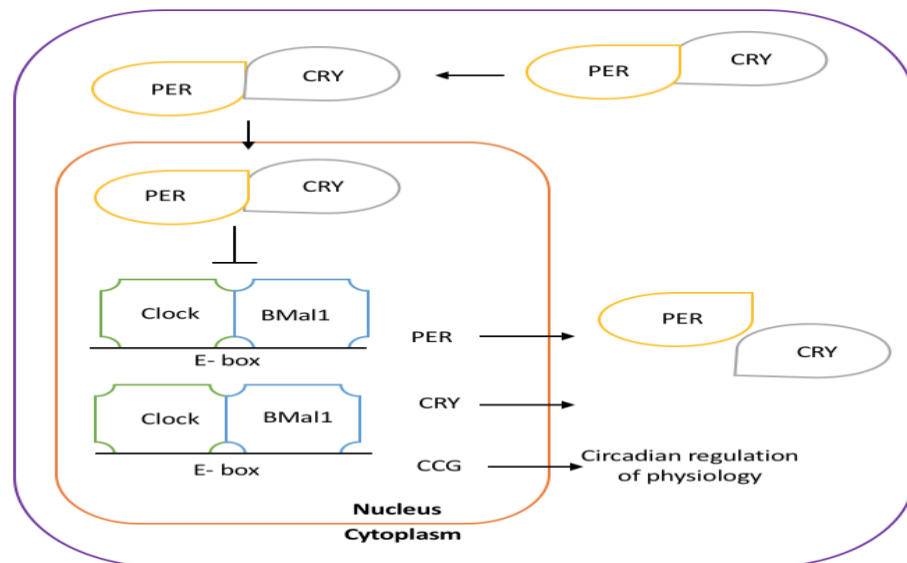


Figure 2.5: Clockwork mechanism of the *Homo sapiens*.

- *Tribolium castaneum* (red flour beetle) CRY2

Yuan et al. suggested that TcCRY2 proteins are not light sensitive in S2 cells, and it functions in the core clock. As CRY1 does not exist in *T. castaneum*, the exact mechanism of light-dependent TIM degradation is not known [5], (Fig. 2.6 is modified from [5]).

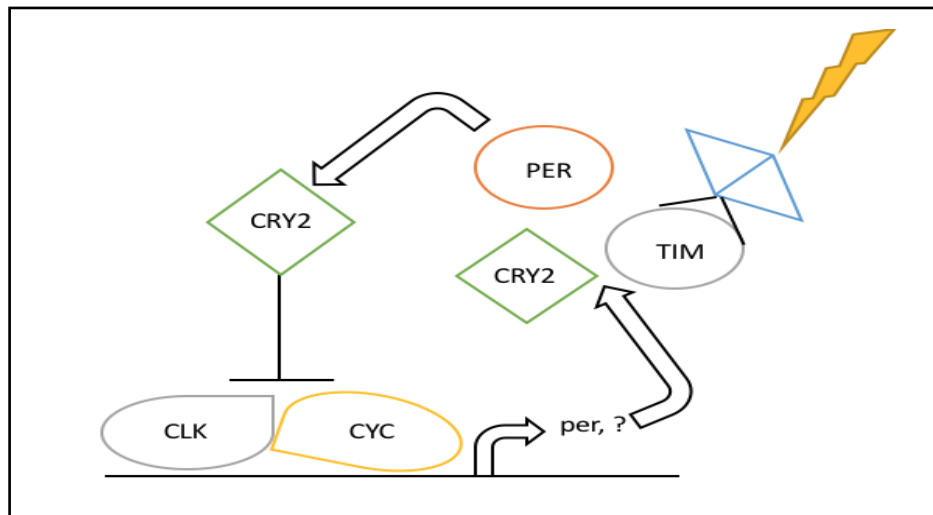


Figure 2.6: Clockwork mechanism of the *Tribolium castaneum*.

2.1.2.2. Type 4 Cryptochromes

- *Danio rerio* (zebrafish) CRY4

Several phylogenetic analysis showed that zebrafish have multiple copies of CRY genes including mammalian-like (Type 2) ones [20].

In zebrafish, ZfCRY4 which is a member of Type 4 CRYs found in some vertebrates such as birds, frogs, turtles and some fish, could be a circadian photoreceptor because it can be purified with stoichiometric amounts of light-sensitive FAD cofactor. On contrary to Type 1 CRYs such as dCRY, ZfCRY4 is not degraded by proteolysis in either S2 cells or zebrafish cell lines after light exposure [20].

ZfCRY4 does not have transcriptional repressor activity while ZfCRY1 has high activity. ZfCRY4 also does not associate with CLOCK and BMAL [22].

2.2. Role of the C-terminus of dCRY for Light-induced Proteolysis

Various CRYs have aligned by using bioinformatics alignment tool show that animal and plant CRYs have variable extended C-terminal. They are indicated to functional diversity because of their variable C-termini extensions. Depending on species, their extensions have ranged from 20 to 300 amino acids (Figure 2.7).

Studies of *Drosophila melanogaster* CRY protein have revealed that C-terminus of dCRY is important for photoreceptor function and mediate blue-light signals [15],[23],[7]. C-terminal 24 amino acids of dCRY is important for the light-induced resetting signal. Blue-light induces a conformational change in the dCRY resulting in opening or relaxing of the C-terminus which is normally bended over the surface of FAD-binding domain in dark. Therefore, it is assumed that the bended C-terminus blocks or masks the binding sites of TIM, JET and/or BRWD3. Upon light exposure, relaxed C-terminus exposes the TIM, JET or BRWD3 binding sites which ends in the an active photoreceptor complex. In this photoreceptor complex, JET ubiquitylates TIM for proteolysis and BRWD3 ubiquitylates dCRY for proteolysis. Degradation of TIM causes the resetting of the circadian clock while degradation of dCRY ensures that photoreceptor complex is dissolved to prevent the resetting of the circadian continuously.

Therefore, the deletion of the C-terminus of dCRY generates a constitutive active form which active under dark or light conditions and cause arrhythmicity in *Drosophila melanogaster* by sending resetting signal continuously.

In this study, we decided to analysis the structural flexibility of the C-terminus of dCRY because its function is seemingly to block the binding sites of dCRY's partners under darkness.

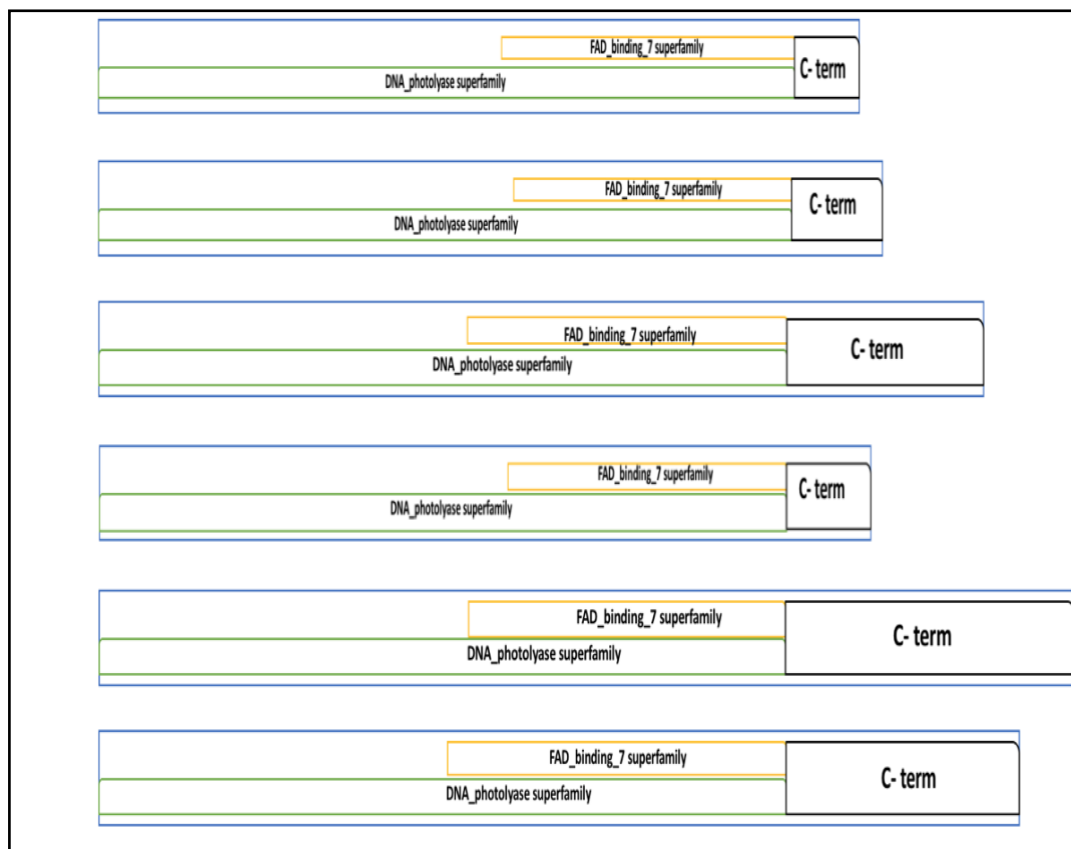


Figure 2.7: Alignments of primary structures of the animal Cryptochromes. The animal cryptochromes consists of a photolyase homology region (PHR) which covers, FAD-binding domain and a variable C-terminus. The C-termini of lengths of the CRYs which are used in this thesis are as follow: dCRY (first), 24 amino acids; DpCRY1 (second), 30 amino acids; DpCRY2 (third), 57 amino acids; TcCRY2 (fourth), 29 amino acids; HsCRY2 (fifth), 85 amino acids; ZfCRY2 (sixth), 69 amino acids.

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1.: List of kits and reagents.

Nucleospin Plasmid Kit	740588.50, Macherey Nagel, Germany
PCR clean-up Gel extraction Kit	740609.50, Macherey Nagel, Germany
Protease Inhibitor Cocktail Tablets	S8830-2TAB, Sigma-Aldrich, USA
Protein Molecular Weight Marker	P7712S, New England Biolabs, USA
PRONASAFE Nucleic Acid Staining	CK130, CONDA, S.A
DNA Ladder	N3232S, New England Biolabs (NEB), USA
DNA Loading Dye	B7021S, New England Biolabs (NEB), USA

3.2. Buffers and Solutions

Table 3.2: List of buffers and solutions.

10X SDS Running Buffer	250 mM Tris-Base (pH:8.3) 1.90 M Glycine 1% SDS
100X Penicillin/Streptomycin	15140-122, Gibco Invitrogen, USA
10X PBS	BE17-517Q, Lonza, Belgium
10X TBE	108g Tris base 55g Boric acid 40 ml 0.5M EDTA (pH 8.0) in 1 L ddH ₂ O
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X TBST	1X TBS 0.1% Tween-20
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol

Table 3.2: Continued.

4X Protein Loading Dye (Laemmli)	250 mM Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 20% β -mercaptoethanol 0.008% Bromophenol Blue
5% Blocking Solution	5% non-fat dry milk in 1X TBS-T
Schneider's Insect Medium	S0146, Sigma-Aldrich, USA
Fetal Bovine Serum (FBS)	10270-076, Gibco Invitrogen, USA
LB Agar	LBP04, Caisson Labs, USA
LB Broth Medium	LBP03, Caisson Labs, USA
0.05% Trypsin-EDTA	25300-054, Gibco Invitrogen, USA

Table 3.3: SDS-PAGE gel recipe.

7% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.775	5.55	8.325
40% Acryl:Bis	0.875	1.75	2.625
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	50	100	150
10% APS	50	100	150
TEMED	4	8	12
10% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.4	4.8	7.2
40% Acryl:Bis	1.25	2.5	3.75
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006
5% stacking gel	2 ml	4ml	6 ml
ddH ₂ O	1.46	2.92	4.38
40% Acryl:Bis	0.25	0.5	0.75
1.5 M Tris pH8.8	0.25	0.5	0.75
10% SDS	0.02	0.04	0.06
10% APS	0.02	0.04	0.06
TEMED	0.002	0.004	0.006

3.3. Antibodies

Table 3.4: List of antibodies used in this study.

Name	Dilution	Source
Anti-V5	1:5000	V8012, Sigma-Aldrich
Anti-mouse, HRP	1:5000	#7076, Cell Signaling Technology

3.4. Equipment

Table 3.5: List of equipments used in this study.

Agarose Gel Electrophoresis	1704406, Bio-Rad, USA
Centrifuges	521-1647 refrigerated, Micro Star 17R, VWR, USA 521-1646 Micro Star 17, VWR, USA 521-2844 Mini Star, VWR, USA NF400-Nuve , 02-1827
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, Bio-Rad, USA
Incubator (CO ₂)	Thermo-Scientific, USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad, USA
Inverted Microscope	(Nikon, Eclipse, E100)
Laminar Flow Cabinet	Hera-Safe Class II Cabinet, Thermo Scientific, USA
Blue light lamb	MLP 58 Multiple ray lamb, UVP, Canada
Power Supply	Bio-Rad, USA
PCR Thermal Cycler	Bio-Rad, 41798
TransBlot Transfer System	1704155, Bio-Rad, USA
NanoDrop	206-26300-48, Shimadzu Biotech
Shaker	Standart Analog shaker, VWR, USA
Vortex	Scientific Industries, USA
Water Bath	Wisd, Germany

4. METHODS

4.1. Molecular Cloning

To replace the C-terminus of dCRY with C-termini from other CRYs, we used classical molecular cloning methods based on the restriction enzyme digestion of PCR products and ligation as explained in detail below. Basically, we amplified dCRY with C-terminus missing (Delta dCRY or Δ dCRY) from full-length dCRY cDNA and inserted an EcoRV site to the C-terminus encoding sequence in order to exploit in further subcloning of C-termini from other CRYs as a fusion to the Δ dCRY.

4.1.1. Construction of Chimeric Cryptochrome Proteins

First, Δ dCRY was generated by molecular cloning method. dCRY was amplified from pAc5.1.dCRY plasmids using primers cDmCRY_F and DelDmCry_R (see Table 4.1 for the sequence information). DelDmCry R had an EcoRV site which converts serine residues to threonine which enabled us to exploit this restriction enzyme for subcloning purposes. We therefore called modified proteins as a chimeric dCRY to differentiate from the wildtype dCRY protein. The Δ dCRY cDNA was inserted into EcoRI-EcoRV sites of pAc5.1.V5/His A vector (ThermoFisher Scientific, USA). C-terminus coding sequences were inserted into EcoRV-XbaI sites of pAc5.1.V5-dCRY Δ C plasmid. All the starting plasmids used in this study were described before [25], [21], [24], [4].

- Generation of pAc5.1HisV5-c.dCRY Δ C

Drosophila CRY was amplified from pAc5.1.HisV5-dCRY plasmids. PCR products were excised from 1% agarose gel, and digested with EcoRI and EcoRV restriction enzymes for 4 hours at 37°C. pAc5.1.V5/HisA plasmid was also digested with EcoRI-EcoRV restriction enzymes, and linearized plasmids were isolated from 1% agarose gel using NucleoSpin Gel and PCR Cleanup from Macherey & Nagel. EcoRI-HisV5-dCRY Δ C-EcoRV fragment was inserted into EcoRI-EcoRV sites of digested pAc5.1.V5HisA by T4

ligase. Ligation products were transformed into *Escherichia coli* strain DH5a, and selected on LB-Agar plates supplemented with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.dCRY

In order to test and show that the change of serine to threonine did not affect the dCRY proteolysis we generated the full-length dCRY by subcloning C-terminus encoding sequence from pAc5.1.V5His-dCRY construct into EcoRV-XbaI site of pAc5.1.HisV5cdCRY Δ C. These plasmids were also called chimeric to differentiate from original pAc5.1.HisV5dCRY constructs because it contained Serine to Threonine modification at codon 518. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.DpCRY1

The C-terminus encoding sequence of DpCRY1 was amplified from pA5.1.DpCRY1-V5His plasmid using relevant oligo pairs listed in Table 4.1, and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.DpCRY2

The C-terminus encoding sequence of DpCRY2 was amplified from pA5.1.DpCRY2-V5His plasmid using relevant oligo pairs listed in Table 4.1, and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.HsCRY2

The C-terminus encoding sequence of HsCRY2 was amplified from pcDNA4.HsCRY2 plasmid using relevant oligo pairs listed in Table 4.1, and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.TcCRY2

The C-terminus encoding sequence of TcCRY2 was amplified from pA5.1.TcCRY2-V5His plasmid using relevant oligo pairs listed in Table 4.1, and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.ZfCRY4

The C-terminus encoding sequence of ZfCRY4 was amplified from pcDNA4.flag-ZfCRY4 plasmid using relevant oligo pairs listed in Table 4.1, and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin.

- Generation of pAc5.1His.V5-c.dCRY.Rless

The C-terminus encoding sequence of dCRY was amplified from pA5.1.dCRY1V5His plasmid using cDmCRY_F and RlessdCRY_R oligos (see Table 4.1) and cloned into EcoRV-XbaI sites of pAc5.1.HisV5.cdCRY Δ C. The ligation products were transformed into DH5 α and selected on LB-agar plates with 100 mg/l ampicillin. The reverse oligo contained a change at the corresponding sequence of codon 532 which changed Arginine (R) into Lysine (K) residue.

- Oligos for Chimeric Proteins

Table 4.1: List of oligos for chimeric proteins.

Oligo ID	Scale/Purification	5' to 3' Sequence
DelDmCry_R	25 nmol/Desalted	GGGGATATCAGCGAATTTCCGGAGAGAC
cDmCRY_F	25 nmol/Desalted	GCTGATATCCCCGCCGCCGCGATTGCCGAC
cDpCRY1_F	25 nmol/Desalted	AAAGATATCCGCTCCTCCTCACTGCTGTCCGTC
cDpCRY1_R	25 nmol/Desalted	AAATCTAGATTATGTGGTGGTGAGCTCAGGCT
cDpCRY2_F	25 nmol/Desalted	GCTGATATCCTTTTACTTCACCGACAATG
cDpCRY2_R	25 nmol/Desalted	AAATCTAGATTAAATTTTCTTTCCGATGAG
cTcCRY_F	25 nmol/Desalted	AAAGATATCCTACCGAAATGTGGATAATTC
cTcCRY_R	25 nmol/Desalted	AAATCTAGATTATTTCTACTGGGGTTA
cZfCRY4_F	25 nmol/Desalted	AAAGATATCCACCGCAGAACTCACGCGAGATGTA
cZfCRY4_R	25 nmol/Desalted	AAATCTAGATTACATGACCTCTCCGCTGAGCTC
cHsCRY_F	25 nmol/Desalted	GCTGATATCCTACCGGGGACTCTGTCTAC
cHsCRY_R	25 nmol/Desalted	AAATCTAGATCAGGCATCCTTGCTCGGC
RlessdCRY_R	25 nmol/Desalted	GCTCTAGATCAAACCACCGTCCGCCAGCCAGA AGAACTGCTTCACTTCCTCCTCGTTG

4.2. The Motif Analysis of C-termini of Chimeric Cryptochrome Proteins

A multiple sequence alignment (MSA) of the homologous sequences was constructed using MUSCLE and using the Bayesian estimation to calculate evolutionary conservation, and confidence intervals for the conservation scores estimations were obtained.

Consurf software was used to obtain patterns in the C-termini regions of the animal CRYs in *Drosophila melanogaster*. The motif analysis revealed that the Arginine residue of CRY proteins are highly conserved. For this reason, Arginine was converted to Lysine via PCR as described in the generation of pAc5.1.HisV5.c.dCRY.Rless plasmid.

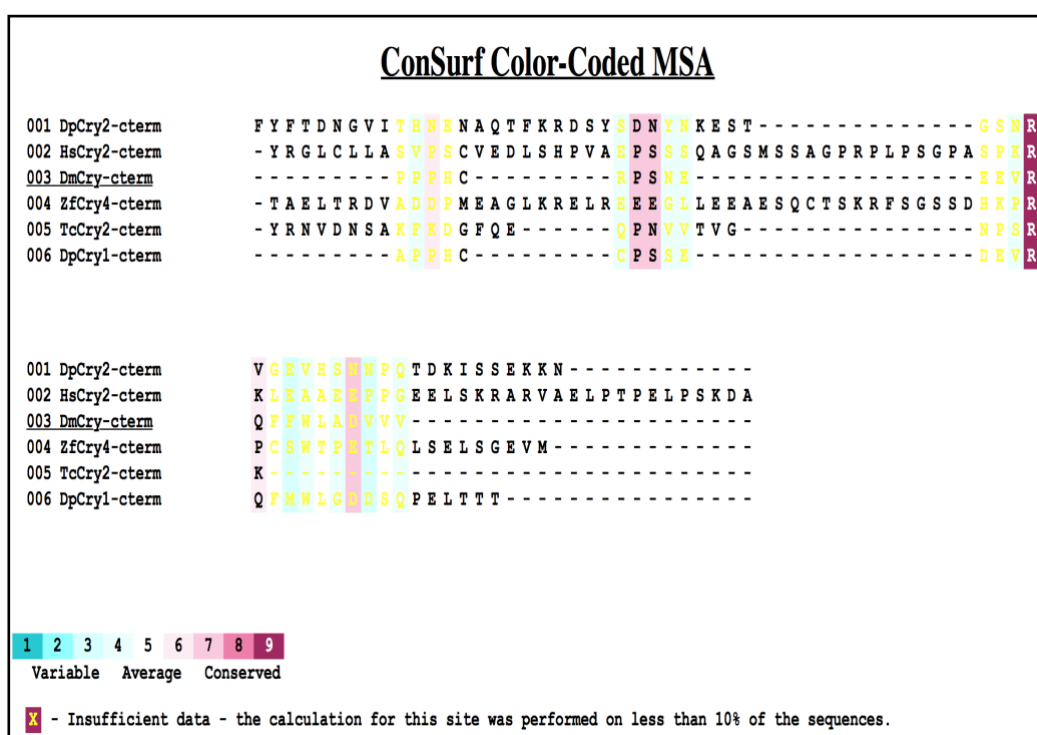


Figure 4.1: The motif analysis of chimeric CRYs. C-termini of cCRYs are aligned by using Consurf. As shown in the fig.4.1, the conservation scores are computed using the Bayesian estimation.

4.3. Restriction endonuclease digestion of DNA

Table 4.2: Restriction endonuclease digestion of DNA.

Restriction Enzyme: XbaI	2 μ l
DNA	5 μ l
XbaI Buffer	5 μ l
Total Reaction Volume	50 μ l
Incubation Time	3-4 hr
Incubation Temperature	37 °C
Restriction Enzyme: EcoRI	2 μ l
DNA	5 μ l
EcoRI Buffer	5 μ l
Total Reaction Volume	50 μ l
Incubation Time	3-4 hr
Incubation Temperature	37 °C
Restriction Enzyme: EcoRV	2 μ l
DNA	5 μ l
EcoRV Buffer	5 μ l
Total Reaction Volume	50 μ l
Incubation Time	3-4 hr
Incubation Temperature	37 °C

4.3.1. Agarose Gel Electrophoresis for the Separation of DNA Fragments

4.3.1.1. Preparing the Agarose Gel

- 0.4 g agarose powder was weighed and it was put into a 250 ml Erlenmeyer flask.
- 40 ml TBE Buffer was added to the flask.
- The agarose was melted in a microwave until the solution becomes clear.
- The solution was cooled to about 50-55°C. 1 μ L of pronasafe (CONDA, S.A) adds to the solution.
- The ends of the casting tray were sealed with two layers of tape.

- The combs were placed in the gel casting tray.
- The melted agarose solution was poured into the casting tray and cool let until it is solid.
- Carefully the combs were pull outed and the tape was removed.
- The gel was placed in the electrophoresis chamber.
- Enough TBE Buffer was added.

4.3.1.2. Running the Gel

- 1 μ L loading dye per 5 μ L sample were added because the dye was 6x DNA loading dye.
- Total sample volume should be 20 μ L.
- 1 kb marker was loaded.
- The voltage was set at $\sim$ 130 V.

4.3.1.3. Imaging the Gel

- To visualize the DNA bands, they were looked under UV light.
- The UV transilluminator was used (ChemiDoc™ XRS+ System).

4.3.1.4. Gel Extraction from Agarose Gel

- A clean scalpel was used excise gel slice which contain the DNA fragment under 366 nm black light exposure. The gel slice was placed into a pre-weighed clean eppendorf tube and weight of the gel slice was recorded.
- Buffer NT1 of NucleoSpin™ Gel and PCR Clean-up was added as much as 3 volumes of gel slice and incubated for 5-10 min at 50 °C heat-block until the slice of gel completely dissolved with very quick vortexing at every two minutes.
- The sample upto 700 μ l was loaded into Clean-up Column which was inserted into a 2-ml collection tube.

- The column was centrifuged for 30 s at 11.000 xg. The liquid in collection tube was discarded and the column was placed back into the tube.
- 700 µL of Buffer NT3 was added into the column and was centrifuged for 30 s at 11.000 x g. The liquid in collection tube was discarded and the column was placed back into the tube.
- The tube was centrifuged for 1 min at 11,000 x g for removing Buffer NT3 completely.
- The column was placed into new 1.5 mL eppendorf tube. 50 µL of ddH₂O was added on the membrane in the column and incubated for 1min at room temperature.
- The tube was centrifuged for 1 min at 11,000 xg, and eluted sample was transferred into a new 1.5-ml microcentrifuge tube.

4.3.2. Ligation: Insertion of Isolated DNA into the Plasmids

- Total reaction was set in a microcentrifuge tube on ice.
- After adding all the components was described above, the tube was vortexed and spined briefly.
- The tube was incubated at 4°C overnight.

Table 4.3: Components of ligation.

Component	10 µL Reaction
T4 DNA Ligase Buffer (5X)	2 µL
Vector DNA	3 µL (50 ng)
Insert DNA	4 µL (150 ng)
T4 DNA Ligase	1 µL

4.4. Bacterial Transformation

- Ultracompetent cells were taken from -80°C freezer and thawed on ice.
- 5 µL of ligation reaction mixture was added into -80 µL of the competent bacterial cells, and incubated for 30 minutes on ice by mixing gently at every 10 minutes.

- Then, the tube was placed into a heat-block set 42 °C for 1 min for heat shock.
- LB medium upto 1 ml was added into the tubes and then incubated on shaker at 37°C for 1 hour.
- Culture was spinned down at 11,000 xg for 1 minute, and supernatant was removed except leaving 100 µl medium on the samples. The pellets were resuspended in the remaining 100 µl medium, and 50 µl of this resuspension was spread onto LB agar plates with ampicillin. LB plates were incubated at room temperature for 5 minutes, and then they were inverted to keep at 37°C for 16 hours to allow grow colonies.

4.4.1. Colony Pick up

- Single colonies were identified and labeled before pick-up.
- Single colonies were taken into 3 ml LB medium containing 100 µg/ml ampicillin by inoculation sterile micropipette tips with the colony and immersing the end of the tip into LB medium. Inoculated LB medium was cultured at 37°C for 16 hours to allow single cell derived bacteria.

4.4.2. Plasmid DNA Purification (Mini and Midi Prep)

This procedure is used to extract plasmid DNA from bacteria.

Miniprep using NM NucleoSpin Plasmid Kit. This protocol was used to analyze candidates colonies by restriction enzyme analysis and sequencing of the plasmids.

- 1-3 ml of overnight LB culture in 15 ml conical tubes was pelleted 4,000 xg for 5 minutes, and supernatant was removed.
- The cell pellet was resuspended completely by vortexing or pipetting up and down in 250 µl of Buffer A1.
- 250 µl of Buffer A2 was added into resuspension, and mixed gently by inverting the tube 6–8 times. The tube was Incubated at room temperature for up to 5 min or until lysate appears clear.

- 300 µl of Buffer A3 was added on the samples and mixed thoroughly by inverting the tube 6–8 times until blue samples turn colorless completely.
- The samples were centrifuged for 5 min at 11,000 x g at room temperature
- A nucleoSpin® Plasmid Column was placed in a Collection Tube (2 mL) and the supernatant from previous step was transferred onto the column. The tubes were centrifuged for 1 min at 11,000 x g, and the flowthrough was discarded and the column was put back into the collection tube.
- 600 µl of Buffer A4 (supplemented with ethanol) was added onto the column and the column tube was centrifuged for 1 min at 11,000 x g. The flowthrough was discarded the column was placed back into the empty collection tube.
- The column in the collection tube was centrifuged for 2 min at 11,000 x g and the column was transferred into a new collection tube (generally into a 1.5-ml microcentrifuge tube after removing the cap).
- 50 µl of 1xTE buffer was added onto the membrane in the column, and incubated for 1 min at room temperature. Then the column was centrifuged for 1 min at 11,000 x g, and eluted plasmid DNA was transferred into a 1.5-ml microcentrifuge tube.

MidiPrep using NM Midi Prep Kit. This protocol was used to isolate the selected plasmids to use in transfection assays and expression in S2R+ cells.

- Bacterial culture (100 ml) was pelleted by centrifugation at 4,000 x g for 10 minutes.
- The cell pellet was completely resuspended in 8 ml of Resuspension Buffer RES + RNase A by pipetting the cells up and down or vortexing the cells.
- Lysis Buffer LYS (8 ml) was added to the suspension and mixed gently by inverting the tube 5 times. The cell lysis was allowed to continue for 5 minutes at room temperature.
- During lysis incubation, a NucleoBond Xtra Column together with the inserted column filter was equilibrated by applying 12 ml of Equilibration Buffer EQU. The equilibration buffer was allowed to pass the column by gravity flow.

- 8 mL of Buffer NEU was added on the lysed suspension and mixed thoroughly by inverting the tube until blue samples turns colorless completely!
- The lysate was simultaneously cleared and loaded onto the column and allowed to pass the column by gravity flow. The column filter and column were washed with 5 ml of the equilibration buffer (Buffer EQU), and then column filter was discarded.
- For the second was, column only was washed with 8-ml of Wash Buffer WASH.
- The plasmid DNA was eluted with 5 ml of Elution Buffer ELU into 30 ml high-speed centrifuge tubes.
- Isopropanol (3.5 ml) was added to the eluted DNA solution and mixed by inverting 3-4 times thoroughly.
- The mixture was centrifuged for 30 min at 21,000 x g, at 4°C.
- After centrifugation, the supernatant was removed. Then, pellet was dissolved with 1 ml of 70 % ethanol, and transferred into a 1.5 ml microcentrifuge tube.
- The resuspension was centrifuge at 17,000 x g for 5 min at room temperature (18–25 °C), and ethanol was completely removed from the tube with a pipette tip. Then, the pellet was allowed to dry at room temperature (18–25 °C).
- Finally, the pellet was dissolved with 1x TE Buffer (10 mM Tris.Cl pH 8.0 and 1 mM EDTA) according to DNA pellet size and the DNA concentration was determined using a spectrophotometer.

4.4.3. Diagnostic Digestion

In order to select the plasmids with right inserts, plasmid DNA from candidates colonies was digested with EcoRV and BamHI restriction enzymes. This digestions releases inserted C-terminal encoding sequences by cutting inside the at the beginning of C-terminus corresponding sequence (EcoRV) and at the plasmid backbone in pAc5.1V5HisA (BamHI). The mixture was set up for each digesting reaction took place in 20 µl reaction volume in a microfuge tube.

Table 4.4: Components of digestion.

Restriction Enzyme: EcoRV	0.5 μ l
Restriction Enzyme: BamHI	0.5 μ l
DNA	2 μ l
Buffer 3.1	2 μ l
ddH ₂ O	15 μ l
Total Reaction Volume	20 μ l
Incubation Time	2-3 hr
Incubation Temperature	37 °C

4.5. Cell Culture

4.5.1. Maintenance of the Cell Lines

- *Drosophila* cell lines were grown at 27°C in the Schneider medium containing 10% FBS and 2.5% Pen-Strep antibiotics.
- S2R+ cells grow semi-adherently, and cells were dislodged by tapping the flask several times.

4.5.2. Transient Transfection of S2R+ Cells

- Appropriate amounts DNA as listed below were added into 75 μ l of a resuspension buffer containing 150 mM NaCl, 10 mM Tris.Cl pH 8.0 and 1 mM EDTA in microcentrifuge tubes.
- For Western blot assays, cells were transfected with several conditions of pAc5.1-V5-TIM (800ng), pAc5.1-V5-JET (800ng), pAc5.1His.V5-cdCRY Δ C (1200ng), pAc5.1His.V5-cdCRY (500ng), pAc5.1His.V5-cDpCRY1 (1200ng), pAc5.1His.V5-cZfCRY4 (1200ng), pAc5.1His.V5-cHsCRY2 (600ng), pAc5.1His.V5-cdCRYRLess (1200ng), β -Gal (200ng).
- PEI was added to diluted DNA solution at 3:1 ratio of PEI:DNA.

- The mixture was incubated for 15 minutes and then was resuspended in the appropriate amount of the fresh medium containing the appropriate density of resuspended S2R+ cells.
- The cells mixed with the transfectant were transferred into 6-well plates and incubated at 27°C.

4.6. Expression of Constructs in S2R+ Cells

To analyze expression levels of cCRYs, both pAc5.1.V5His-cdCRYs and β -Gal plasmids were transiently co-transfected with polyethyleneimine (PEI) in S2R+ cells. 0.5 μ g total DNA for cdCRY, 1.2 μ g total DNA for cZfCRY4, cdCRY Δ C, cDpCRY1 and cdCRY-Rless, 0.6 μ g total DNA for cHsCRY2 and 0.2 μ g total DNA for β -Gal were used for co-transfections. After 24 hours following transfections, cells were resuspended in total 4 ml of complete Schneider's medium, and split into 12-well plates (1 ml into each well) for 0, 45, 90 minute blue-light treatment experiments. Light source was MLP 58 Multiple ray lamp, UVP. At the end of the light exposure at 1 mW.cm⁻² for 0, 45, 90 minutes, the cells were resuspended in 1 ml culture medium under dim light conditions, and transferred in 1.5 ml microcentrifuge tubes.

4.7. Western Blotting (immunoblotting)

4.7.1. Sample Preparation

- Co-transfected S2R+ cells with cCRY(s) and β -Galactosidase were irradiated with 366 nm light at a fluence rate of 1 mWcm² for the 45 and 90 minutes.
- After light exposure, cells were removed from 12 well plate by the aid of pressure of micropipette and were transferred to the properly labeled microcentrifuge tubes.
- The tubes were centrifuged for 3 min at 8500 x rpm.

- The supernatant was discarded and 500 μ l of 1X PBS (optional) was added or the supernatant was not discarded completely from tubes and centrifuge step was repeated for 1 min.
- Then 1X PBS was added, the tubes were centrifuged for 1 min at 8500 x rpm.
- In this step, the supernatant was completely removed from tubes.
- 1X Lysis Buffer which contains 1X Laemmli Buffer added 1% Beta-Mercaptoethanol was added onto pellet according to cell confluency.
- Protein samples were placed at 95 °C for 10 min.

4.7.2. SDS-PAGE Gel Preparation

- SDS-PAGE gel was prepared according to standard protocols.
- Biorad Mini-PROTEAN® Tetra Cell System was used following the manufacturer's instructions. The acrylamide gels were prepared by using the appropriate amounts of buffers as listed in Table 3.3
- After polymerization of the gel, the gel preparation was completed.

4.7.3. SDS-PAGE Gel Electrophoresis

- The gel cassette was removed from the casting system and placed to clamping frame and then the clamping frame was placed into the electrophoresis tank.
- 1X running buffer was poured to fill the wells of the polyacrylamide gel and filled to outside of the frame as well.
- Samples and protein marker were loaded slowly and gel was run in 1X SDS Running Buffer.
- The electrophoresis tank was connected to the power supply.
- SDS-PAGE was run at 80 V for 10 minutes, and then at 120 V until Bromophenol Blue tracking dye (in the Laemmli loading buffer) reaches to the exit of the gel.

4.7.4. Electrotransfer

- After SDS-PAGE was completed, the gels were removed from glasses and were put into the transfer buffer.
- Also, nitrocellulose membrane and filter papers were wet in transfer buffer for 10 min.
- The 2 pre-wet filter papers were placed onto a semi-dry transfer chamber. The nitrocellulose membrane was put on the first filter paper, and then gel was carefully placed onto the membrane. A second filter paper was then placed onto the gel and air bubbles were removed using a roller.
- Then Trans-Blot Turbo Cassette was closed and put into the Trans-Blot® Turbo™ Base system. Semi-dry transfer was performed at 12V for 60 min for 1.5 mm gels.

4.7.5. Blocking

- After transfer, the membrane was incubated in the blocking solution containing 5% w/v milk powder in 1x TBS-T for 1 hour.

4.7.6. Antibody incubation

- The membrane was placed to the 15 mL blocking solution with anti-V5 antibody (3 µl) (Sigma-Aldrich) and incubated overnight.
- The membrane was washed with 1X TBS-T for 5, 15, 5 and 5 min, respectively.
- Then the membrane was incubated with the secondary antibody (3 µl Anti-mouse, HRP-Cell Signaling Technology in 15 ml of blocking solution), and then incubated for 1 hour at room temperature.
- The membrane was then washed with 1X TBS-T for 5, 15, 5 and 5 min, respectively.

4.7.7. Chemiluminescence Imaging

- Protein bands were detected by using homemade ECL (~5 min) which contained 100mM Tris pH8.5, 2.5mM Luminol, 0.4mM p-Coumaric acid, and 2.6 mM hydrogenperoxide.
- The chemiluminescent signals were captured by the aid of ChemiDoc™ XRS+ Imaging Systems (BioRad).
- Exposure time was set from 1 sec to 5 min.

5. RESULTS

5.1. Preparation of Chimeric Expression Vectors

To construct chimeric Cryptochrome (CRY) expression vectors, we first generated pAc5.1HisV5dCRY Δ C plasmid by amplify in HisV5dCRY Δ C coding sequence from pAc5.1.V5dCRY plasmid as described in Methods section. We then amplified C-termini of different CRYs and cloned into pAc5.1HisV5dCRY Δ C plasmid by exploiting EcoRV-XbaI cloning sites both in plasmids and PCR-amplified C-termini. We generated R-less mutant of pAc5.1c.dCRY in the same manner except a mutation at codon 518 of dCRY was inserted by inserting this mutation in the reverse PCR primer. All the expression vectors was confirmed by sanger sequencing and results are given in Appendices C to J. Original plasmids where we amplified C-termini and β -galactosidase plasmid were described previously [21].

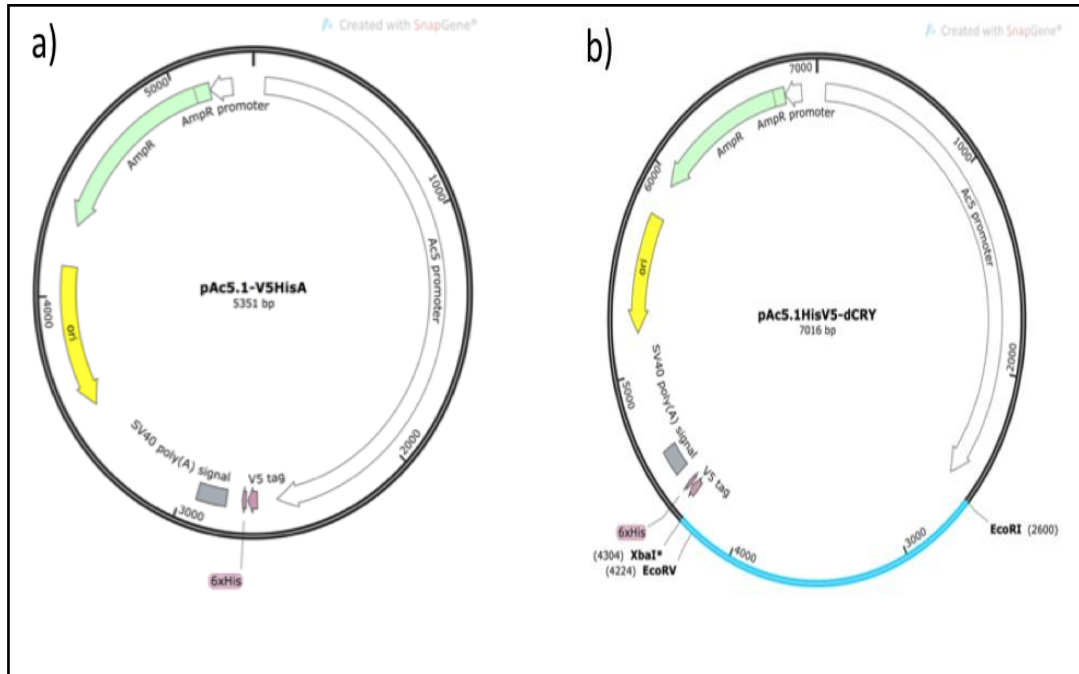


Figure 5. 1: a) pAc5.1-V5HisA and b)pAc5.1V5HisA-dCRY and the blue color represents to cdCRY Δ C. Plasmids are created by SnapGene program.

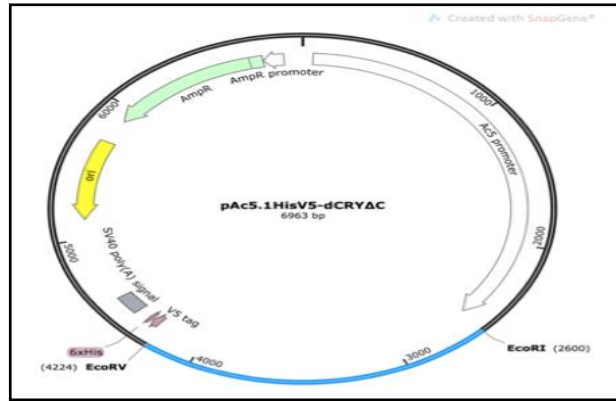


Figure 5.2: pAc5.1V5HisA-cdCRYΔC, the blue color represents to cdCRYΔC. Plasmid is created by SnapGene program.

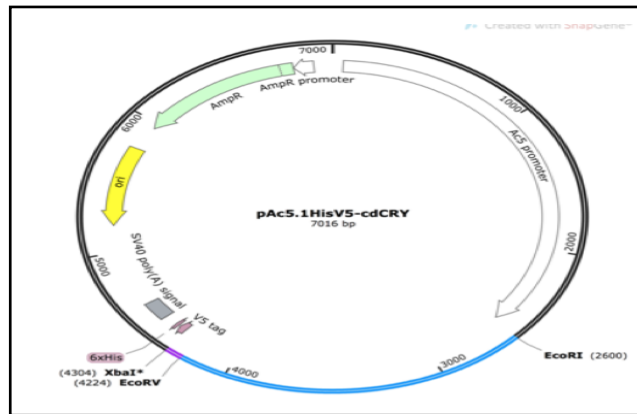


Figure 5.3: pAc5.1V5HisA-cdCRY, the purple color represents to C-terminus of cdCRY and the blue color represents to cdCRYΔC. Plasmid is generated by SnapGene program.

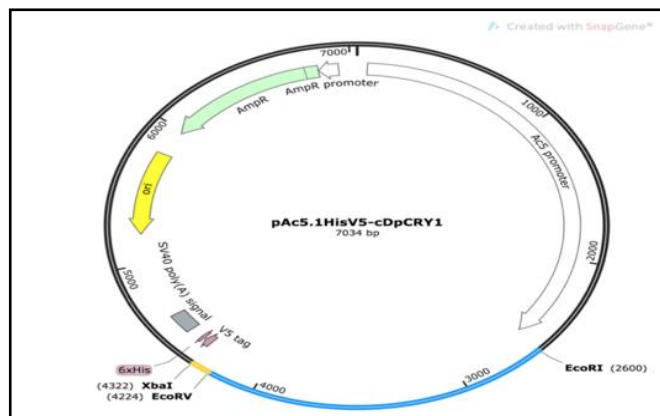


Figure 5.4: pAc5.1V5HisA-cDpCRY1, the yellow color represents to C-terminus of cDpCRY1 and the blue color represents to cdCRYΔC. Plasmid is generated by SnapGene program.

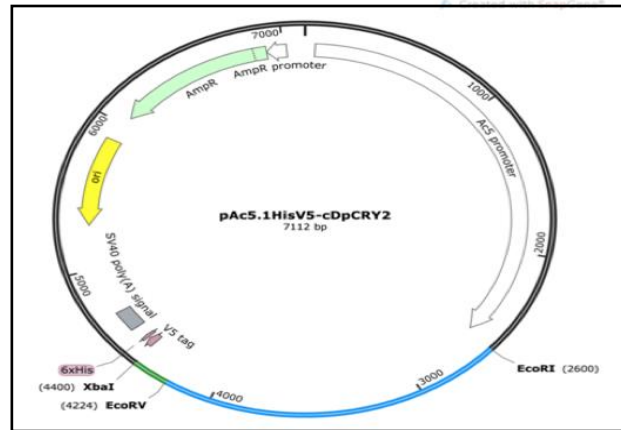


Figure 5.5: pAc5.1V5HisA-cDpCRY2, the green color represents to C-terminus of cDpCRY2 and the blue color represents to cdCRYΔC. Plasmid is generated by SnapGene program.

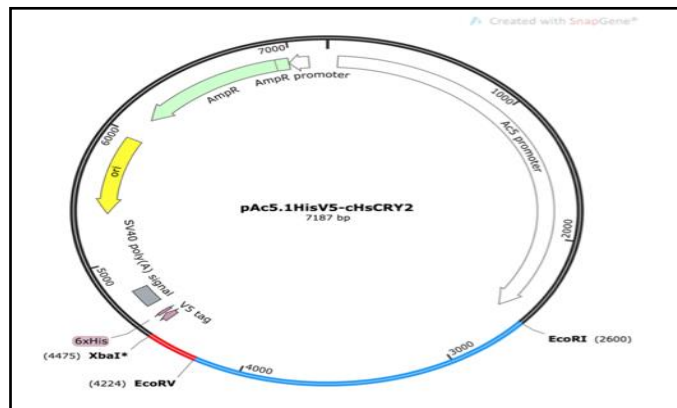


Figure 5.6: pAc5.1V5HisA-cHsCRY2, the red color represents to C-terminus of cHsCRY2 and the blue color represents to cdCRYΔC. Plasmid is generated by SnapGene program.

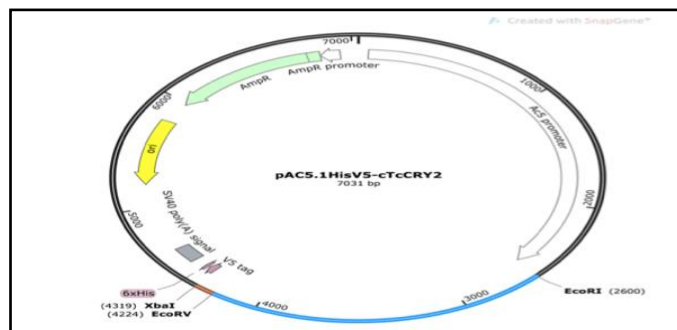


Figure 5.7: pAc5.1V5HisA-cTcCRY2, the brown color represents to C-terminus of cTcCRY2 and the blue color represents to cdCRYΔC. Plasmid is created by SnapGene program.

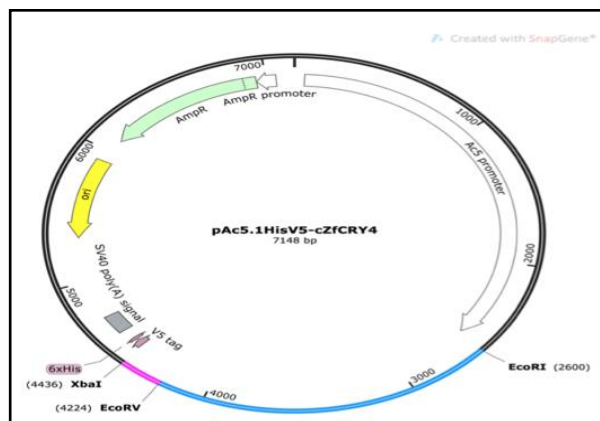


Figure 5.8: pAc5.1V5HisA-cZfCRY4, the pink color represents to C-terminus of cZfCRY4 and the blue color represents to cdCRY Δ C. Plasmid is generated by SnapGene program.

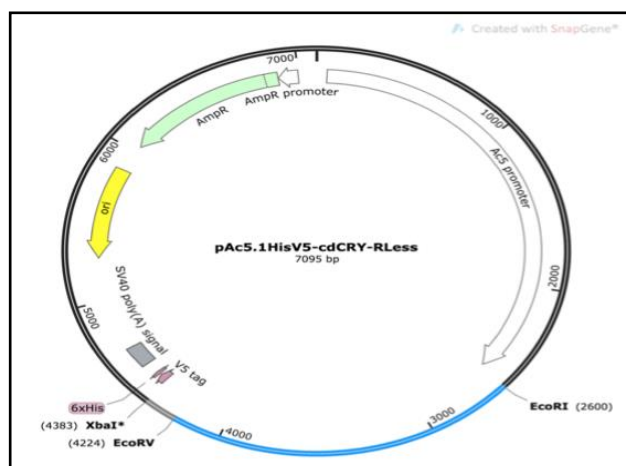


Figure 5.9: pAc5.1V5HisA-cdCRY-RLess ligations plasmid is generated by SnapGene program.

5.2. Light-induced Degradation of Chimeric Cryptochrome Proteins in Insect Cells

Cells were transfected with the plasmids encoding the chimeric CRYs, and β -galactosidase which served as an internal loading control, and 48 h later, the cells were irradiated with 366 nm light at a fluency rate of 1 mWcm² for the 45 and 90 minutes. The cells were then collected and the level of CRYs and β -galactosidase were analyzed with immunoblotting using anti-V5 antibody for cCRYs in S2R+ cells. Figure 5.10 show representative blots. "c." letter before CRYs indicates that dCRY sequence contains a serine to threonine replacement before the start of C-terminus.

Species name such as Dp, Hs, Zf, d, indicate that chimeric CRYs contains C-termini from these species fused to the cdCRY Δ C. Therefore, these experiments are designed to measure if these C-termini from other species' CRYs support the light-dependent proteolysis of dCRY after replacing original C-terminus of dCRY.

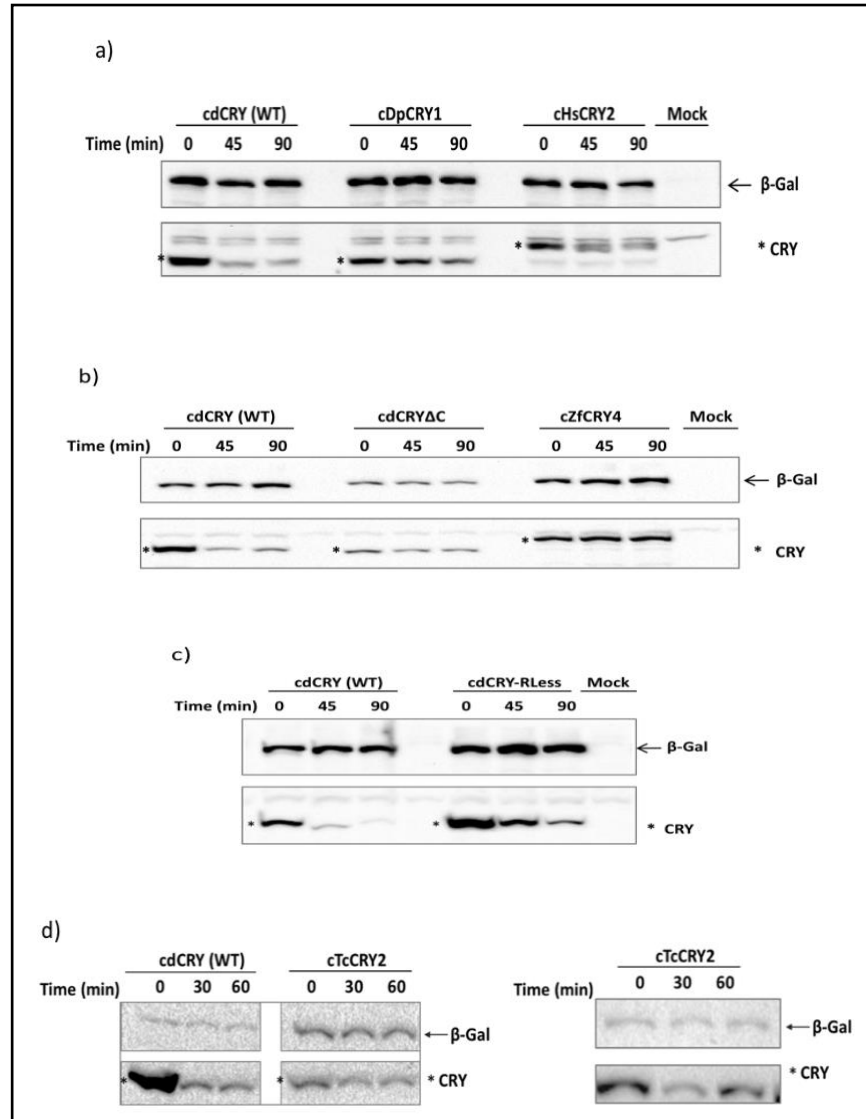


Figure 5.10: Western blot analysis of chimeric CRYs. Light-induced degradation of cCRYs. a) Western blot analysis showing the effects of cdCRY (WT), cDpCRY1 and cHsCRY2 on light-induced CRY degradation. Three independent experiments were performed for each test group. b) Western blot analysis showing the effects of cdCRY (WT), cdCRY Δ C and cZfCRY4 on light-induced CRY degradation. c) Western blot analysis showing the effects of cdCRY (WT), cdCRY-Rless on light-induced CRY degradation. d) Western blot analysis showing the effects of cdCRY (WT) and cTcCRY2 on light-induced CRY degradation. β -Gal (top) levels were determined as a loading control. β -Gal and CRY(bottom) were visualized by Western blot analysis using V5 antibody.

5.3. Quantification of chimeric Cryptochrome Proteins Expression by Light-induction

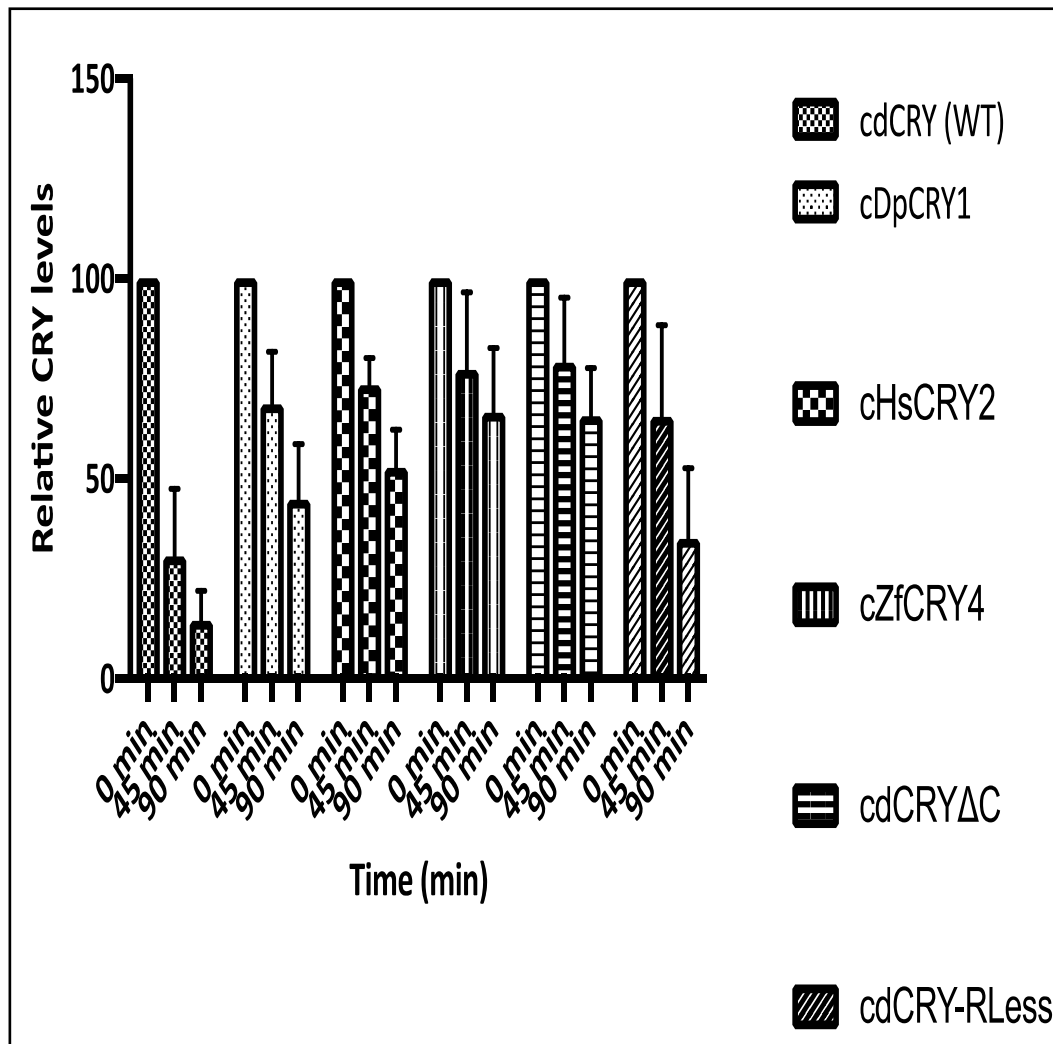


Figure 5.11: Quantitative graphs of light-induced degradation of chimeric CRYs. Relative cCRYs levels from three (for cDpCRY1, cHsCRY2 ad cdCRY-RLess) and five (for cdCRYΔC and cZfCRY4) independent experiments were used for the quantitation. cCRYs levels were normalized to β -Gal levels, and the average cCRYs level in the dark conditions was set to 100. We have also observed slight degradation in cdCRYΔC samples. Levels of CRY were significantly reduced in the cCRYs ($P < 0.001$ s), ($F(10, 20) = 6.485$). Two-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.00 for Mac OS X, GraphPad Software, La Jolla California USA.

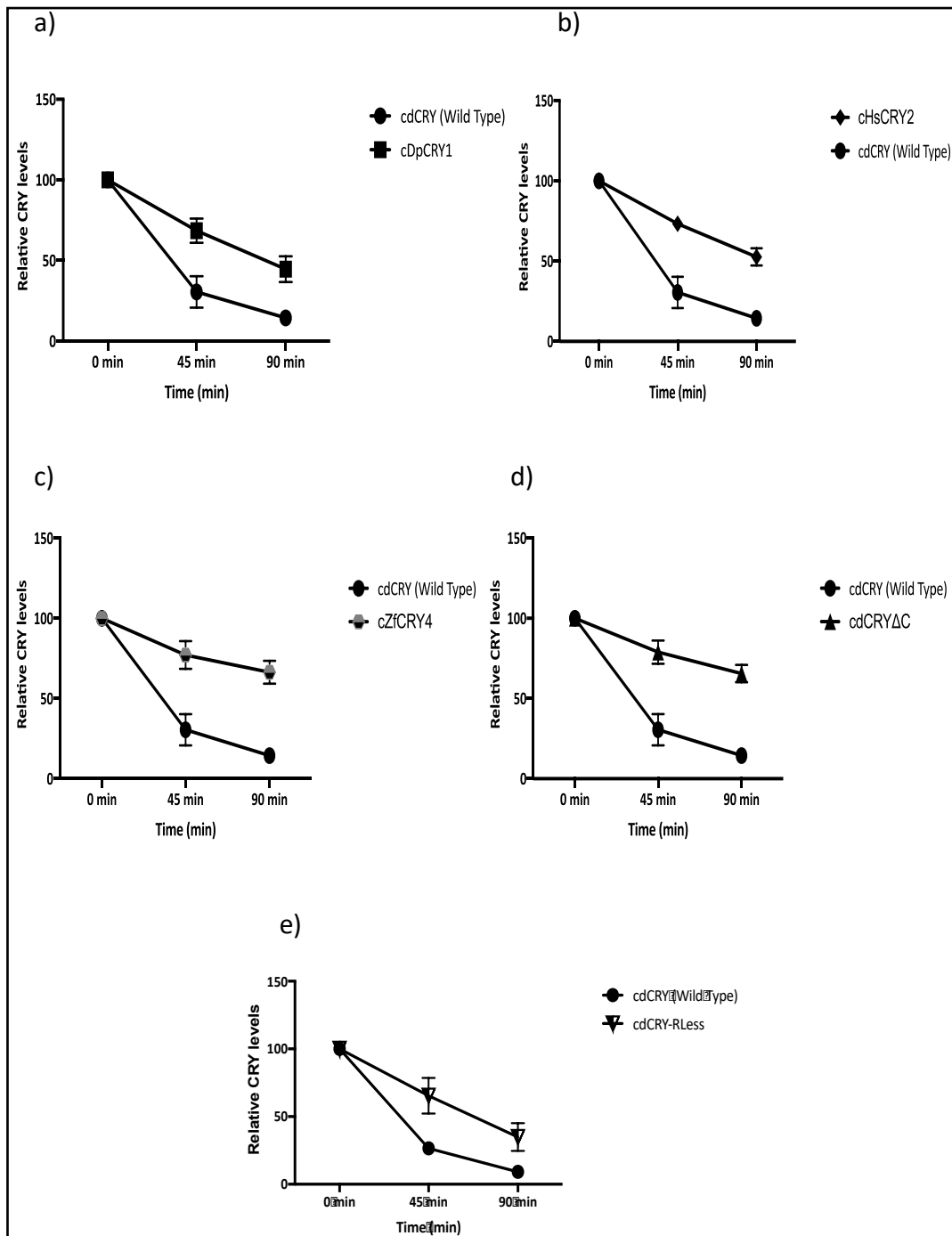


Figure 5.12: Quantification of Wild Type vs Chimeric CRYs using points and connecting line with error bars graphs. a) cdCRY (Wild Type); circle, cDpCRY1; square. b) cdCRY (Wild Type); circle, cHsCRY2; diamond. c) cdCRY (Wild Type); circle, cZfCRY4; hexagon. d) cdCRY (Wild Type); circle, cdCRYΔC; triangle. e) cdCRY (Wild Type); circle, cdCRY-RLess; an inverted triangle. Graphpad prism 7 program was used.

We quantitated the remaining cCRY signals relative to β -Gal signal in each immunoblot to find out light-induced degradation of chimeric CRYs. Quantification showed that the effects of chimeric CRYs degradation following a 45-min and 90-min light exposure. Error bars represent standard error of the mean (SEM). Data shown were averaged from at least three independent experiments. (Fig.5.12. a, b, c, d, e).

Statistical analysis was conducted using two-way ANOVA. Multiple comparisons (Dunnett's test (the difference between the two points at each given time points)) show that CRY degradation; the 45-min light pulse was significantly less ($***P<0.001$) in the all chimera except c.dCRY.RLess ($*P=0.03$), the 90-min light pulse was significantly less ($***P<0.001$) in all chimera except ($**P=0.003$) in c.DpCRY1 and c.dCRY.RLess ($**P=0.004$), as compared with the cdCRY (WT) control. Even though, the c.dCRYs such as c.HsCRY2, and c.DpCRY1 are degraded less efficiently by light compared to c.dCRY, our main marker or test is the degradation of c.DpCRY1. As we know that DpCRY1 is a Type 1 CRY, and it is less efficiently degraded, this can be explained by the heterologous or chimeric expression of them in S2 (Drosophila) cell line but not in the cells from species where the C-termini come.

These data suggest that the total variation of chimeric CRYs according to the time-dependent chimeric CRY degradation was significantly reduced in 11.26% c.DpCRY1 ($***P=0.001$), 16.43% c.HsCRY2 ($*P=0.02$), 24.87% c.ZfCRY4 ($**P=0.002$), and 25.79% c.dCRY Δ C ($***P=0.001$) as compared with wild-type control. 8.592% c.dCRY.RLess ($^{ns}P=0.09$) was non significant. Significance was established at $P<0.05$.

More-detailed information about percent of total variations were significantly increased in 76.08% c.DpCRY1 ($***P<0.001$), 70.51% c.HsCRY2 ($***P<0.001$), 54.49% c.ZfCRY4 ($***P<0.001$) and 78.99% c.dCRY.RLess ($***P<0.001$) and 55.44% c.dCRY Δ C ($***P<0.001$) as compared with its dark.

In summary, our results are as follow:

- c.dCRY is degraded by light at the expected rate

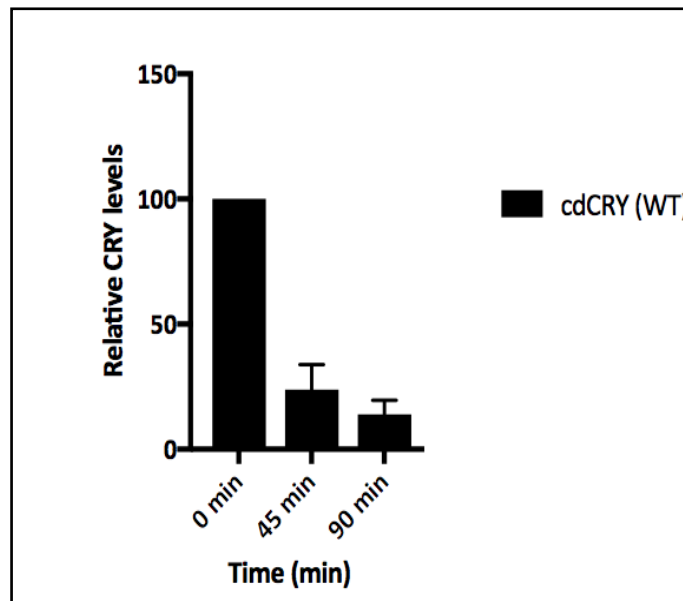


Figure 5.13: Quantification of c.dCRY. Graphpad prism 7 program was used.

- c.DpCRY1 C-terminus support the c.dCRY degradation because the C-terminus of dCRY is replaced and chimeric c.DpCRY1 (dCRY PHR domain fused to the C-terminus of DpCRY1) is efficiently degraded by light. Hence, c.DpCRY1 is light sensitive.
- cHsCRY2 (chimeric dCRY with C-terminus of HsCRY2) is efficiently degraded by light, even as much as cDpCRY1 is degraded.
- c.dCRY Δ C is degraded very little. This can be attributed to the high light dose we used or excess transfection conditions that we had to use in order to get signal with c.dCRY Δ C because if continuously degraded by constitutively active nature of the protein.
- Mutation of a conserved arginine (R) residue to lysine (K) did not interfere with dCRY degradation by light.
- Even though, c.ZfCRY4 does not look to be degraded, we believe that C-terminus of ZfCRY4 renders c.ZfCRY4 constitutively active, and therefore we cannot see further light-dependent degradation (This is explained in the Discussion section).

6. DISCUSSION

All members of Cryptochrome (CRY) protein family have C-termini with variable sizes [15]. In Type 1 CRYs such as dCRY, it is known that the C-terminus plays a crucial role in the phototransduction leading to the resetting of the circadian clock. It was shown that deletion of the C-terminus of dCRY generates a constitutively active form of dCRY which can generate a signal to reset circadian clock continuously, and this repeated resetting signal causes arrhythmicity in flies. On the other hand, the C-termini of mammalian CRYs are thought to be involved in interactions with mammalian BMAL1:CLOCK factors. Therefore, the C-terminal extension is so expected to give distinctive properties and have significant functions [8].

The main aim of this was to analyze the flexibility of C-terminus of dCRY in respect to the light-dependent proteolysis. We expressed dCRY without a C-terminus or infusion to C-terminus from different species of CRYs. These CRYs represents Type 1 (DpCRY1), Type 2 (HsCRY2), and Type 4 (ZfCRY4). Even though dCRY Δ C is seemingly slightly responsive to light, this could be due to transient transfection of dCRY Δ C. We had to really overexpress c.dCRY Δ C and c.ZfCRY4 using an excess amount of plasmids compared to other chimeric CRYs. Because c.dCRY Δ C is continuously degraded and light exposure might inhibit the expression of new proteins by inducing the formation of the reactive oxygen species in culture media which induces unfolded protein response. For ZfCRY4, Ozturk *et al.* suggested that ZfCRY4 adopts an open structure similar to the light-activated dCRY structure [24]. In agreement with this notion, we had to really overexpress c.ZfCRY4 to obtain any detectable signal. In other words, c.ZfCRY4 adopts a light-activated constitutive form which is already degraded under darkness continuously and cannot be further degraded by light. Two-way ANOVA by Dunnett's multiple comparisons was showed that between within chimeric CRYs degradation variation the 90-min light pulse was significantly less (** $P < 0.001$) in all chimera, except (** $P = 0.003$) in c.DpCRY1 and c.dCRY-Rless, comparing with the cdCRY (WT) control. Thus it would say c.DpCRY1 and cdCRY-Rless have similarly degradation profile with cdCRY and so the mutation of the arginine residue, which was conserved in all types of CRYs, did not affect dCRY function.

Secondly, within-group total variation according to time-dependent degradation was significantly less c.DpCRY1 (11.26%), c.HsCRY2 (16.43%), c.ZfCRY4 (24.87%), and c.dCRY Δ C (25.79%), while c.dCRY-Rless (8.592%) was non-significant as compared with wild-type control. It should be emphasized that c.dCRY-Rless has same degradation profile, c.DpCRY1 and c.HsCRY2 have similarly degradation profile, c.dCRY Δ C and c.ZfCRY4 have oppositely degradation profile as compared with c.dCRY. Significance was established at $P < 0.05$.

As a result, according to different evaluation that percent of total variations were significantly increased in c.DpCRY1 (76.08%), c.HsCRY2 (70.51%), c.ZfCRY4 (54.49%), c.dCRY-Rless (78.99%) and c.dCRY Δ C (55.44%) as compared with its dark condition.

Even though some studies suggested that HsCRY2 might have a photosensory function in some cases, these suggestions could not be confirmed by others [15]. Even though HsCRY2 could not be purified with stoichiometric flavin and it is the core component of the circadian clock its C-terminus can support the c.dCRY degradation in S2R+ cells. Therefore, the light-dependent degradation of c.HsCRY2 suggests that C-terminus of dCRY is solely required to occupy the binding sites of BRWD3 E3 ligase. The release of HsCRY2 C-terminus to unmask the binding site of BRWD3 after light exposure also indicates that light-induced conformational change might occur before the C-terminus. The degradation of c.DpCRY1 by light is expected because DpCRY1 is also a Type 1 CRY.

Moreover, CRY Δ C is constitutively active, which is a substrate for the ubiquitin-proteasome system (UPS) [24]. In other words, because it does not have C-terminus this makes accessible of UPS system in light and dark. There is a slight induction of c.dCRY Δ C by light in contrary to previous findings [3]. But degradation is significantly reduced compared to light-induced c.dCRY degradation. Briefly, c.dCRY Δ C degradation was significantly reduced as to c.dCRY.

Even though we had preliminary data for c.TcCRY2 and c.DpCRY2 supporting the notion that they are degraded by light, further controlled experiments are required for statistical analysis. Actually, we have not analyzed if c.CRYs support the light-dependent degradation of TIMELESS protein. As the E3 ligases for dCRY and TIM proteins (BRWD3 and JET, respectively), it is important to analyze TIM degradation in

the presence of c.CRYs. It is known that S2R+ cells do not express enough dCRY or JET to support TIM degradation. Moreover, these cells do not express TIM at detectable levels. Therefore, the cotransfection of c.CRYs, JET, and TIM would reveal if these c.CRYs support Tim degradation by light.

In summary, this work suggests that the C-terminus of dCRY is structural very flexible and light-dependent degradation of dCRY can be supported if the core PHR (dCRYDC) of dCRY is fused to c-termini from even Type 2 CRY such as HsCRY2. A follow-up study to assay if c.dCRY support the light-dependent TIM degradation would reveal if the flexibility of C-terminus is true for the resetting function through TIM degradation.

Further study is required to test if the c.CRYs support the light-dependent degradation of TIM protein. One major obstacle with the expression of c.CRYs is the very variable level of basal (under darkness) of them. This may complicate the interpretation of our findings. Foreexample, high expression of c.HsCRY may, in fact, results less degradation compared to real degradation potential. A high amount of c.HsCRY2 may not have a stoichiometric amount of E3 ligase (BRWD3)-proteasome system to support the ubiquitylation and degradation of c.HsCRY2. On the other hand, c.CRYs which were expressed at lower rate will have about E3 ligase-proteasome system around them, which easy their degradation. The best solution to these technical problems is to obtain stable lines of S2 cells which express cCRYs at comparable levels. It will only possible to really compare light-dependent degradations of cCRYs under lower light fluency to avoid reactive oxygen species.

As future perspective, we also see the possibility of obtaining shorter dCRY Δ C forms to eliminate minor light-dependent degradation that we observed in our c.dCRY Δ C protein. We also see an immediate need to have stable S2 lines of chimeric CRYs which also express TIM and JET in order to analyze light-dependent degradation of TIM in the presence of chimeric CRYs.

We believe that this initial study opened a new avenue in respect to the structural flexibility of dCRY, and as explained above follow-up studies are required to understand this flexibility in respect to the resetting of the circadian clock in *Drosophila* in addition to light-dependent degradation of dCRY.

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BIOGRAPHY

Bihter MURATOĞLU was born in Istanbul, May 2, 1992. She received her BS in Molecular Biology and Genetics from the Bilecik Şeyh Edebali University (Bilecik, 2014). In 2016, Bihter joined the MSc. Program in Gebze Technical University, Graduate School of Natural and Applied Science, Molecular Biology and Genetics Department. Her research interests lie in the area of the biological clock that clarifies to function and structure of C-Terminus of *Drosophila* cryptochrome protein. She worked for 2.5 years under the supervision of Assoc. Prof. Dr. Nuri ÖZTÜRK.

APPENDICES

Appendix A: ANOVA test

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value	P value summary	Significant?
Time	25069	2	12535	F (2, 4) = 135.7	P<0.001	***	Yes
cCRYs	7589	5	1518	F (5, 10) = 8.169	P=0.003	**	Yes
Interaction: Time x cCRYs	4714	10	471.4	F (10, 20) = 6.485	P<0.001	***	Yes

Appendix B: Dunnett's multiple comparisons test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
0 min					
cDpCRY1 vs. cdCRY (Wild Type)	0	-19.04 to 19.04	No	ns	>0.99
cHsCRY2 vs. cdCRY (Wild Type)	0	-19.04 to 19.04	No	ns	>0.99
cdCRYΔC vs. cdCRY (Wild Type)	0	-19.04 to 19.04	No	ns	>0.99
cdCRY-Rless vs. cdCRY (Wild Type)	0	-19.04 to 19.04	No	ns	>0.99
cZfCRY4 vs. cdCRY (Wild Type)	0	-19.04 to 19.04	No	ns	>0.99
45 min					
cDpCRY1 vs. cdCRY (Wild Type)	38.09	19.05 to 57.13	Yes	***	<0.001
cHsCRY2 vs. cdCRY (Wild Type)	42.82	23.78 to 61.86	Yes	***	<0.001
cdCRYΔC vs. cdCRY (Wild Type)	37.46	18.42 to 56.5	Yes	***	<0.001
cdCRY-Rless vs. cdCRY (Wild Type)	34.9	15.86 to 53.94	Yes	***	<0.001
cZfCRY4 vs. cdCRY (Wild Type)	52.05	33.01 to 71.08	Yes	***	<0.001
90 min					
cDpCRY1 vs. cdCRY (Wild Type)	30.34	11.3 to 49.37	Yes	**	0.001
cHsCRY2 vs. cdCRY (Wild Type)	38.33	19.29 to 57.37	Yes	***	<0.001
cdCRYΔC vs. cdCRY (Wild Type)	52.2	33.16 to 71.24	Yes	***	<0.001
cdCRY-Rless vs. cdCRY (Wild Type)	20.51	1.472 to 39.55	Yes	*	0.03
cZfCRY4 vs. cdCRY (Wild Type)	62.48	43.44 to 81.52	Yes	***	<0.001

Appendix C: cdCRYΔC cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTGCA
GCAGCTGCCCACGCCGGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGAATTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT**GATATC**CAGCACAGTGGCGGCCGCT
CGAGTCTAGAGGGCCCTTCAAGGTAAGCCTATCCCTAACCTCTCCTCGGTCTCGATTCTA
CGCGTACCGGTCATCATCACCATCACCATTGAGTTTAAACCGCTGATCAGCCTCGACTGTG
CC

- cdCRYΔC Protein Sequence

SEFATMHHHHHHGKPIPNLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIALL
IPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVR
LHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTV
QIIGLPPRPRTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVYGDNMGFLAKINWRGGETQ
ALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGCLSVRRFYWSVHDLFKNVQL
RACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSW
RLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWS
VCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTYIKQYVPELMNVPKFVHEPWARM
SAEQQEYQECLIGVHYPERIIDLSMAVKRNLMLAMKSLRNSLI**SSTVAAARV**-Blue comes from
MCS.

Appendix D: cdCRY cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTGCA
GCAGCTGCCCACGCCGGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGATTTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT**gatatc**CCCGCCCGCATTGCCGACC
ATCCAACGAGGAGGAAGTGCGTCAGTTCTTCTGGCTGGCCGACGTGGTGGTTTGA**CTAG**
AGGG

- cdCRY Protein Sequence

MHHHHHHGKPIPNPLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIALIPVFIF
DGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVRLHRICI
EQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTVQIIGLP
PRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVDNMGFLAKINWRGGETQALLLLD
ERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGLSVRRFYWSVHDLFKNVQLRACVR
GVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSWRLGQT
GFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWSVCAG
NWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTIYQYVPELMNVPKEFVHEPW RMSAEQ
QEYQECLIGVHYPERIIDLSMAVKRNMLAMKSLRNSLSPPPHCRPSNEEEVRQFFWLADVVV

Appendix E: cTcCRY2 cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTGCA
GCAGCTGCCCACGCCGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGATTTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCGAAATTCGCT**gatatc**ctaccgaaatgtggataattcGGCG
AAATTTAAAGACGGATTTCAAGGAGCAACCGAATGTTGTAA**CCGTTGGTAACCCAGTAGG**
AAATAATCTAGATT

- cTcCRY2 Protein Sequence

SEFATMHHHHHHGKPIPNLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIALL
IPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVR
LHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTV
QIIGLPPRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVDNMGFLAKINWRGGETQ
ALLLLDERLKVEQHAFERGFYLPNQALPNIHDSKMSAHLRFGCLSVRRFYWSVHDLFKNVQL
RACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSW
RLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWS
VCAGNWMWVSSSAFERLLDSSLVTCVVALAKRLDPDGTYIKQYVPELMNVKPEFVHEPWRM
SAEQQEYQECLIGVHYPERIIDLSMAVKRNLMLAMKSLRNSLSYRNVDNSAKFKDGFQECPNV
VTVGNPSRK

- **Appendix F: cDpCRY1 cDNA Sequence**

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCCGAGTTCTGCCGAAGTCTTAAGTTGTTGCA
GCAGCTGCCCACGCCGGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGAATTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT**gatatc**cgctcctcctcactgctgtccgtcATCT
GAAGACGAGGTGCGGCAGTTCATGTGGCTTGGAGACGActcgagcctgagctcaccaccacaTA
ATCTAGATT

- **cDpCRY1 Protein Sequence**

SEFATMHHHHHHGKPIPNLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIAL
IPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVR
LHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPTYQMFLHTV
QIIGLPPRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVYGDNMGFLAKINWRGGETQ
ALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKMSAHLRFGCLSVRRFYWSVHDLFKNVQL
RACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSW
RLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWS
VCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTYIKQYVPELMNVPKFVHEPWPM
SAEQQEYQECLIGVHYPERIIDLSMAVKRNMLAMKSLRNSLI**SAPPHCCPSSSEDEV**RQFMWL
DDSQPELTTT-

Appendix G: cZfCRY4 cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTCTGA
GCAGCTGCCCACGCCGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGATTTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCTAGA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT**gatatc**caccgcagaactcacgcgagatgta
GCAGATGACCCGATGGAGGCGGGGCTTAAGCGTGAGCTCCGGGAGGAGGAGGGGCTTCT
GGAGGAGGCGGAGTCACAGTGCACCTCCAAGCGTTTCAGTGGCAGCTCAGACCATAAGCC
CCGCCCTGCAGCTGGACTCCAGAGACTCTACAGCTATCAgagctcagcggagaggtcatgTAATC
TAGATTT

- cZfCRY4 Protein Sequence

SEFATMHHHHHHGKPIPNPLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGI
ALIPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQ
VRLHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHT
VQIIGLPPRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVYGDNMGFLAKINWRGGET
QALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGLSVRRFYWSVHDLFKNV
QLRACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQS
WRLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDAD
WSVCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTIYKQYVPELMNVPKEFVHEPW
RMSAEQQEQYECLIGVHYPERIIDLSMAVKRNMLAMKSLRNSLI**S**TAELTRDVADDPMEAGLK
RELREEEGLLEEAEQCTSKRFSGSSDHKPRPCSWTPETLQSELSGEVM-

Appendix H: cHsCRY2 cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCGGTAAGCCTATCCCTAACcctctcctcg
gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
CCGTTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTACAATCGGATGC
GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
TGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTCTGA
GCAGCTGCCCACGCCGGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
GAGCAGCATGCGTTTGAGCGTGGAATTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCAGA
TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
GGTGAACAATCCAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGT
ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCTgatatcctaccggggactctgtctacGGAGC
GTGTGCGCCGGCAACTGGATGTGGGTGAGCAGCAGCGCCTTCGAGAGGCTGCTGGACAG
CAGCCTGGTGACCTGCCCCGTGGCCCTGGCCAAGAGGCTGGACCCCGACGGCACCTACAT
CAAGCAGTACGTGCCCGAGCTGATGAACGTGCCCAAGGAGTTCGTGCACGAGCCCTGGAG
GATGAGCGCCGAGCAGCAGGAGCAGTACGAGTGCCTGATCGGCGTGCACTACCCCGAGA
GGATCATCGACCTGAGCATGGCCGTGAAGAGGAACATGCTGGCCATGAAGAGCCTGAGG
AACAGCCTGATCAGCTACAGGGGCCTGTGCCTGCTGGCCAGCGTGCCAGCTGCGTGGAG
GACCTGAGCCACCCCGTGGCCGAGCCCAGCAGCAGCCAGGCCGGCAGCATGAGCAGCGC
CGGCCCCAGGCCCTGCCAGCGGCCCGCCAGCCCCAAGAGGAAGCTGGAGGCCGCCG
AGGAGCCCCCGGCGAGGAGCTGAGCAAGAGGGCCAGGGTGGCCGAGCTGCCACCCCC
GAGCTGCCAGCAAGGACGCC

- cHsCRY2 Protein Sequence

SEFATMHHHHHHGKPIPNPLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIALL
IPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVR
LHRICIEQDCEPIWNERDESIRSLCRELNIDFEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTV
QIIGLPPRPRTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVYGDNMGLAKINWRGGETQ

ALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGCLSVRRFYWSVHDLFKNVQL
 RACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSW
 RLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWS
 VCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTYIKQYVPELMNVKPEFVHEPWARM
 SAEQQEQYECLIGVHYPERIIDLSMAVKRNMLAMKSLRNSLSYRGLCLLASVPSCVEDLSHPVA
 EPSSSQAGSMSSAGPRPLPSGPASPKRKLEAAEPPGEELSKRARVAELPTPELPSKDA-

Appendix I: cdCRYRLess cDNA Sequence

AGC**GAATTC**GCCACCATGCACCACCACCACCACCACGGTAAGCCTATCCCTAACcctctcctcg
 gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
 CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
 CCGTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTTACAATCGGATGC
 GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
 TGGACGCCTCCTGGTCTTCGAGGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
 GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
 GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
 CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
 AATGTTCTGACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
 TAGAAGACGCCACCTTTGTCGAGCTGGACCCCGAGTTCTGCCGAAGTCTTAAGTTGTTCTGA
 GCAGCTGCCCACGCCGGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAG
 ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
 GAGCAGCATGCGTTTGAGCGTGGAATTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
 ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTGGTACGTCGCTTCTAC
 TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCTCAGA
 TGACTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
 GGTGAACAATCCAAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
 GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCCTC
 ATCGACGGCGCCATGCGACAACCTCCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
 CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
 ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCTGGCTGCGCTGGCAACTGGATGTGGGT
 ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
 GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
 TGCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
 AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
 AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT**gatatc**CCCGCCGCCGATTGCCGACC
 ATCCAACGAGGAGGAAGTG**AAG**CAGTTCTTCTGGCTGGCCGACGTGGTGGTTTGAT**TCTAG**
 AGGG

- cdCRYRLess Protein Sequence

SEFATMHHHHHHGKPIPNLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGI
 ALIPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRR
 LHEQVRLHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIP
 PLTYQMFLHTVQIIGLPPRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVDN
 MGFLAKINWRGGETQALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGC

LSVRRFYWSVHDLFKNVQLRACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGN
 DICLSIPWAKPNENLLQSWRLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLW
 QSWEHGLQHFLKYLLDADWSVCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTI
 KQYVPELMNVPKEFVHEPW RMSAEQQEQYECLIGVHYPERIIDLSMAVKRNM LAMKSLRN
 SLISPPPHCRPSNEEEVQFFWLADV VV

Appendix J: cDpCRY2 cDNA Sequence

AGC GAATTC GCCACCATGCACCACCACCACCACCGTAAGCCTATCCCTAACcctctcctcg
 gtctcgattctacgATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCT
 CCATGATAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTC
 CCGTTTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTTACAATCGGATGC
 GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACG
 TGGACGCCTCCTGGTCTTCGAGGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAA
 GTGCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGAT
 GAAAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACA
 CGCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA
 AATGTTCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCGAC
 TAGAAGACGCCACCTTTGTCGAGCTGGACCCGAGTTCTGCCGAAGTCTTAAGTTGTTCTGA
 GCAGCTGCCCACGCCGAGCACTTCAATGTGTATGGAGACAACATGGGCTTCTGGCCAAAG
 ATTAAGTGGCGCGGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTG
 GAGCAGCATGCGTTTGAGCGTGGATTTTATCTGCCCAACCAGGCACTGCCCAATATCCACG
 ACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGCCTTTCCGTACGTCGCTTCTAC
 TGGAGCGTCCACGATCTCTTCAAGAATGTCCAGTTGCGCGCCTGTGTGCGGGGCGTTTCTAGA
 TGAAGTGGCGGCGCGCACATCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTC
 GGTGAACAATCCAAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCCGTG
 GGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAACGGGATTCCCGCTC
 ATCGACGGCGCCATGCGACAACCTCTGGCCGAGGGATGGCTCCACCATACGCTGCGCAACA
 CCGTGGCCACCTTTCTCACGCGCGGCGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGC
 ACTTTCTGAAGTATCTGCTGGATGCGGATTGGTCTGGTCTGCGCTGGCAACTGGATGTGGGT
 ATCCAGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGTGGCATTG
 GCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACGTCCCGGAGTTGATGAATG
 TGCCCAAGGAATTTGTTACGAGCCCTGGCGAATGTCTGCCGAGCAGCAGGAGCAGTACG
 AGTGCCTGATCGGAGTCCATTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCG
 AAACATGCTGGCCATGAAGTCTCTCCGAAATTCGCT GATATC Cttttacttcaccgacaatggagtta
 taactcacaatgaaaatgcacaaacttttaaaaggatagttattcagataattataataaagagagtaccgggtctaa
 cagagttggtgaggtacacagtaataaccaccaaacagataagatctcatcgaaaagaaaaattaaTCTAGATT
 T

- cDpCRY2 Protein Sequence

SEFATMHHHHHHGKPIPNPLLGLDSTMATRGANVIWFRHGLRLHDNPALLAALADKDQGIALL
 IPVFIFDGESAGTKNVGYNRMRFLLDSLQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVR
 LHRICIEQDCEPIWNERDESIRSLCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTV
 QIIGLPPRPTADARLEDATFVELDPEFCRSLKLFELPTPEHFNVYGDNMGFLAKINWRGGETQ

ALLLLDERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGCLSVRRFYWSVHDLFKNVQL
RACVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSW
RLGQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWS
VCAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTIKQYVPELMNVPKEFVHEPWRM
SAEQQEYECBIGVHYPERIIDLSMAVKRNMLAMKSLRNSLSFYFTDNGVITHNENAQTFKRD
SYSDNYNKESTGSNRVGEVHSNNPQTDKISSEKKN-