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A THESIS SUBMITTED FOR THE DEGREE OF

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CRYPTOCHROME PROTEİN TABANLI OPTOGENETİK ARAÇ GELİŞTİRME

GÖZDE ÖZÇELİK
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. NURİ ÖZTÜRK

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ÜYE
(TEZ DANIŞMANI) : Doç. Dr. Nuri ÖZTÜRK

ÜYE : Prof. Dr. Halil KAVAKLI

ÜYE : Prof. Dr. Sedef TUNCA GEDİK

ONAY

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SUMMARY

Light is used to control signaling pathways via photosensitive proteins, this technology is called optogenetics. Adoption of new light sensitive proteins into optogenetic methods improves the control and observation of cellular events with high temporal precision. Cryptochrome proteins which have function to reset circadian rhythms in many organisms are highly attracted as possible optogenetic tools. *Drosophila* Cryptochrome (CRY) is photosensory receptor; therefore introduction of *Drosophila* CRY into mammalian cells can render these cells sensitive to light. In this study, it is aimed the development of a new optogenetic tool by using *Drosophila* CRY protein.

To develop a molecular tool to control endogenous gene expression and protein activity by light in mammalian cells, it was exploited that the light-dependent interaction between *Drosophila* CRY and Jetlag proteins. To test regulation of an endogenous gene expression with this light system, Retinoid X Receptor (RxR) protein was used without dimerization domain, and it was expected that light induced interaction between CRY and JET allow dimerization of truncated RxR, then activate p21 gene expression. Both Rxr-dCRY and Rxr-JET constructs were expressed in HEK293T cells and these cells were exposed the black light at 0,02mW/cm^2 intensity. Firstly, p21 mRNA upregulation was shown by qPCR light-dependently. Then, immunoblotting assays up to 36 hrs. showed that p21 protein expression enhanced upon light exposure.

In conclusion, it was found that *Drosophila* CRY is good candidate as an optogenetic tool. This system offers an alternative to classical gene regulation systems and may find large application area for research and biotechnological purposes.

Keywords: Optogenetics, photoreceptor, biological clock, light, gene regulation, Cryptochrome, Jetlag.

ÖZET

Işık, ışığa duyarlı proteinler aracılığı ile sinyal yolaklarının kontrolünü sağlamak için kullanılır, bu sistem optogenetik olarak adlandırılır. Işığa duyarlı yeni aday proteinlerin bilinen optogenetik araçlara uyarlanması hücresel aktivitelerin ışık ile daha kesinlikte gözlemlenmesini ve kontrol edilmesini sağlayabilir. Çoğu organizmada sirkadiyen ritimleri resetleyen Cryptochrome proteinleri potansiyel optogenetik araçlar olarak oldukça ilgi çekicidir. *Drosophila* Cryptochrome (CRY) proteini fotoreseptördür, dolayısıyla, *Drosophila* CRY proteininin memeli hücrelerine aktarımı bu hücreleri ışığa duyarlı yapabilir. Bu çalışmada, *Drosophila* CRY proteini kullanılarak yeni bir optogenetik araç geliştirmek amaçlanmıştır.

Memeli hücrelerinde endojen gen ekspresyonu ve protein aktivitesini ışıkla kontrol etmek için, *Drosophila* CRY proteini ve onun ışık partneri Jetlag proteini kullanılmıştır. Bu ışık ışık sistemini kullanarak, endojen bir genin ifadesini kontrol etmek için Retinoid X Reseptör (RxR) proteinin ligand bağlanma domaini silinerek dimerizasyonu engellenmiş ve ışık verildiği zaman CRY ve JET proteinlerinin bir araya gelerek RxR ın dimerize olmasını sağlaması ve p21 ekspresyonunu aktive edebilmesi beklenmiştir. RxR-CRY ve RxR-JET konstraktları HEK293T hücrelerinde olarak eksprese edilmiş ve bu hücreler 0,02mW/cm^2 ışık şiddetine maruz bırakılmıştır. Öncelikle, p21 mRNA artışı kuantatif PCR (qPCR) ile gösterilmiştir. Ardından 36 saate kadar ışık muamelesine bırakılan hücrelerle yapılan immunoblot sonuçları p21 proteinin ışığa bağımlı olarak arttığını göstermiştir.

Sonuç olarak, *Drosophila* Cryptochrome proteininin iyi bir optogenetik araç adayı olduğu bulunmuştur. Bu sistem klasik gen regülasyon sistemlerine alternatif önermektedir ve araştırma ve biyoteknolojik amaçlar için yaygın bir kullanım alanı bulabilir.

Anahtar kelimeler: optogenetik, fotoreseptör, biyolojik saat, ışık, gen regülasyonu, Cryptochrome, Jetlag.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
µg	:	mikrogram
µl	:	Mikrolitre
mW/cm2	:	Milliwatt per centimeter squared
APS	:	Amonium persulfate
CRY	:	Cryptochrome
dCRY	:	*Drosophila* Cryptochrome
CRY Δ	:	CryDelta
Clk	:	Clock gene
Cyc	:	Cycle gene
FAD	:	Flavin adenin dinucleotide
JET	:	JETLAG
HEK293T	:	Human embriyonic kidney 293T cells
HRP	:	Horseradish peroxidase
LB	:	Luria Bertani
LOV	:	Light–oxygen–voltage
PCR	:	Polymerase chain reaction
Per	:	Period gene
PHY	:	Phytochrome
RT-qPCR	:	Real-time Quantative Polymerase chain reaction
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
RxR	:	Retinoid X Receptor
Tim	:	Timeless gene
TTFL	:	Transcription Translation Feedback Loop

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1. INTRODUCTION

The subject of this thesis is to control signaling pathways via light sensitive proteins to take advantages against classical chemical-based regulation systems. Adoption of new light sensitive proteins into current optogenetic methods can improve sensitivity of these methods and would find a large application in the field of molecular biology. Cryptochrome proteins which reset the circadian rhythms in many organisms have attracted attention as optogenetic tools. The circadian photoreceptor of fruit fly *Drosophila* Cryptochrome can be novel optogenetic system to regulate gene expression.

The light-dependent interaction between Cryptochrome and Jetlag proteins can be exploited to control regulation of gene expression in mammalian cells. *Drosophila* and mammalian molecular circadian clocks are essentially the same; however their photoreception mechanisms are different. Mammalian CRY proteins are not light sensitive thus introduction of *Drosophila* CRY and its light partner Jetlag into mammalian cells can became these cells sensitive to light.

1.1. The Purpose, Contribution and Content of Thesis

We aimed to keep light dependent interaction between *Drosophila* CRY and JET proteins in mammalian cells while overcome CRY degradation problem in these cells. Then we will either express transcription factors that are functional only when they are dimers in fusion to CRY and JET. We expect that light bring monomers into close proximity through CRY and JET interaction to make a functional transcription factor. We anticipate to the control expression of cell cycle inhibitor protein p21 by using a transcription factor called Retinoid X Receptor (RxR).

In chapter 2 gives detailed information about optogenetic systems, light-activated proteins and circadian clock mechanism of fruit fly. Chapter 3-4 explain all experimental procedures and materials. Chapter 5 presents the results. The thesis is finalized in chapter 6 by giving concluding remarks and discussion part.

2. LITERATURE REVIEW

2.1. Optogenetics

Regulation of gene expression includes many mechanisms that are used by cells to increase or decrease the production of a specific gene. There are different tools to regulate the gene expression and protein activity. These tools use either a chemical (such as antibiotics or chemicals) or physical (such as heath) agents to regulate some events in living cells. In the chemical-based gene regulation systems, a controllable transcription factor or pieces of transcription factors are expressed in mammalian cells which generally control target gene expression by binding to promoter regions in the presence of the chemical [Clackson, 2000]. Another approach is to regulate oligomerization of protein to regulate the function. These systems generally require a chemical (such as IPTG, tetracyclin, so on) or a physical pressure (such as heat) [Lee and Mapp, 2010]. However, these applications (antibiotics or heat) give some stress and even sometimes cause a shock or toxicity in the cells. Also, these tools are invasive and resolution of regulation is so low that it takes hours. Therefore, scientists are looking for less invasive methods to regulate the gene expression or protein function.

It is an attractive area to develop non-invasive and precise agents such as light to regulate the gene or protein activity in cells. Light offers unique spatiotemporal resolution. Recently development of light-controlled system has attracted much attention. Even though, there are some new systems which use light to regulate specific signaling pathways in living cells, they generally require sophisticated equipment or special light sources [Muller and Weber, 2013]. Therefore, a simple light controllable tool would find a large application in the field of molecular biology and genetics.

The first light controlled molecular tools were photo-responsive chemically caged inducers include nucleic acids and transactivators that could be cleaved off in response to UVB light [Gardner and Deiters, 2012]. However, an important disadvantage of this method is the need to exogenous caged molecules or introduction of non-natural amino acids into proteins. This disadvantage has been

overcome by optogenetics technology which is combination of optical and genetic methods to control defined events of specific cells [Muller and Weber, 2013].

Optogenetics is a method which utilizes light to control molecular events in a targeted manner in living cells. It depends on the usage of genetically-encoded light sensitive proteins that change their conformation in the presence of light to activate signaling pathways. The first breakthrough in optogenetics was the application of microbial opsins to activate single neuronal cells with high spatiotemporal resolution [Boyden et al., 2005], [Muller and Weber, 2013]. Channelrhodopsin, the first opsin was applied in mammalian neurons, later, bacteriorhodopsin and then halorhodopsin all had allowed to switching neural activity on and off by diverse colors of light impulse [Deisseroth, 2011].

The non-neural optogenetic applications have been realized in recent progress to control diverse signaling processes in mammalian cells. Photoreceptors from different organisms promoted the genetically encoded light responsive systems. Adoption of genetically encoded light responsive proteins provides reversible, easy delivered, less invasive control of cellular function because of expression of protein rather than injection [Tischer and Weiner, 2014]

The light inducible gene expression systems for mammalian cells are briefly introduced below.

2.1.1. Optogenetic Systems

The key components of optogenetic technique are light-sensitive proteins. There are different strategies to regulate intracellular signals with photosensitive proteins. Light induced conformational changes promote heterodimerization of light-sensitive protein and its effector and this system is widely used to manipulate internal signals thereby activation or inhibition. Cell signaling also can be controlled by homodimerization of light sensitive proteins by localizing to a promoter to initiate transcription of gene of interest. Oligomerization of some proteins in response to light or sequestering protein away from site of action alternatively to clustering can be used to activate signaling cascades. Finally, conformational changes in the protein can generate active signaling protein upon light induction by revealing signaling

domain or relieving protein from autoinhibited state (Figure 2.1) [Tischer and Weiner, 2014].

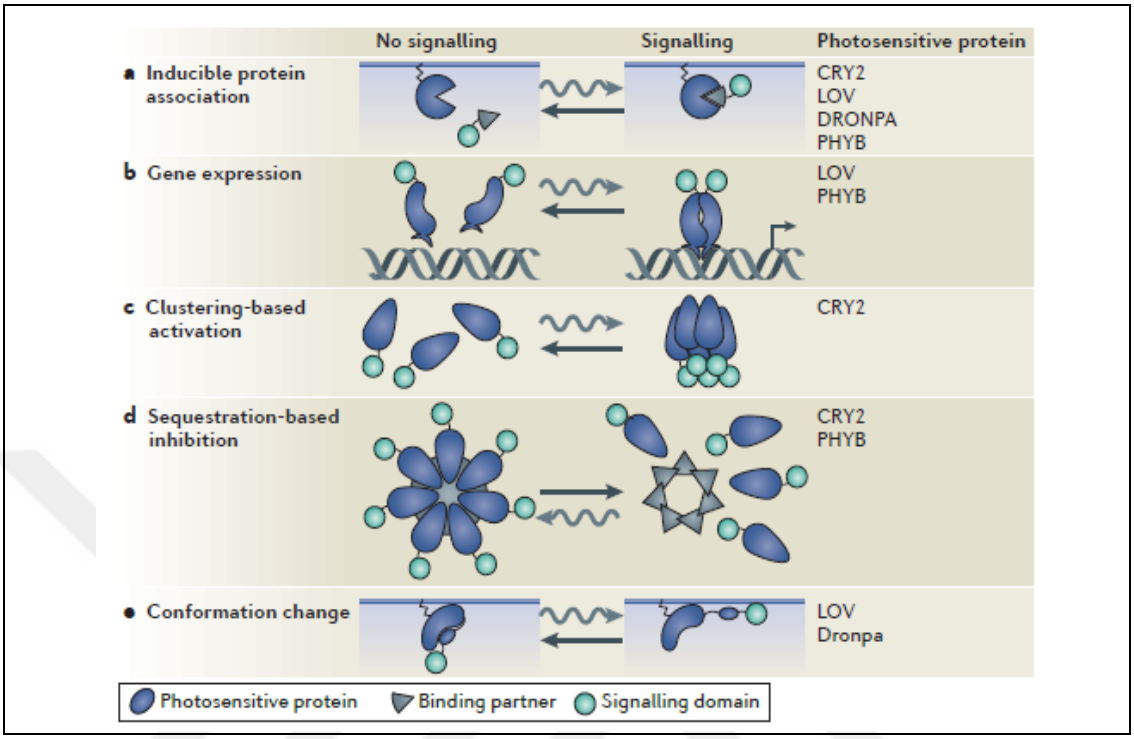

Figure 2.1: Different strategies to manipulate intercellular signals with optogenetic system proteins. a) Inducible protein association by heterodimerization. b) Homodimerization and heterodimerization recruit transcriptional activators or other DNA-modifying proteins. c) Cryptochrome 2 (CRY2) naturally clusters when it is activated with light. d) Signaling can be inhibited by sequestering signaling protein away from its site of action. e) Conformational change of photosensitive protein can activate intercellular signaling.

These common ways in reversible optogenetic systems use main photosensitive proteins which will be described below.

- The LOV domains:

Light–oxygen–voltage (LOV)-domains are small, blue light-sensing domains that are conserved in prokaryotes, fungi and plants. They are all sensitive to blue light (440–473 nm), subclass of Period-ARNT-Singleminded (PAS) domain family and use an endogenous Flavin (FMN,FAD or riboflavin) as a chromophore [Pudasaini et al., 2015]. There are different LOV-based optogenetic platforms and they differ in how each one uses the light induced change to regulate cell signaling.

One approach is conformational changes that upon blue light absorption, flavin mononucleotide (FMN) covalently linked to the LOV domain and triggered downstream signaling pathways. In the dark, the bound FMN detaches spontaneously and the LOV domain returns to the inactive state.

One other mechanism is homodimerization that blue light-induced dissociation of a C-terminal alpha-helix (Ja) that releases steric inhibition of an effector domain and homo-dimerization is initiated in response to blue light to activate effector molecule. In additionally, in some optogenetic systems LOV domains heterodimerize with a partner; flavin-binding kelch repeat F-box1 (FKF1) that contains a LOV domain binds Gigenta (GI) following blue light activation [Pudasaini et al., 2015].

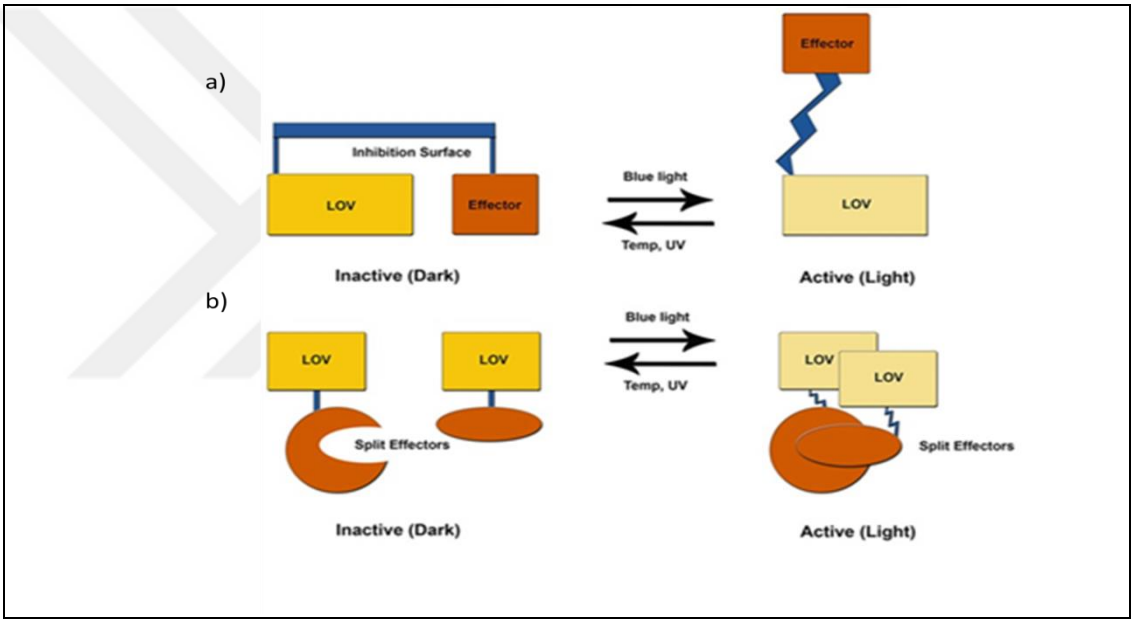

Figure 2.2: Schematic of two mechanisms of LOV tools**.** a) An effector is attached through a helical linker to create an inhibitory surface; after photoexcitation it is released to activate function of protein. b) An effector molecule is split into two inactive components. Light induces LOV-mediated dimer formation to activate the effector molecule.

- The Cryptochrome 2 protein:

Cryptochrome 2 (CRY2) is a protein from *A. thaliana* that is sensitive to blue light (405–488 nm), uses the ubiquitously expressed flavin as its chromophore to absorb. Upon exposure to blue light, CRY2 can both undergo homooligomerization and heterodimerization with its dimerization partner CIB1 (CRYPTOCHROME-INTERACTING BASIC HELIX–LOOP–HELIX 1) (Figure 2.3) [Che et al., 2015].

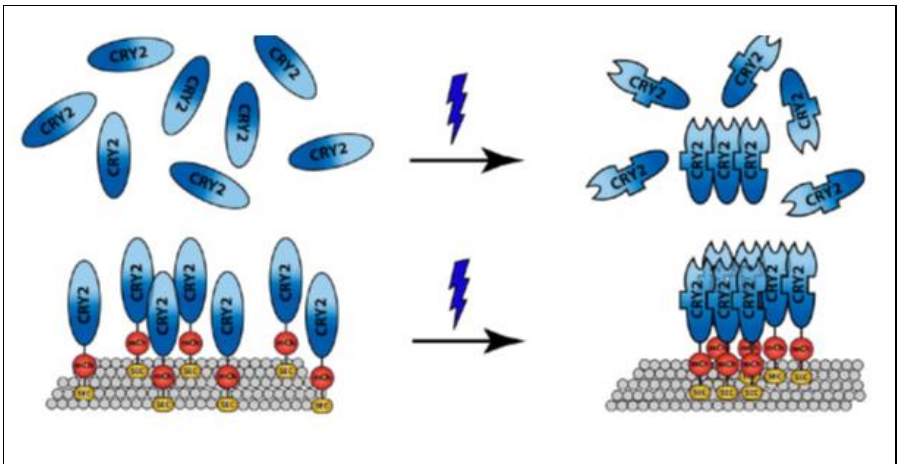

Figure 2.3: Proposed mechanism of CRY2-CRY2 oligomerization and CRY2-CIB1 heterodimerization.

- The Phytochrome B protein:

The phytochrome B protein is red (650nm)-far red (750nm) light absorbing photoreceptor in *Arabidopsis thaliana*. The phytochrome optogenetic system exploits the red light–mediated heterodimerization of the phytochrome B protein (PHYB) with its partner phytochrome interaction factor (PIF) in the presence of the chromophore PCB. Upon exposure to red light, PHYB that is bound to PCB changes conformation and binds to a PIF protein within seconds and this interaction is reversed upon exposure to infrared light within seconds (Figure 2.4) [Tischer and Weiner, 2014].

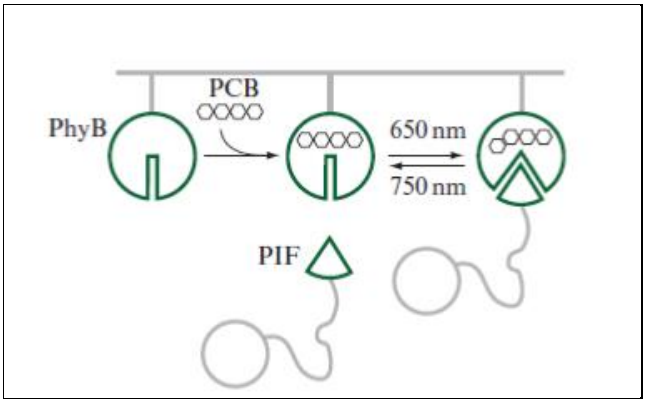

Figure 2.4: Schematic of Phytochrome B- PIF interaction.

- The Dronpa Protein:

Dronpa is a reversibly switchable photoactivatable monomeric fluorescent protein. Fluorescence of Dronpa switches off under cyan light (~500 nm) and switches on under violet light (~400 nm). The optogenetic system is most robustly used by fusing a copy of Dronpa to the amino and carboxyl termini of a protein of interest because it has low affinity for itself in dimeric state. The mutant Dronpa145N of the monomeric Dronpa that is tetrameric at low micromolar concentrations with Lys145 changed to Asn [Zhou et al., 2012]. Upon illumination with 500nm light Dronpa145N became monomeric and lost its green fluorescence, inversely, upon illumination with violet light (400 nm) tetramers were formed and the protein regained its green fluorescence (Figure 2.5) [Zhou et al., 2012].

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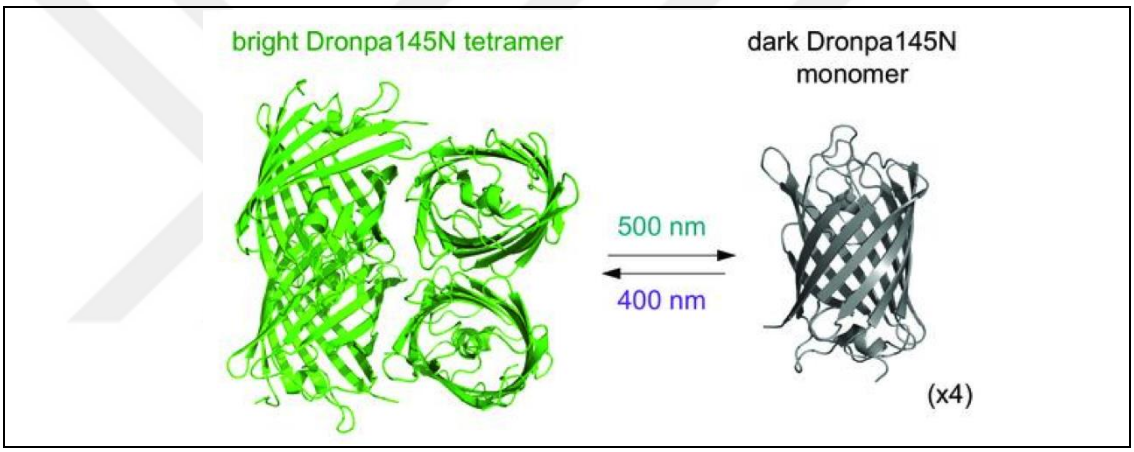

Figure 2.5: Schematic of switchable fluorescent protein Dronpa.

Optogenetic tools based on photosensitive proteins which have been developed to control diverse signaling processes in mammalian cells described above. Figure 2.6 shows the one of each optogenetic applications in mammalian cells [Bacchus et al., 2013]. A good candidate for the alternative to these applications is the circadian photoreceptor system of fruit fly, *Drosophila melanogaster* which will be explain next chapter.

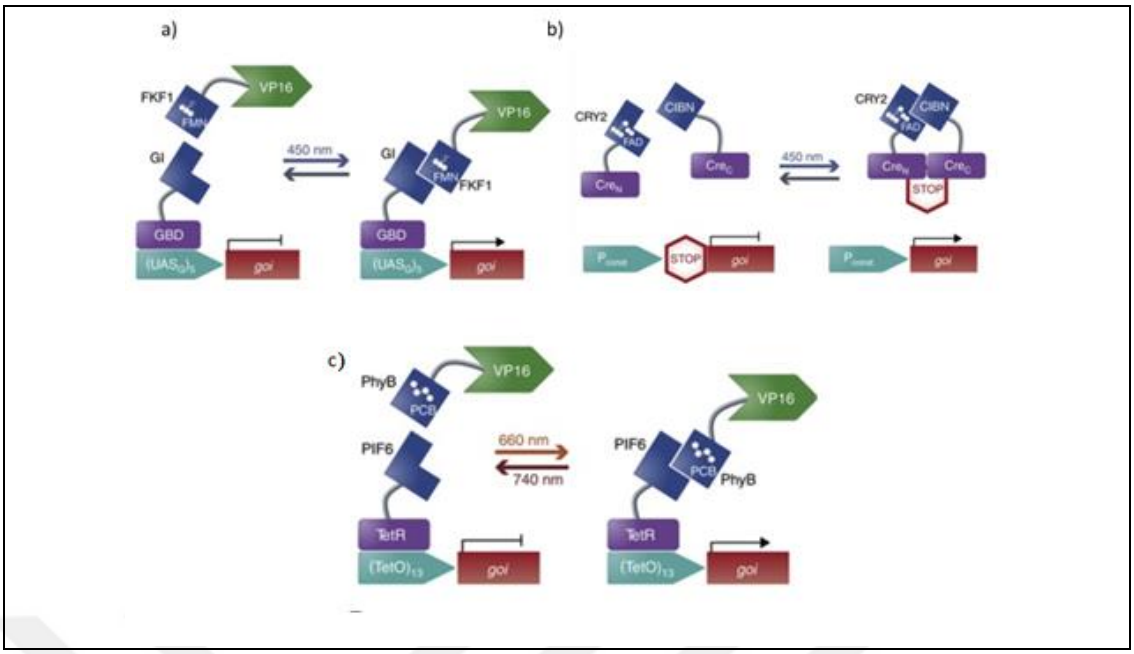

Figure 2.6: Optogenetic gene switches systems for mammalian cells. a) One of LOV-based system: Blue light inducible GI-FKF1 interaction.
b) Cryptochrome based system: Blue light inducible CRY2-CIB interaction enabled Cre recombinase lacking enzymatic activity (Cre_N and Cre_C) c) One of Phytochrome based system: Red far red light inducible PhyB-PIF6 interaction induces activation of the gene expression tethered via TetR to the $(tetO)_{13}$ operator site. Absorption of a far-red (740 nm) induces dissociation from PIF6 resulting in de-activation of the target promoter.

2.2. The Circadian Clock

The almost all organisms have circadian rhythms in their physiological functions. Circadian rhythms are oscillations which occur with a periodicity of approximately 24 h. [Sancar, 2004]. The functional biological clock occurs from core clock and photoreceptor system. While core clock is a genetic system which oscillates with a period of 24 hours, photoreceptors reset the circadian clock early in the morning with the sun light thereby keep the internal circadian clock and geological clock in the same phase [Partch et al., 2014]. The mammalian cells and *Drosophila* cells use different systems as photoreceptor even though they have similar core clock systems. Both organisms circadian core clock is composed of transcriptional-translational feedback loop (TTFL) which consist negative and positive arms [Ueda, 2007]. The positive arm regulators activate the transcription of the negative arm genes and negative arm regulators form a complex and suppress

their own gene expression by inhibiting the positive arm heterodimer-centered transcription. This feedback loop leads to a cycle in gene expression that takes approximately 24 h to complete [Bell-Pedersen et al., 2005].

In *Drosophila*, TTFL loop involves Clock (Clk) and Cycle (Cyc) transcription factors in positive arm; Period (Per) and Timeless (Tim) in negative arm. Expression of the Per (period) and Tim (timeless) genes is promoted by the heterodimeric CLK–CYC (Clock-Cycle) transcription factors. Translation of PER and TIM RNAs leads to the gradual accumulation and dimerization of PER and TIM proteins within the cytoplasm. When protein levels peak, they enter the nucleus to inhibit further CLK–CYC transcriptional activity.

In mammalian cells, in the positive arm of TTFL loop, Clock (Clk) and Bmal1 (homolog of Cycle in *Drosophila*) transcription factors trans-activate the Period(Per) and Cryptochrome (Cry) genes of the negative arm as well as circadian clock regulated genes, which represent 10-20% of all transcripts. Then, translated Period and Cryptochrome translocate into the nucleus where they inhibit Clock-Bmal1 activity by protein-protein interactions, progressively degraded, allowing the circuit to start again.

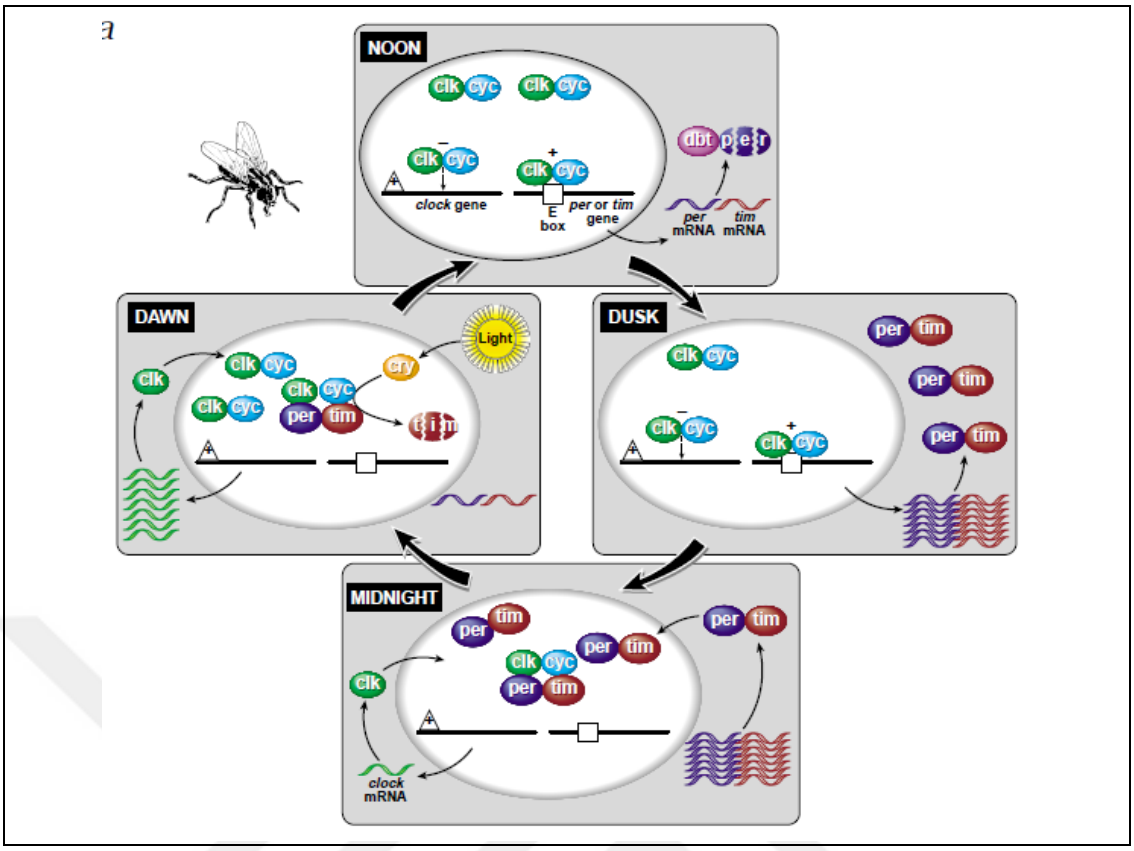

Figure 2.7: Sequence of intracellular events at the core of the circadian clock in *Drosophila*.

The biggest difference between mammalian and *Drosophila* molecular clock lies at the resetting mechanism. *Drosophila* Cry (dCRY) molecule and its mammalian homolog have different functions in the circadian clock system. *Drosophila* Cryptochrome (Cry) protein is the photosensory receptor which is covalently bounded light sensitive Flavin adenine denucleotid (FAD) cofactor and photoreception completely based on cryptochrome molecule. On the other hand, mammalian cryptochrome is not light sensitive protein; mammals use melanopsin-based photoreceptor system which is expressed only in the very low number of retinal ganglion cells. In mammals, the master clock is located in the suprachiasmatic nucleus of the brain, while peripheral clocks exist in other organs. Sunlight resets the master clock every morning through retinal ganglion cells in the eyes. In turn, the master clock resets and synchronizes the peripheral clocks.

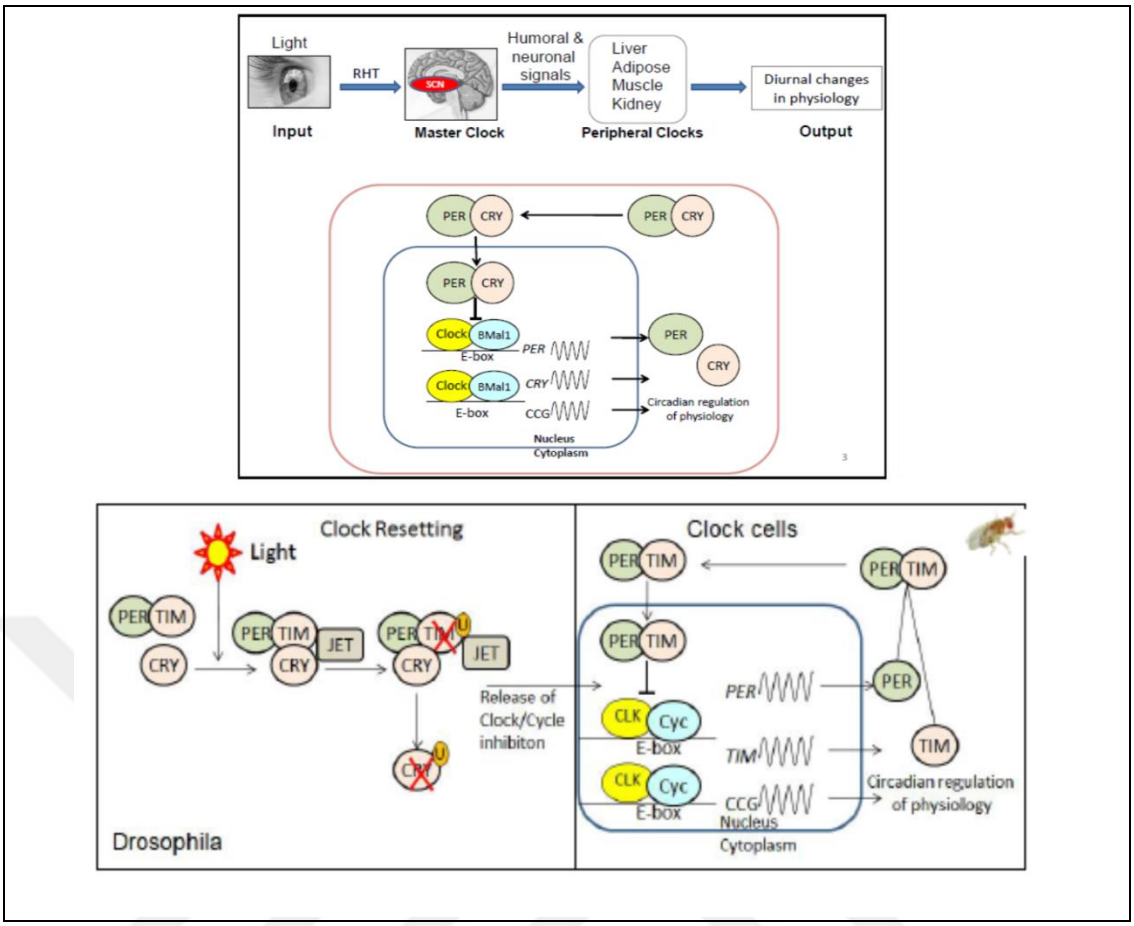

Figure 2.8: Molecular clock mechanisms comparison between mammals and *Drosophila*. Clock resetting mechanism and TTFL loops are shown at both organisms.

2.2.1. Light Dependent Interactions of *Drosophila* Cryptochrome

Drosophila cryptochrome (dCRY) is a blue light sensitive flavoprotein related to photolyases and it is primary circadian photoreceptor. Absorption of light by the Flavin cofactor in the dCRY causes conformational changes which is extension of C-terminal 20 amino acids beyond photolyase homology region (PHR) [Ozturk et al., 2011]. This conformational change allows dCRY binding to Timeless (TIM) and Jetlag (JET) allowing degradation of TIM by targets for ubiquitination which resets the circadian clock. In addition to TIM, dCRY also degrade itself upon light induction, thus allow newly synthesized TIM and PER and verify the repressive phase of circadian clock cycle [Busza et al., 2004].

The flavin cofactor of cryptochrome and photolyases families is photoreduced by electron transfer via three evolutionarily conserved tryptophan residues known as

the Tryptophan Triad. In *Drosophila* the 3 tryptophan that make up the triad are Trp-342, Trp-397, and Trp-420, respectively [Zoltowski et al., 2011]. When Cryptochrome is purified under dark conditions its cofactor FAD is found in oxidized form (FAD_{ox}). When this purified protein is exposed to light, FAD is reduced to anionic radical ($FAD^{\cdot-}$) [Ozturk et al., 2008]. This photoreduction has been suggested as the action mechanism of Cryptochrome.

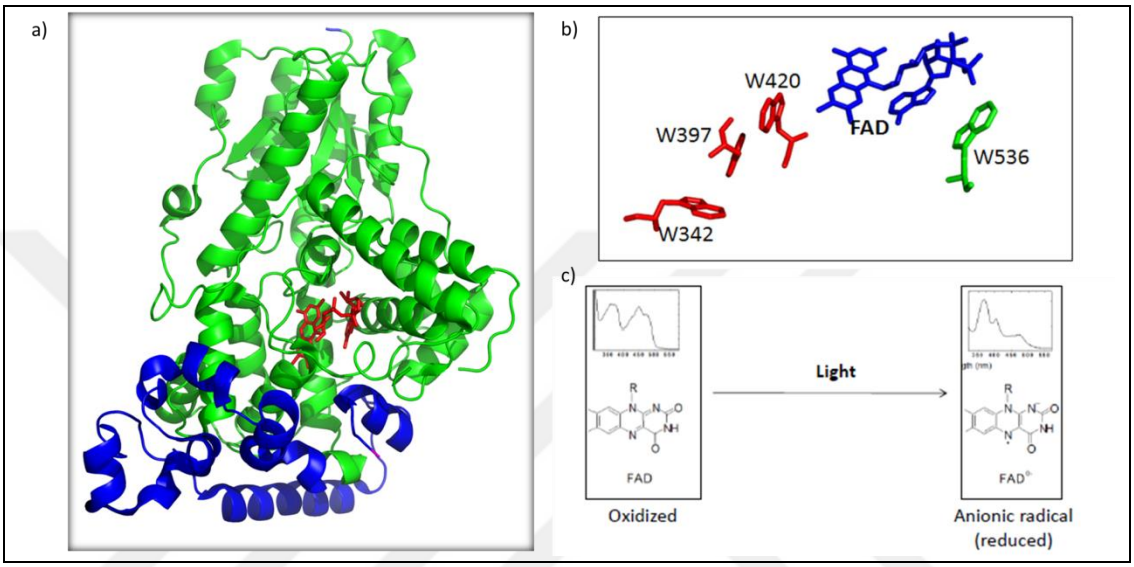

Figure 2.9: Crystal structure of *Drosophila* Cryptochrome. a) Flavin Adenin Dinucleotid shown in red. b) Flavin cofactor and Trp residues involved in photoexcitation mechanism of dCRY. c) Oxidized and reduced form of FAD.

A new tryptophan at 536 was observed in close proximity of FAD when crystal structure of Cryptochrome was published, and has been implied that this tryptophan can work as alternative photoreduction pathway. The mutation of this new residue in adition to classical trp-triad did not affect change kinetics of Timeless or Cryptochrome light-induced proteolysis. Therefore, it is suggesting that photoreduction is not part of photocycle [Ozturk et al., 2014]. Surprisingly, it is observed that both oxidized and reduced forms of Cryptochrome are able to change its conformation at similar rate, and moreover, both forms can make light-dependent interactions with Jetlag similarly. Based on these findings, it is proposed that excitation of oxidized or reduced FAD in vivo causes a conformational change in apoprotein which increases the affinity for JETLAG protein [Ozturk et al., 2014].

Light causes a conformational change in the C-terminal extension of dCRY as revealed by partial trypsinization. It is compared the fragments of both light-treated

wild-type and CryDelta (CRY**Δ)** which is C-terminal deletion mutant by probing C-terminus and N-terminus tagged. Based on experimental results, it appears that light exposure causes a small structural change in Cryptochrome at the end of the C-terminus. This conformational change is observed via enhanced trypsinization at cut sites III and IV (Figure 10a). Overall, it is concluded that light exposure opens the c-terminus of Cryptochrome. It is also shown that light exposure enhanced the interaction between Jetlag and Cryptochrome whereas CryDelta (CRY**Δ)** binds Jetlag strongly even in the absence of light. These results suggest that the light-induced conformational change observed in the C-terminus of Cryptochrome is important for the Jetlag-Cryptochrome interaction necessary for clock resetting [Ozturk et al., 2011].

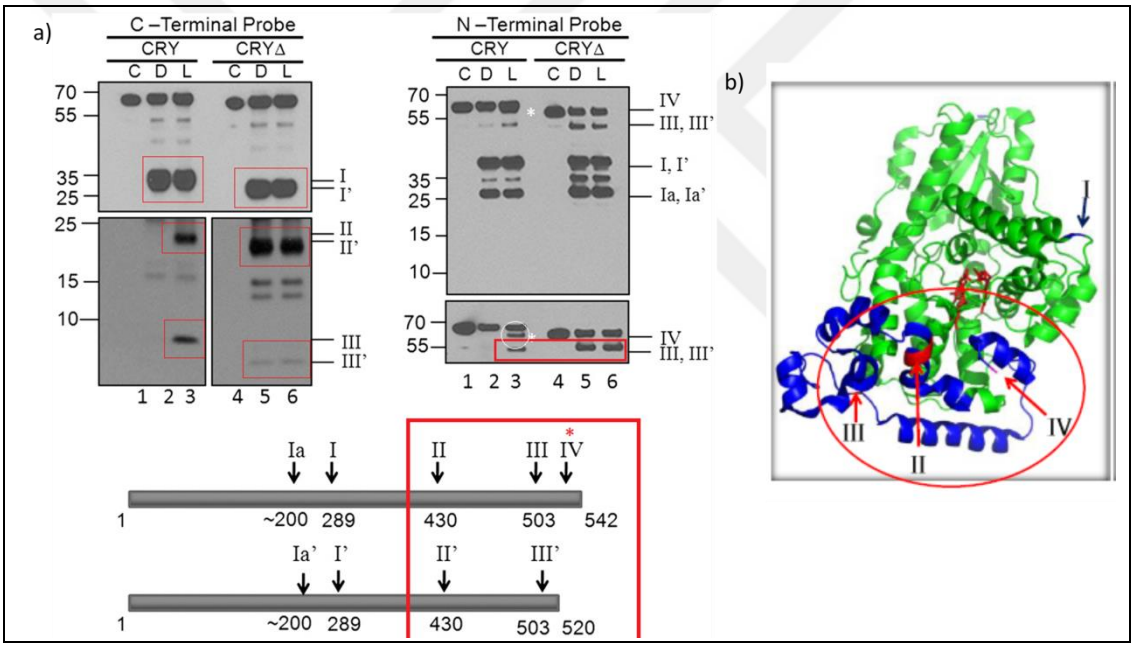

Figure 2.10: Light induced conformational change of *Drosophila* Cryptochrome. a) Partially trypsinized recombinant Cryptochrome molecules tagged at the N- or C-terminus under dark or light conditions and probed the cleaved peptides using immunoblotting. b) The cut sites identified in Cryptochrome as having light dependent accessibility for trypsinization are localized at 3D structure of *Drosophila* Cryptochrome.

Jetlag (JET) is the E3 ligase responsible for the light dependent ubiquitination and proteolysis of Timeless (TIM). However, initially it was not known whether JET was responsible for ubiquitination and degradation of dCRY. To identify the E3 ligase for dCRY proteolysis, 25 f-box protein candidates were screened and it is

found that WD40 protein, Bromodomain and WD repeat domain containing 3 (Brwd3) markedly attenuate dCRY degradation following light induction by BRWD3/DDB1/CUL4/ROC1 E3 ligase [Ozturk et al., 2013]. Light opens a docking site for JET binding by releasing the C-terminal extension of dCRY. Cryptochrome also binds to TIM at the same time; thereby bringing TIM and JET into close proximity with each another to ubiquitylate TIM for degradation by UPS system resulting in the circadian clock resetting. Light also promote dCRY binding to Brwd3 and this bindings lead to degradation of dCRY at slow rate following the TIM degradation, thus the clock resets only once in a day (Figure 11).

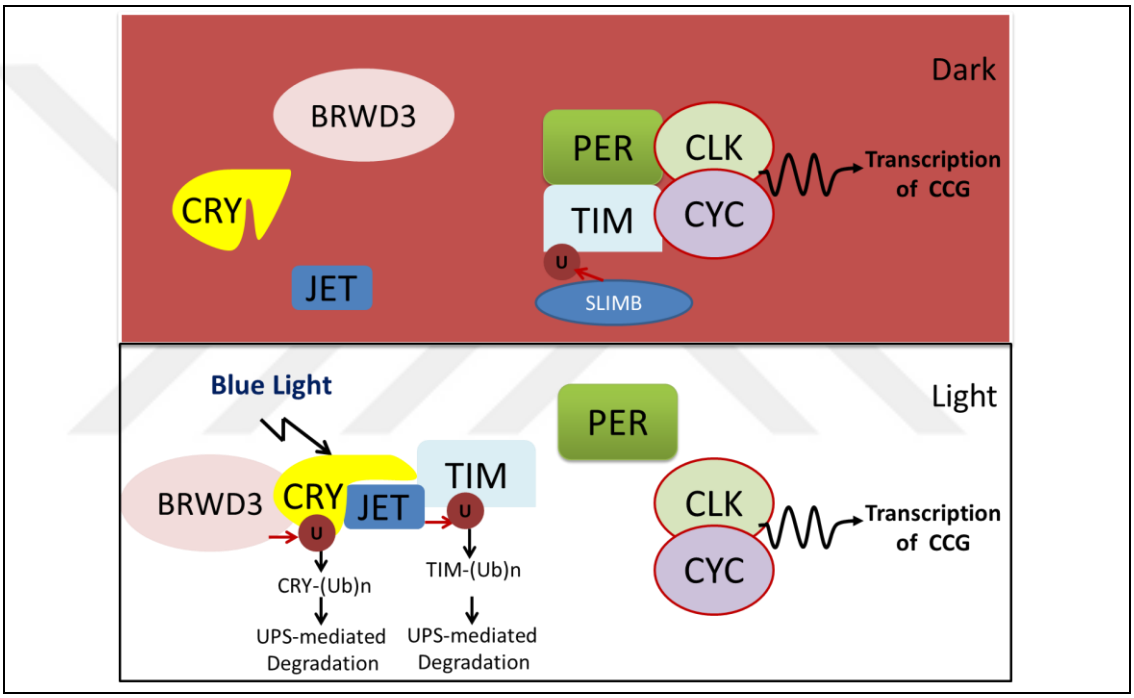

Figure 2.11: Clock resetting model in *Drosophila* by ubiquitin E3 ligases. Under dark conditions, CRY is not in complex with either JET or BRWD3. Under condition of light exposure dCRY change conformation enables it to bind to TIM, JET and BRWD3.

2.2.2. Light-induced Interaction Between dCRY and JET

Physical association between dCRY and JET proteins is observed in yeast two-hybrid system and *Drosophila* cells [Peschel et al., 2009] (Figure 12a).

Light dependent interaction between these proteins can be shown with in vitro recombinant proteins. Recombinant GST-JET bound to sepharose beads incubated with CRY under dark or exposed to blue light (1 $mWcm^{-2}$,) then, pull-down

performed and bound proteins were visualized by immunoblotting (Figure 12b). Mutant Cry used as positive control and it binds JET strongly even in dark condition. Wild type CRY interacts weakly with JET under dark, however light exposure promotes strong interaction between these proteins. Next, it is measured the half-life of the conformational change as well as the half-life of the CRY-JET interaction. Both half-lives were approximately 15 min and in agreement with each other (Figure 12c) [Ozturk et al., 2011].

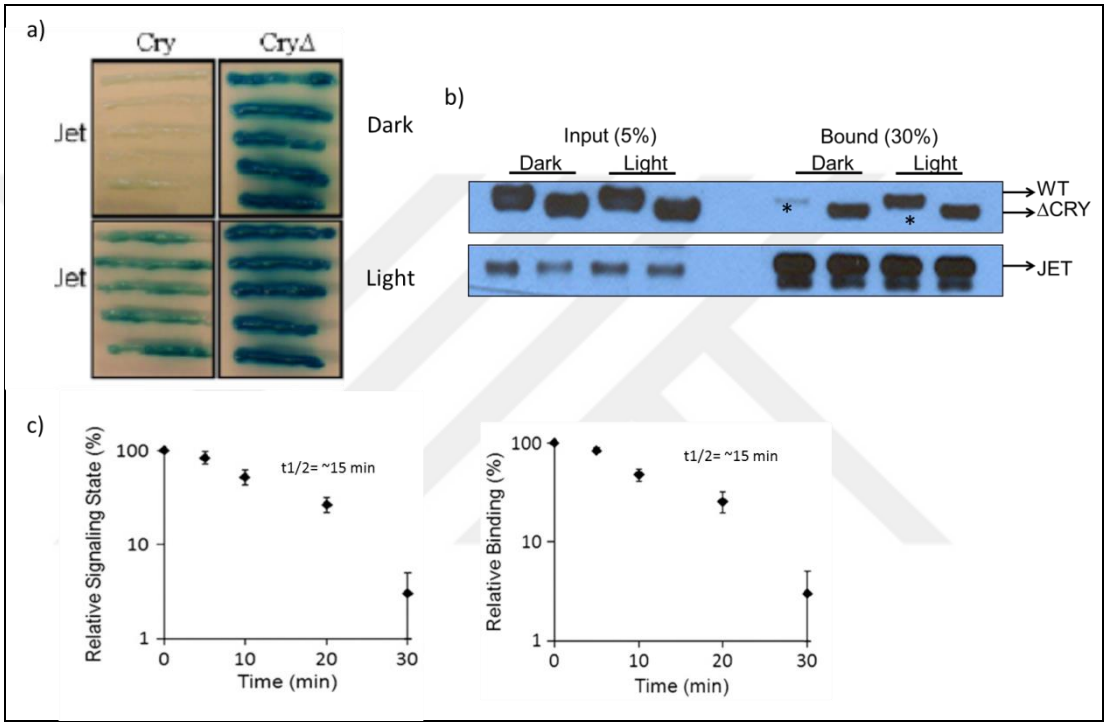

Figure 2.12: Cryptochrome (dCRY) and Jetlag(JET) proteins light dependent interactions. a) In yeast cells, JET and dCRY interaction is observed by substrate color change following galactosidase gene expression. Mutant Cry form used as positive control. b) In vitro light activated interaction of dCRY and JET. c) Quantitative analysis of decay of the light induced signaling state and dCRY amount bound to JET.

Previous studies revealed that increasing doses of 366nm light up to 120min cause the degradation of dCRY at 50% percentage in *Drosophila* S2 cells. Interestingly, it found that even a human embryonic kidney cell line (HEK293T) is capable of photoinduced proteolysis of dCRY. In contrast, no degradation of ZfCRY4 is detected within the dose range used [Ozturk et al., 2009]. However, subsequent studies showed that low intensity of light (0,1 $mWcm^{-2}$) is enough to

observe light manner protein-protein intracellular interactions [Ozturk et al., 2014] and would expected to cause very little dCRY degradation.

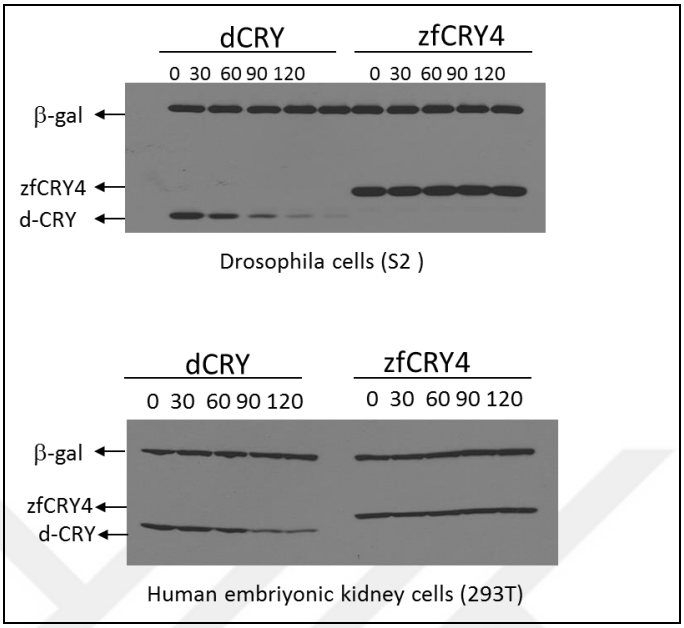

Figure 2.13: Light-induced proteolysis of CRYs in different cell lines.

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1: List of kits and reagents.

CheckMate™ Mammalian Two-Hybrid System	E2440, Promega, USA
Dual-Glo® Luciferase Assay System	E2920, Promega, USA
FuGENE® Transfection Reagent	E2691, Promega, USA
Effectene® Transfection Reagent	301425, QIAGEN, USA
Nucleospin Plasmid Kit	740588.50, Macherey Nagel, Germany
PCR clean-up Gel extraction Kit	740609.50, Macherey Nagel , Germany
Nucleospin RNA Kit	740955.50, Macherey Nagel, Germany
cDNA synthesis Kit	E6300S, New England Biolabs (NEB), USA
Maxima SYBR Green/ROX qPCR Master Mix	K0221, Thermo Scientific, USA
Bradford Protein Assay Dye	5000006, Bio-Rad, USA
Protease Inhibitor Coctail Tablets	S8830-2TAB, Sigma-Aldrich, USA
Protein Molecular Weight Marker	P7712S, New England Biolabs,USA
Chemiluminescent HRP Substrate	K-12043-D10, Advansta, USA
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
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol
1X TBST	1X TBS 0.1% Tween-20
5% Blocking Solution	5% non-fat dry milk in 1X TBS-T
10X TBE	108g Tris base 55g Boric acid 40 ml 0.5M EDTA (pH 8.0)
4X Protein Loading Dye (Laemmli)	250 mM Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 20% β-mercaptoethanol 0.008% Bromophenol Blue
Cell Lysis Buffer (RIPA)	750 mM NaCl 250 mM Tris (pH: 8.0) 0.1% SDS 1.0% NP-40
10X PBS	BE17-517Q, Lonza, Belgium
Dulbecco's Modified Eagle Medium (DMEM)	41965-039, Gibco Invitrogen, USA
0.05% Trypsin-EDTA	25300-054, Gibco Invitrogen, USA
Fetal Bovine Serum (FBS)	10270-076, Gibco Invitrogen, USA
100X Penicillin/Streptomycin	15140-122,Gibco Invitrogen, USA
Dulbecco's Modified Eagle's Medium - low glucose	D2902, Sigma-Aldrich, USA
HEPES Buffer	P05-01100, PAN, Germany
LB Broth Medium	LBP03, Caisson Labs, USA
LB Agar	LBP04, Caisson Labs, USA

Table 3.3: List of SDS-PAGE gel recipe.

12% separating gel	5 ml	10 ml	15 ml
ddH2O	2.15	4.3	6.45
40% Acryl:Bis	1.5	3	4.5
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

8% separating gel	5 ml	10 ml	15 ml
ddH2O	2.65	5.3	7.95
40% Acryl:Bis	1	2	3
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	0.003	0.006	0.009

5% Stacking Gel	2 ml	4 ml	6 ml	8 ml
ddH2O	1.46	2.92	4.38	5.84
40% Acryl:Bis	0.25	0.5	0.75	1
1 M Tris pH 6.8	0.25	0.5	0.75	1
10% SDS	0.02	0.04	0.06	0.08
10% APS	0.02	0.04	0.06	0.08
TEMED	0.002	0.004	0.006	0.008

3.3. Antibodies

Table 3.4: List of antibodies used in this study.

Name	**Dilution**	**Source**
Anti-V5	1:2000	V8012, Sigma-Aldrich
Anti-p21/Waf1/Cip1	1:1000	#2946, Cell Signaling Technology
Anti-βactin	1:5000	#4967, Cell Signaling Technology
Anti-mouse, HRP	1:3000	#7076, Cell Signaling Technology
Anti-rabbit, HRP	1:3000	#7074, Cell Signaling Technology

3.4. Equipment

Table 3.5: List of equipment.

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 (CO2)	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
Microplate Reader	Varioskan Flash, Thermo Scientific, USA
Blue light lamb	MLP 58 Multiple ray lamb, UVP, Canada
Power Supply	Bio-Rad, USA
PCR Thermal Cycler	Bio-Rad, 41798
RT-qPCR system	Agilent Technologies Mx3005P QPCR
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

4.1.1. Digestion of DNA with Restriction Enzymes

In separate tubes, the plasmid vector and the DNA were digested with the appropriate restriction enzyme(s) and buffer(s). In a microfuge tube, the mixture was set up for each digesting reaction took place in 50 µl reaction volume: 2µl DNA (1-2 µg), 2 µl 10x restriction enzyme buffer, 1 µl restriction enzyme(s), ddH2O to a final volume of 50 µl. The reaction was performed in microfuge tubes at 37°C for 1-2 hours.

4.1.2. Agarose Gel Electrophoresis

Double digested vectors and PCR amplifications were run on 1% agarose gel, in horizontal electrophoresis tank filled with 1X TBE.

In order to isolate the double digested vector from the digestion solution, entire digested vector was run in agarose gel and then was extracted. Gel was cut from the borders of digested vector with a scalpel under UV light, cutting it as small as possible. To extract DNA from gel, Macherey-Nagel Nucleospin Gel and PCR Clean-up kit was used according to manufacturer's instructions.

4.1.3. Ligation of Inserts into Plasmids

The enzyme used to ligate DNA fragments into vectors called T4 DNA ligase. The insert calculation was done by applying 1:3 molar vector to insert ratio and reaction was performed in 10 µl with a final concentration of 1X T4 ligation buffer at 4^{o}C overnight. The reaction mixture: 3µl Plasmid vector- 4µl Insert DNA(depend on DNA amount), 2µl 5x T4 DNA ligase Buffer, 1µl T4 DNA ligase.

4.1.4. Transformation to Competent Bacteria

Escherichia coli DH5α competent strain was used for transformations. Competent cells were taken from -80^{o}C freezer and thawed on ice. 5 µl of ligated plasmids were added on competent cells and were incubated for 30 minutes on ice before heat shock at 42^{o}C for 1 minute. Samples were then cooled on ice for 1,5 minutes, ~700 µl Luria broth (LB) was added on tubes and they were incubated in shaker at 37^{o}C for 1 hour to give them enough time to express the antibiotic resistance gene. 200 µl of the samples were spread at ampicillin containing LB agar plates and incubated overnight. Single colonies were taken into 5 ml ampicillin containing LB medium by micropipette tip and were incubated overnight for plasmid isolation.

4.1.5. Plasmid Isolation

To isolate the plasmid, Macherey-Nagel Nucleospin Plasmid Isolation kit protocol was followed. This protocol begins with centrifuging the lysate of bacteria pellet from the overnight solution, followed by binding the DNA to a filter column. The filter was then washed and DNA eluted. To quantify concentration and purity of the isolated plasmid, NanoDrop spectrophotometer was used to measure concentration and read OD260 and OD280 values.

4.1.6. PCR Amplification of Constructs with Cloning Primers

Platinum Pfx DNA Polymerase was used in the PCR reactions and the PCR master mix for one sample was as follows: 5 µl 10X Pfx buffer; 1 µl MgSO4 (50 mM final concentration); 1,5 µl dNTP (10 mM final concentration); 1 µl forward and reverse primer mix (10 µM final concentration); 1 µl Pfx DNA polymerase; 200ng template DNA; ddH2O final volume to 50 µl . The PCR reaction was as: initial denaturation at 94^{o}C for 5 minute; 33 cycles of denaturation, annealing and elongation at 90^{o}C for 15 seconds, 60^{o}C for 30 seconds and 68^{o}C for 1,5 minutes respectively; final elongation at 68^{o}C for 5 minutes.

4.2. Cell Culture

4.2.1. Maintenance of the Cell Lines

All the cell lines were incubated at 37^{o}C and 5% CO2 environment, provided by the CO2 incubator, and were cultured in complete high glucose DMEM: 10% FBS, 100U/ml penicillin & 100µg/ml streptomycin.

Passaging was done by first detaching the adherent cells from the flask using 0.05% trypsin-EDTA solution and incubating the flask at 37^{o}C then the trypsin activity was inhibited by adding complete DMEM. In order to keep the cells healthy, they were sub-cultivated at a ratio of 1:6, and the process was repeated every 3 to 4 days.

4.2.2. Transfection of HEK293T Cells

HEK293T cell line was used for transient and stable transfection purposes. PEI, polyethyleimine is a transfection method that condenses DNA into positively charged particles that bind to anionic cell surfaces, consequently the DNA:PEI complex is endocytosed by the cells. To transiently transfect HEK293T cells, they were seeded into 6-well plate at 60% confluency 1 day before transfection. On the day transfection, in an Eppendorf tube, appropriate concentration of DNA is diluted with serum-free media and mix well pipetting. PEI is added to diluted DNA solution at 3:1 ratio of PEI:DNA (e.g., 9 µl PEI for 3 µg DNA). The mixture was incubated for 15 minutes and then was added to the fresh medium of cells by dropwise.

Effectene Transfection Reagent-QIAGEN and FuGENE HD Transfection Reagent-Promega were used, especially stable transfections. Suggested volumes for both transfection reagents with various multiwell formats were indicated at manual instructions of kits.

4.2.3. Generation Stable Cell Line and Limiting Dilution

Stably-transfected cells can be selected by using culture medium with drugs, if the expression plasmid carries a drug resistance gene. Transfection was done at 6-well plate. Antibiotic treatment was applied after 36 hrs.

Transfected cells at 6well plate transfer to 96-well plate by serial dilution. 100 µl medium in each well is added. 100 µl of cell suspension is added to the first column, 100 µl is carried over to the next column after gentle up and down pipetting, thereby diluting in a ratio of 1:2. This procedure is repeated for each consecutive column and finally 100 µl from the last column is discarded.

Cells are feed at every 2–3 days with selection medium. For the selection of stably expressing cells, cells are growth in medium with the appropriate amount of antibiotic. Cell death is checked after 7–14 days by light microscopy.

- Light Treatment:

In light conditions, cells were held under blue light multiple ray lamb (365 nm). Filters were used to adjust exposure dose of light. Cells were incubated with Atmospheric Medium at 37^{o}C warm room during light or dark treatment.

4.3. Mammalian Two-Hybrid Assay

Mammalian Two-Hybrid System provides a method for detecting interactions between two proteins in mammalian cells. The CheckMate™ Mammalian Two-Hybrid System was used to show interaction between *Drosophila* Cryptochrome and Jetlag which results transcription of the firefly luciferase reporter gene. Dual-Glo Luciferase Reporter Assay System () was used quantitated luciferase reporter gene.

4.3.1. The Dual-Glo® Luciferase Assay System

This system is used to high-throughput analysis of mammalian cells containing genes for firefly and Renilla luciferases grown in 96- or 384-well plates.

HEK293T cells were seeded 2x10^{4} at 96-well luciferase plate and next day they were transfected. 2 days after transfection, a volume of Dual-Glo® Luciferase

Reagent was added equal to the culture medium volume to each well and mix (For 96-well plates, 75µl is appropriate).After 10 minutes of incubation at room temperature, the firefly luminescence was measured at microplate reader until generate optimal results. A volume of Dual-Glo® Stop & Glo® Reagent was equal to the original culture medium volume to each well and mix. After 10min. incubation Renilla luminescence was measured. The ratio of luminescence from the experimental reporter to the control reporter was calculated and ratios were normalized. Relative Response Ratios can then be calculated from the normalized Ratios

4.4. Western Blotting

4.4.1. Sample Preparation

Cell lysates were collected first by removing the medium over the cell monolayer in well plates or culture dishes and the cells were detached using a cell scraper after adding 1ml 1X PBS. Cell suspension was taken into 1.5ml tube and centrifuged at 5000 rpm for 3 minutes. Supernatant was discarded and cell pellet re-suspended in cold 1X RIPA buffer according to pellet volume with freshly added protease inhibitors. The tube was incubated on ice 20min. dissolve the pellet by gently pipetting or vortex gently every 10 minutes, and centrifuged at 13,000rpm for 15 minutes at 4^{o}C after 20 minutes of incubation. The total protein lysate, which was in the supernatant, was transferred into a new 1.5 ml tube.

4.4.2. Determine of Protein Concentration

With a small volume (1-2 µl) of supernatant, Bradford assay was performed. Protein concentrations of each cell lysate were measured with Bradford Assay. BSA samples which concentrations are known are used to determine unknown protein concentrations via absorbance values. Absorbance is measured at 595nm. (Biorad Protein Assay Dye Reagent Concentrate, Cat: 500-0006 is used).

The concentrations of lysates are equalized by diluting them with the appropriate amounts of RIPA lysis buffer. ~50 µg of each sample are loaded SDS-

PAGE gel with 4X Laemmli loading buffer that includes 5% volume of beta Mercaptoethanol (Final concentration of laemmli buffer should be 1X into samples)

- Sample preparation- 2:

In some conditions, cells were lysed directly in 1X Lysis Buffer which contains 1X Laemmli Buffer added 1% Beta-Mercaptoethanol. After the medium was removed, the cells attached to monolayer were washed with 1X PBS, and then 1 X Lysis Buffer added onto wells according to cell confluency. After incubation 10 min at room temperature with shaking, cells were transferred into Eppendorf tubes from plate.

4.4.3. SDS-PAGE Gel Preparation

10ml Separating SDS-PAGE gel (Table 3.3) was prepared in a falcon tube and poured between the glasses which were placed in the gel casting system. 2-propanol was layered top of the gel to prevent any bubbles and drying. After separating gel was frozen, stacking gel was prepared (Table 3.3) and comb was inserted on the gel and shaken if necessary to get rid of any bubbles that could have been stuck between the gel and the comb. After the polymerization of the gel (approximately 30 minutes), comb was removed and the gel was moisten with dH2O and stored in 4°C for later use or was put in the vertical electrophoresis system which was filled with 1X running buffer (Table 3.2).

4.4.4. SDS-PAGE Gel Electrophoresis

Protein samples were incubated at 95°C for 10 minutes, and then they were shortly spun. Samples were loaded each well of the gel respectively. 1X SDS Running Buffer was used for electrophoresis. It is prepared as 10X Stock Solution and diluted to 1X with distilled water before using.

The gel run at 80 V until proteins reach separating gel, then the voltage might be increased up to 120-150V. Electrophoresis stop when loading dye reached end of the gel.

4.4.5. Transferring the Protein from the Gel to the Membrane:

Trans-blot Turbo Transfer System (Biorad Transblot Turbo Transfer System, Serial No: 690BR011760). 4 blotting papers and 1 nitrocellulose membrane with the same dimensions of SDS PAGE gel were cut. The blotting paper and the nitrocellulose membrane were incubated with 1X transfer buffer and then a transfer sandwich was created.

Assemble the Blotting Sandwich: The components were assembled on the cassette in the order as follow: Top(-) Cassette electrode (cathode)-Two Blotting papers -Gel-Blotting Membrane-Two blotting papers-Bottom (+) cassette electrode (Anode). A roller was used to remove any air trapped between the layers. Transfer at 12V constant voltage for 90 min at Transblot transfer system.

4.4.6. Antibody Incubation

Membrane was taken into a case filled with 5% blocking solution for 1 hour at room temperature after transfer was completed. The primary antibody was diluted in 5% blocking solution as recommended in its datasheet and the membrane was incubated with it over-night at 4^{o}C. The membrane was washed three times (5-15-5-5min. respectively) with 1X TBST and was then incubated in horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBST as recommended in it datasheet) for 1 hour at room temperature.

4.4.7. Chemiluminescence Imaging of the Membrane

By the end of the secondary antibody incubation, the membrane was washed three times with TBS-T. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation (In our laboratory, Western Blotting Detection Kit Advansta Cat: K-12043-D10 and Signal Fire ™ ECL Reagent , Cell Signaling Cat: 6883S substrates are used).

Western Blotting Detection Kit Advansta includes Western Bright Peroxide (Component 1) and Western Bright Sirius (Component 2). Working solution mix is prepared with equal aliquot of component 1 and 2. The membrane is exposed to the

mix for 2 minutes.Signal Fire ™ ECL Reagent includes ECL Reagent A and B. Working solution mix is prepared with equal aliquot of Reagent A and B. The membrane is exposed to the mix for 1 minute.

The chemiluminescent signals were captured by using an imager (BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545 is used).

4.5. RT-qPCR

4.5.1. RNA Isolation from Cell Lines

The cells were collected from 6-well plates by detaching them using trypsin, then they were centrifuged, supernatant was discarded and the pellet was suspended with 1X PBS. To lyse the cells and isolate total RNA, Machery Nagel NucleoSpin RNA Isolation kit was used and the manufacturer's instructions were followed. Freshly isolated RNA's were stored at -80°C freezer.

4.5.2. cDNA Synthesis

Total RNA samples, isolated from the cells, were used as a template for cDNA synthesis. ProtoScript® First Strand cDNA Synthesis kit was used and the manufacturer's instructions were followed. 850ng of RNA and oligo d(T) primer was used at the beginning of the reaction. Oligo d(T) binds to the poly(A) tails of the mRNA's and starts their reverse transcription in the presence of reverse transcriptase, resulting in the production of the total cDNA.

4.5.3. Real Time PCR (qPCR)

In order to compare the target gene expression level differences between the cells, mRNA levels of target gene was measured by real time PCR. Maxima SYBR Green qPCR Master Mix was used according to the manufacturer's instructions. One typical reaction includes 12,5 µl 2X master mix, 2 µl primer mix, 2 µl cDNA (diluted) and 8,5 µl dH2O. Reaction was run Agilent Technologies Mx3005P QPCR

system. Steps of PCR reaction was as follows: initial denaturation step first at 50°C for 2 minutes then at 95°C for 10 minutes, then 40 identical cycles of denaturation at 95°C for 15 seconds followed by annealing step at 60°C for 30sec. and elongation 72°C for 30sec. then 72°C for 10min. After every cycle, fluorescence data was acquired by real time PCR system. Analysis of the raw data was done by ΔΔct method, by basically calculating the median of the replicates (for both p21 mRNA and GAPDH mRNA), then exponentiation was done by taking number 2 as base and using the calculated median as exponent. (This calculation for p21 mRNA was then divided to the GAPDH mRNA calculation.).

5. RESULTS

The main purpose of this thesis is to develop a molecular tool to control endogenous gene expression and protein activity by light in mammalian cells. For this purpose, we exploited the light-dependent interaction between *Drosophila* Cryptochrome (dCRY) and Jetlag(JET) proteins in mammalian cells.

As a first step, this light dependent interaction was studied with two-hybrid mammalian system and Dual-Luciferase Reporter system.

5.1. Mammalian Two-Hybrid Assay

Mammalian two-hybrid assay is one of the most powerful methods to investigate protein-protein interactions. In this system, the pBIND vector that contains the yeast GAL4 DNA-binding domain and the pACT vector that contains the herpes simplex virus VP16 activation domain upstream of a multiple cloning region are used with pG5*luc* vector that contains GAL4 binding sites which in turn is upstream of the firefly luciferase gene (luc+). One protein of interest is cloned into a pBIND plasmid, while the other interacting partner protein is cloned into pACT plasmid. PG5*luc* plasmid carries firefly luciferase reporter gene fused with of five GAL4 DNA-binding domains. When the two candidate protein interact, their fused partners GAL4 and VP16 comes in close proximity, thus it leads to recruitment of RNA polymerase II complex onto the promoter of luciferase gene. Consequently, this binding increases the firefly luciferase reporter gene transcription.

To observe the light dependent interaction between *Drosophila* CRY and JET, HEK293T cells were co-transfected with pACT-dCRYV5H, pBIND-V5-JET and pG5*Luc* plasmids. The interaction between dCRY and JET by light, expressed as GAL4-dCRY and VP16-JET fusion constructs, resulted in an increase in luciferase expression under the light over dark conditions (Figure 5.1).

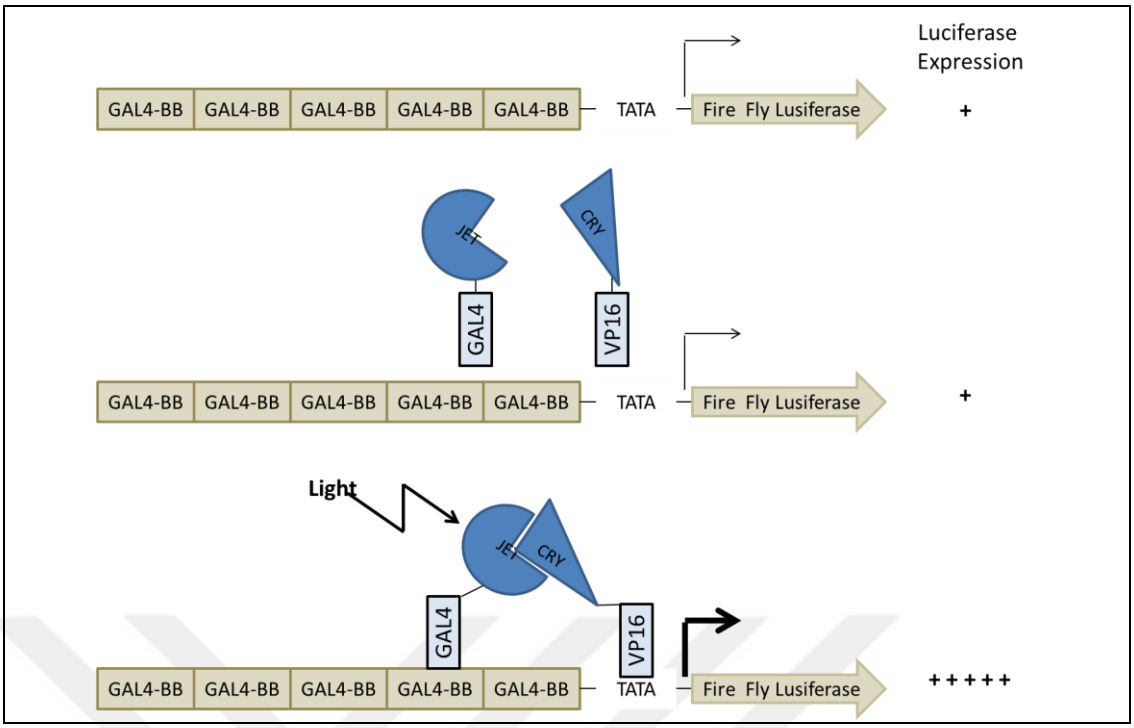

Figure 5.1: Schematic representation of dCRY:JET interaction system in the mammalian cells.

5.1.1. Gene Cloning of Two-Hybrid Mammalian System Constructs

To make pBIND-V5-JET construct, pAc5.1-V5-JET was amplified with V5-Jet primers. In order to insert V5-JET into pBIND vector, we isolated BamHI/XbaI- V5-Jet fragment by restriction enzyme digestion of pAc5.1-V5-JET. The same enzymes were used to linearize pBIND vector.

To make pACT-V5-dCRY construct, pcDNA3-dCRYV5His was cut with XhoI/XbaI. To insert V5-dCRY into pACT, vector was cut with SalI/XbaI. SalI site on pACT fused with XhoI site.

The digested vectors were loaded 1% agarose gel and DNA was eluted from the gel with Macherey-Nagel Nucleospin Gel and PCR Clean-up kit (Figure 5.2).

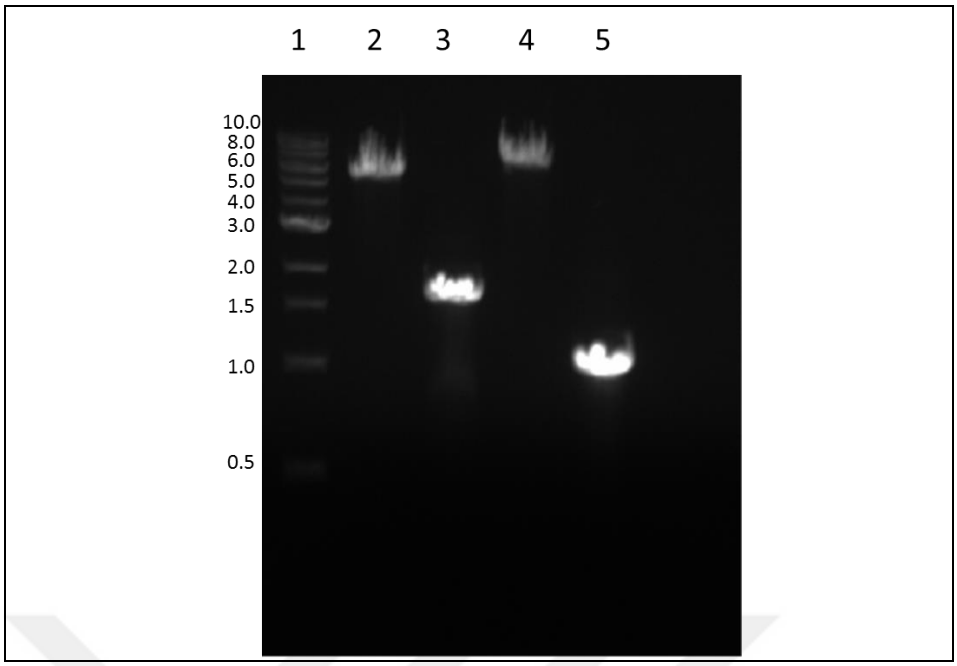

Figure 5.2: Restriction Digestion Analysis of pACT and pBIND vectors; V5-dCRY and V5-JET inserts. Lane 1: 1 kb DNA size marker Lane 2: SalI-pACT-XbaI Lane 3: XhoI-V5-dCRY-XbaI Lane 4: BamHI-pBIND-XbaI Lane 5: BamHI-V5-JET-XbaI.

The purified XhoI-V5-dCry-XbaI fragment was ligated into the SalI-pACT-XbaI vector and BamHI-V5-Jet-XbaI was then ligated into BamHI-pBIND-XbaI vector (Figure 5.3).

The ligation reaction was transformed into competent *E. coli* DH5α cells as described in methods section, and plated onto media with ampicillin.

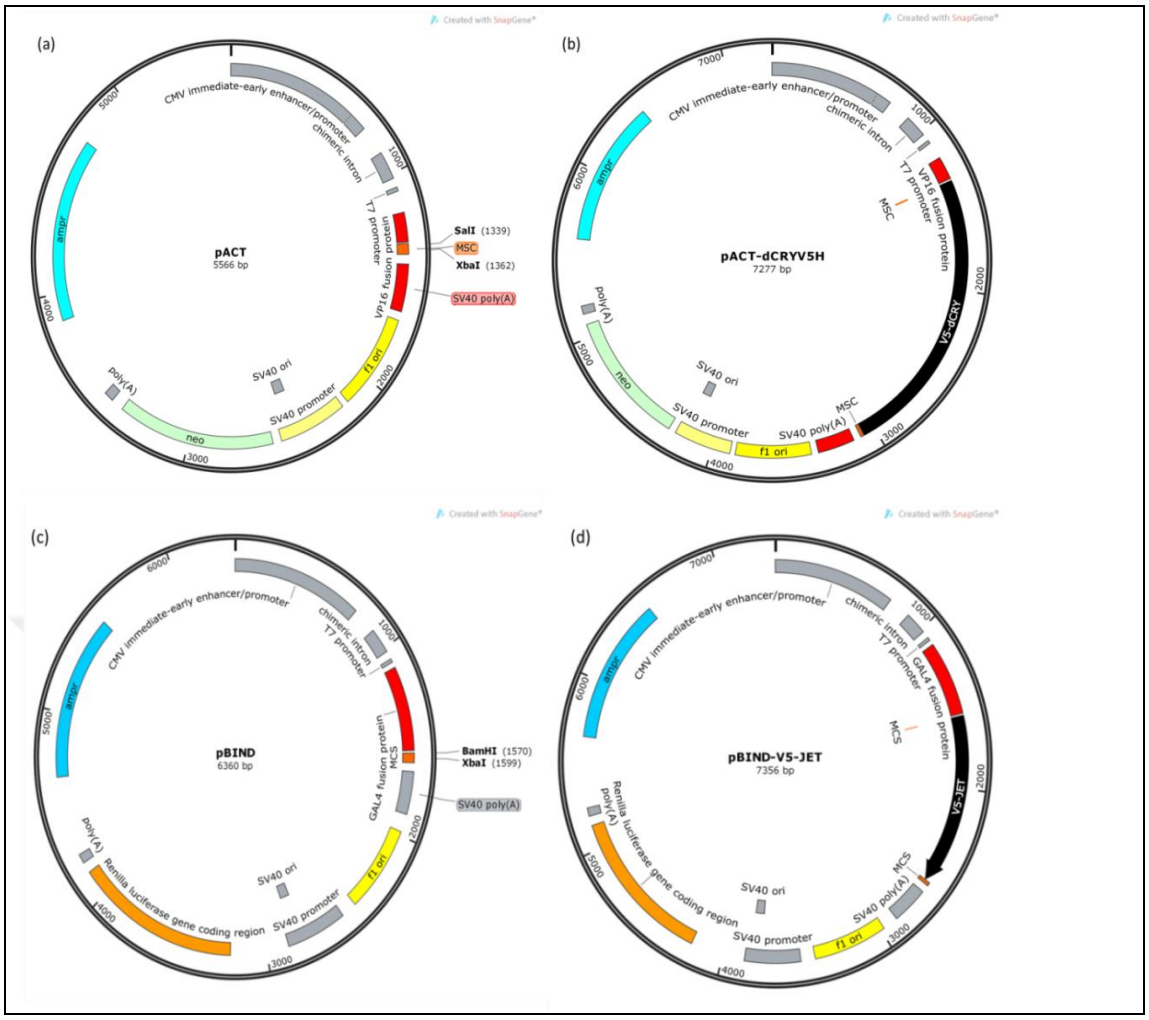

Figure 5.3: a) pACT and c) pBIND vector maps, represention of b) pACT-dCRYV5H and d) pBIND-V5-JET ligations. Plasmids are created by SnapGene.

The single colonies were picked from the plates and were grown over night, then plasmid DNA was purified using Nucleospin Miniprep Kit. To test correct insertion into plasmids, each purified plasmid DNA was restricted (Figure 5.4).

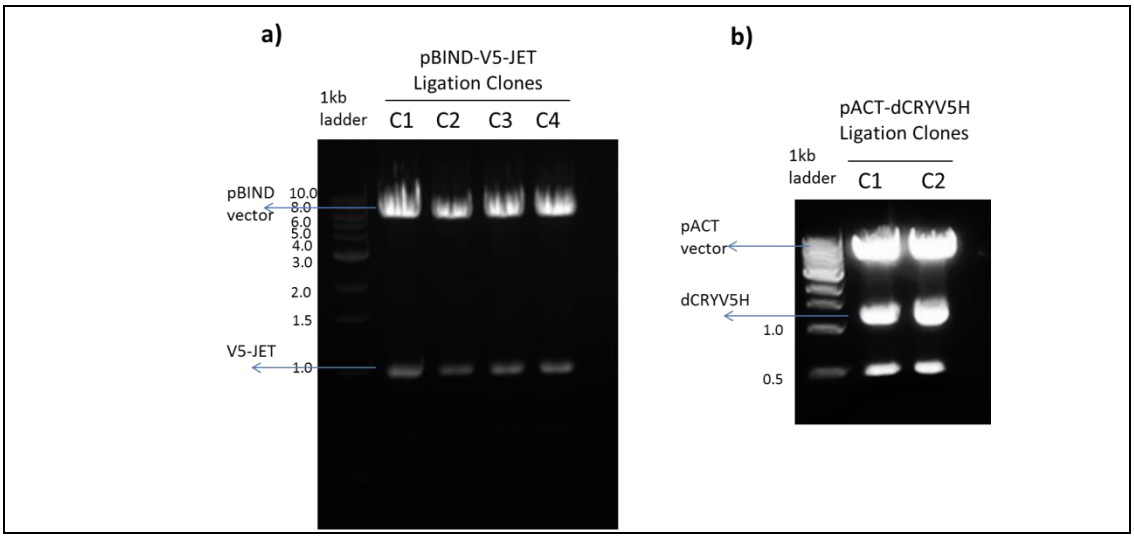

Figure 5.4: Agarose gel images of digested ligation products. a) pBIND-V5-Jet clones were digested with BamHI-XbaI. V5-JET insert size ~1.0kb b) pACT-V5-dCry was digested with NotI-BamHI. The dCRYV5H insert size ~1.7kb.

5.1.2. Luciferase Gene Reporter Assay

In order to confirm the interaction between dCRY and JET, HEK293T cells were co-transfected with pACT-V5-dCRY, pBIND-V5-JET and pG5*luc* plasmids. The 2:2:1 plasmid-DNA ratio was used as pACT-V5-dCRY: pBIND-V5-JET: pG5*luc*, respectively. FuGENE HD Transfection reagent was used according to manufacturer instructions. 48 hours after transfection, cells were exposed to blue light or kept in dark at 37^0C during 6 hours. Then, the firefly luciferase activity is quantified by Dual-Glo® Luciferase Assay System. According to result, the cells which were exposed the light had 2, 5 fold increased luciferase activity compared to the cells under dark condition. Thus it is indicated that JET-dCRY interaction upon light treatment induced the luciferase expression (Figure 5.5).

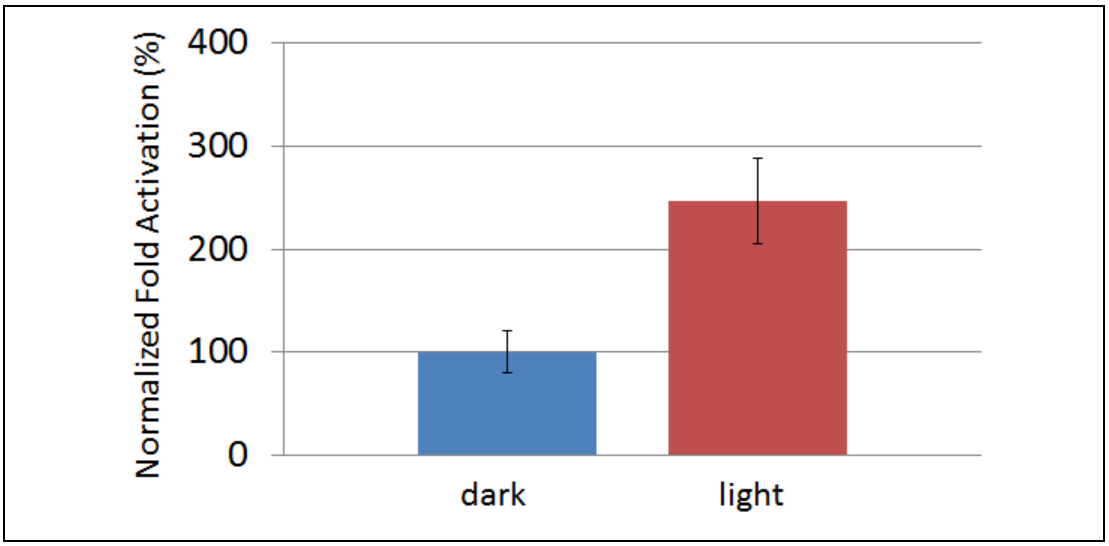

Figure 5.5: Luciferase assay with HEK293T cells showed that dCRY and JET interact upon light exposure. Graph shows the 2, 5 fold increased luciferase activity during 6 hours in the light treatment over dark conditions.

5.2. Gene Cloning of Retinoid X Receptor (RxR) Constructs

In the first step of this study, we showed that the transfected reporter gene expression was regulated by light induction. As second step, we aimed that this light regulated system can arrange an endogenous gene expression. We expected that light brings monomers into close proximity through dCRY and Jetlag interaction to make a functional protein. For this purpose, we chose Retinoid X Receptor (RxR) transcription factor and its target cell cycle inhibitor protein p21 to test our light-dependent system. We fused the Retinoid X Receptor (RXR) protein without dimerization domain to CRY and JET. Therefore we expected that the light induced interaction between CRY and JET cause dimerization of truncated RXR proteins and activate p21 gene expression (figure 5.6).

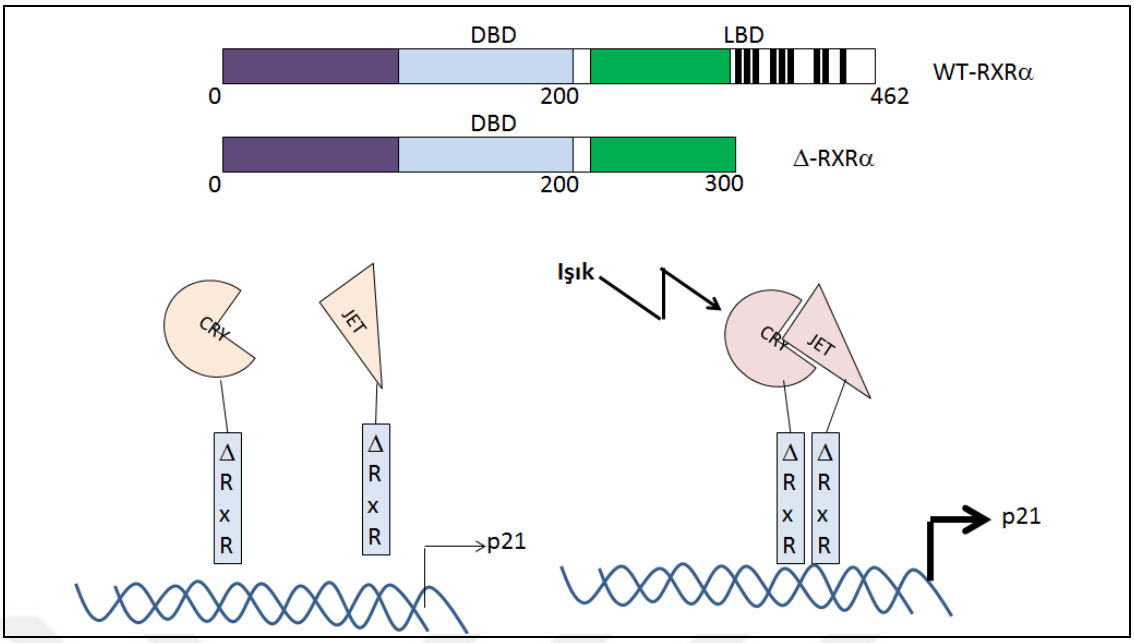

Figure 5.6: Control of an endogenous pathway by CRY and JET interaction in mammalian cells.

5.2.1. Making RXR-V5-JET and RXR-dCryV5His

To make pcDNA3.1-V5-JET construct, V5-JET was taken from pAc5.1V5-JET with EcoRI/XhoI and insert into EcoRI/XhoI site of pcDNA3.1 (+).

pAc5.1-dCRYV5H was cut with EcorI/PmeI to cut out dCryV5H, then inserted into pcDNA3.1 (+) that digested with EcorI/EcorV.

DeltaRXRa (ΔRxRa) sequence from human cell line cDNA fragment which does not have dimerization domain was amplified with primers containing NheI and EcorI sites. PCR product RxR was digested with NheI and EcoRI, and then inserted into pcDNA3.1-dCRYV5His and pcDNA3.1-V5-JET digested with NheI and EcoRI (Figure 5.7).

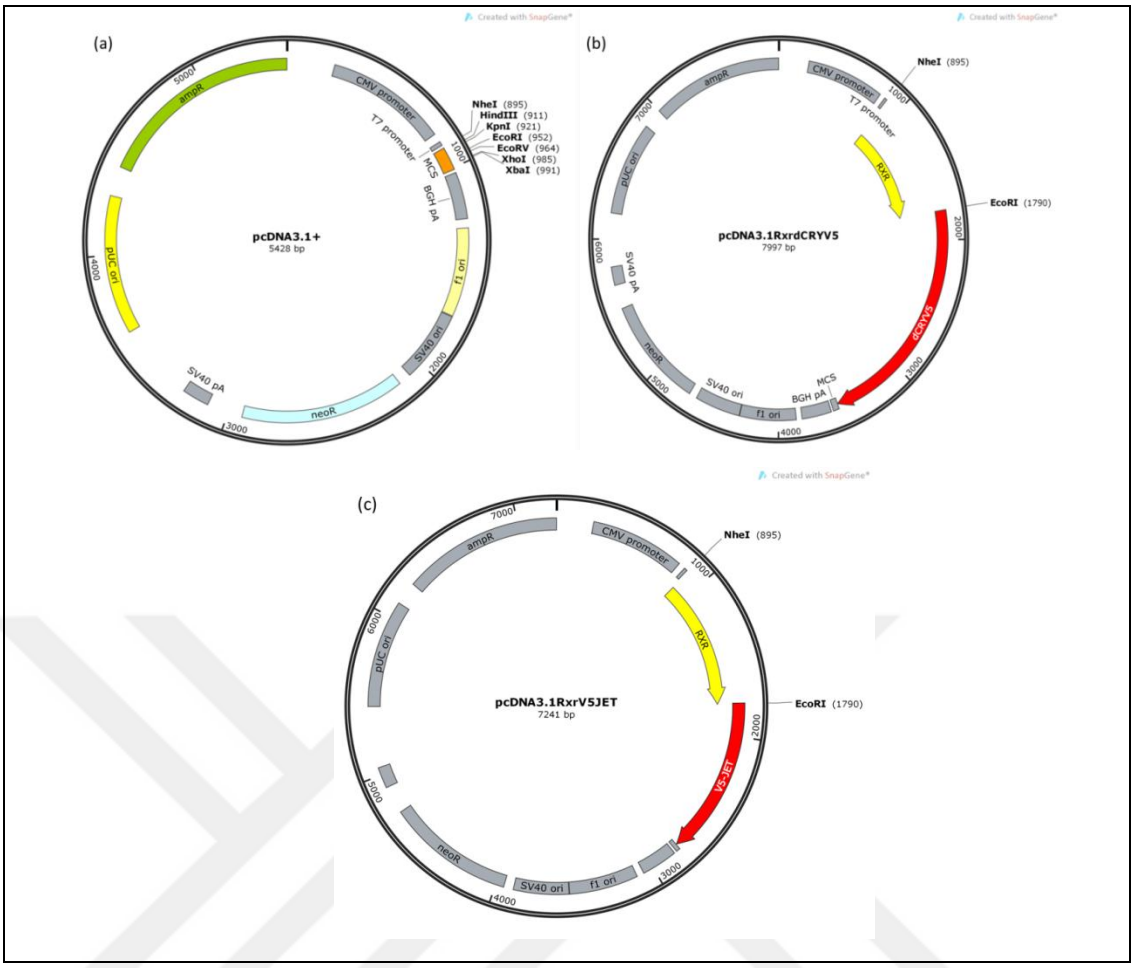

Figure 5.7: a) pcDNA3.1(+) vector map. Representation of b) pcDNA3.1Rxr-dCRYV5H c) pcDNA3.1Rxr-V5-JET ligations. Created by SnapGene.

The ligation reaction of each constructs was setup in 10µl and then ligation product was transformed into DH5α cells and plated out to LB agar containing ampicillin. The single colonies were picked up from agar and were grown in liquid LB medium with ampicillin and plasmid DNA was isolated with Macherey-Nagel Nucleospin Plasmid Isolation Kit.

5.2.2. Expression of Constructs in HEK293T Cells

To analyze expression levels of Rxr-dCRYV5H and Rxr-V5-Jet, both pcDNA3.1.Rxr-V5dCry and pcDNA3.1.Rxr-V5Jet plasmids were transiently co-transfected with polyethylenimine (PEI) in HEK293T cells. One day before transfection ~$5x10^5$ cells were seeded per well at 6-well plate. Next day, transfection was applied as described in Methods Section, 1, 2 µg total DNA was used at each

well. 48 hours after transfection, the cells were prepared for western blot analysis as described in Methods Section.

To equalize Rxr-Jet and Rxr-dCry levels for co-transfection, first we optimized co-transfection condition at HEK293T cells.

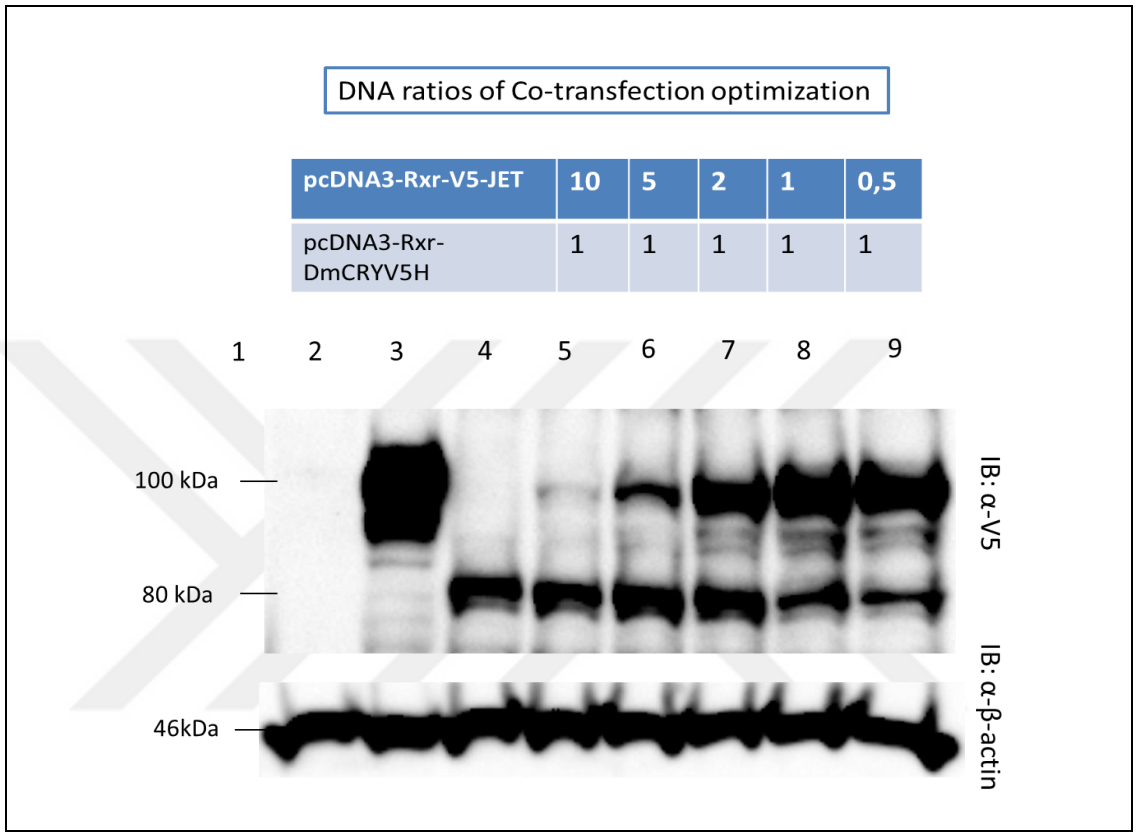

Figure 5.8: Western Blot analysis of transfected 293T cells at 8% SDS-PAGE gel. Actin is internal control. Lane 1: Protein ladder. Lane 2: pcDNA3.1 transfected as negative control. Lane 3: pcDNA3.1-Rxr.V5-dCry transfection. Lane 4: pcDNA3.1-Rxr-V5-Jet transfection. Lane 5-6-7-8 represents pcDNA3.1-Rxr-V5-Jet: pcDNA3.1-Rxr-V5dCRY co-transfection ratios, respectively. Lane 5: (10:1) Lane 6: (5:1) Lane 7: (2:1) Lane 8: (1:1) Lane 9: (0,5:1).

Twofold Rxr-JET to Rxr-dCRY amount in the co-transfection was seemed appropriate to equal expression in the cells (Figure 5.8 Lane 7).

5.2.3. Making Stable Cell Line for Rxr-dCRYV5H

The transient transfection peaked gene expression between 24-48 hours post transfection. However, if sustained gene expression is required for longer periods of time, generation of stable cell lines is a useful method. In addition, stable cell lines

which are selected by limited dilution provide a genetically homogenous and clonal population**.**

We considered making stable cell line expressing Rxr-dCRYV5H and Rxr-V5-JET. Positive selection marker was delivered by use of antibiotic resistance which comes from plasmid carried gene of interest. The pcDNA3.1 vector which carries our inserts, Rxr-DmCRYV5H and Rxr-V5-JET, has Neomycin selectable marker. However, HEK293T mammalian cell line that was used in our experiments is resistant to Neomycin. Hence, we used pcDNA4-MycHisA vector which has Zeocin selection marker to generate stable Rxr-dCry cells.

To generate Rxr-dCRY-HEK293T cell line, pcDNA3.Rxr-dCRY plasmid containing target gene was used 20 part (500 ng) and pcDNA4-MycHisA containing antibiotic selection marker plasmid was used 1 part (25ng). This plasmid ratio provides that the selected cells will express both gene of interest and selection marker. First of all, antibiotic concentration for selecting stable colonies was determined by dose-dependent experiment with HEK293T cells. In this experiment, increasing amounts of antibiotic concentrations were used to determine the minimum antibiotic concentration that needed to kill all the cells during one week. The concentrations of Zeocin used to select stable cell lines may range from 50 to 1000 µg/mL, with the average being around 250 to 400 µg/mL (Invitrogen User Guide, Catalog no: R250-01, R250-05). After the performing a kill curve, 200ug/ml Zeocin concentration was decided for selection.

The HEK293T cells were co-transfected at 6-well plate with Effectene Transfection Reagent as described in Method Section. 48 hours after transfection, 200µl/µg Zeocin was added onto cells and medium containing Zeocin was changed every 3-4 day. Meanwhile, expression of transfected cells can be tested or they can be frozen as polyclonal line.

One week later, transfected cells at 6-well plate were diluted by limited dilution method at 96-well plate. The goal of using this method is to isolate positive monoclonal cells by plating them at very low cell densities. 96-well plate wells were filled with 100 µl Zeocin containing medium, 2X volume medium was added last columns. Rxr-dCRYV5 transfected cells were trypsinized and 100 µl cell suspension was added into first well of first column (A1) then transferred to next row (B1) and repeated 1:2 dilution down through the entire column, discarding to 100 µl from last well. 100 µl medium from last column was added first column wells then 100 µl

transfers to wells of second column and repeated this dilution across entire plate, discarding 100 µl from last column. All the wells end up with 100 µl volume, containing different cell number (Figure 5.9).

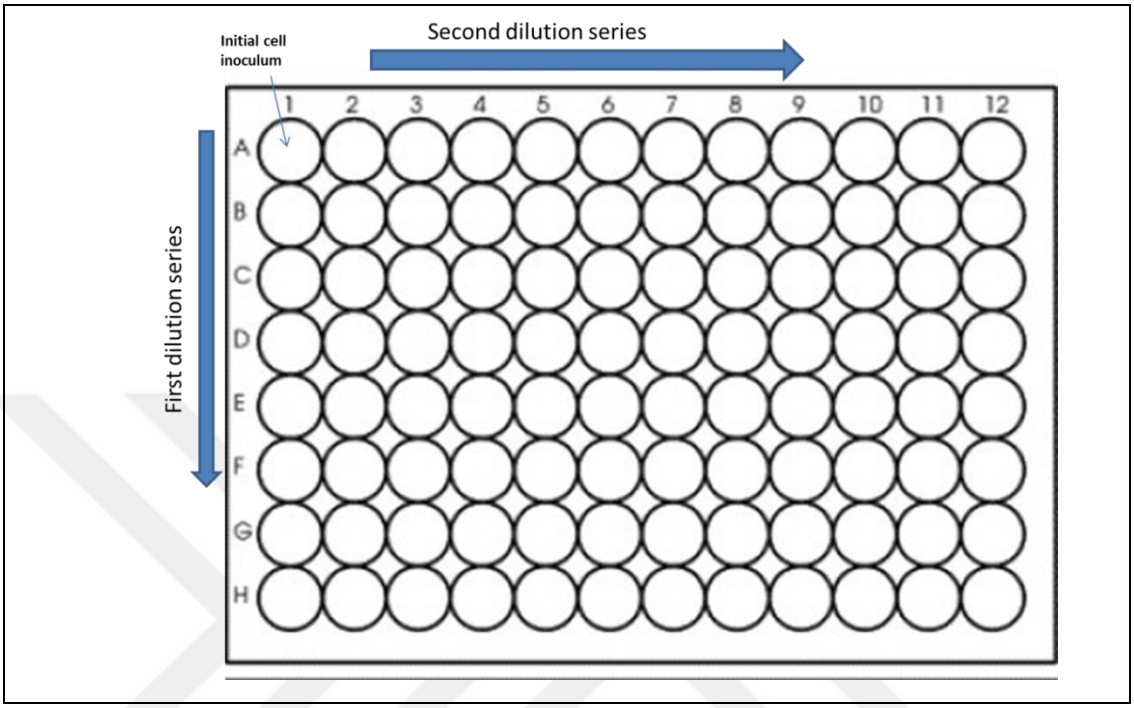

Figure 5.9: Schematic representation of limited dilution method at 96-well plate.

The medium of cells containing 200µl/µg Zeocin was changed every 4 days. The cells were analyzed until one colony at per well grown up. When any single colony appeared, the cells in the selected wells were transferred to 12-well plate and resistance clones were expanded until they reached enough confluency. In order to ensure that selected cells are clones derived from a single cell, another round of limiting dilution processes can be applied. When the cells were grown sufficiently, stable clones were analyzed for Rxr-dCRY expression.

5.2.4. *Drosophila* Cryptochrome (dCRY) Degradation in Mammalian Cells

Drosophila CRY is degraded in *Drosophila* cells upon light exposure in one hour. It was shown that *Drosophila* CRY is also degraded in mammalian cells [Ozturk et al., 2009]. To overcome this problem, we applied to very low intensity

light to Rxr-dCRY-HEK293T cells. First, we used different light doses to optimize degradation of dCRY at different times.

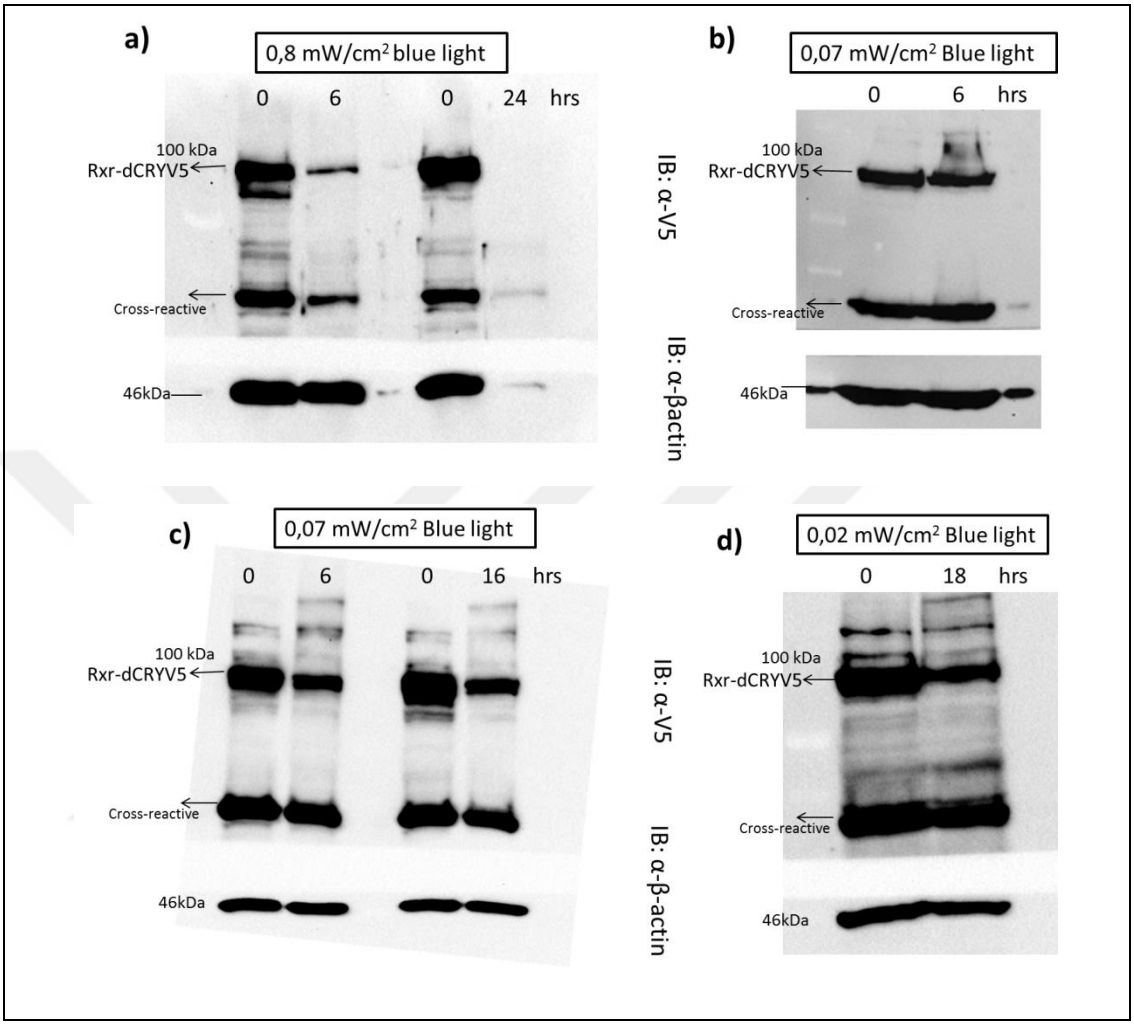

Figure 5.10: Western Blot analysis of Rxr-dCryV5H expression at Rxr-dCry 293T cells under dark or light conditions according to time and dose dependent. Time lines indicate light exposure hours, 0 hr means dark condition. Expecting Rxr-dCRYV5 size is ~100 kDa. Actin is internal control.

The Rxr-dCRY-HEK293T cells at 35x10 mm petri dishes were incubated with Atmospheric Medium in the warm room (37^0C) under dark condition covered with aluminum foil and under light condition that exposed to light at the various times. Then, dCRY expression of these cells was analyzed with Western Blot (Figure 5.10). The Rxr-dCRYV5, which was detected with V5 antibody at the 100kDa marker line, was degraded upon high intensity light exposure. However, it could be prevented in some degree by using low density blue light (Figure 5.10d). To get long time

interaction between dCRY and JET to manipulate gene or protein expression, 0, 02 mW/cm^2 blue light induction was chosen for further experiments.

5.2.5. Making Rxr-dCRY-Rxr-JET 293T Stable Cell Line

We considered generating stable cell line expressing both Rxr-JET and Rxr-dCRY at the same time. Firstly, we have been established Rxr-dCRY-HEK293T cell line, then we stably transfected Rxr-V5-JET construct into these cells.

Both pcDNA3.Rxr-V5-JET plasmid and pTRE2 plasmid containing Hygromycin selection marker were introduced into Rxr-dCRY -HEK293T cells at 6-well plate. 20:1 plasmid ratio was used as like generating Rxr-dCRY stable line.

A kill curve dose dependent experiment was performed at HEK293T cells with suggested concentrations of Hygromycin B and 50µl/µg was decided to selection. 36 hours post-transfection, the medium of transfected cells were changed with medium containing 50µl/µg Hygromycin. After 7-10 days, same limited dilution processes in Rxr-dCRY line was applied to subcloning.

The 20 colonies were selected at 96-well plate were transferred to 24-well plate and when they were reached enough confluency, analyzed with western blot assay for Rxr-dCRY and Rxr-JET expression. The colonies at 24-well plate were lysed with 1X Lysis Buffer for Western Blot Assay (mentioned as in the methods section.) In the first Western Blot analysis, the clones indicated with asterisk were selected according to their Rxr-dCRY and Rxr-JET expression (Figure 5.11a). Then, second Western Blot analysis was done with these clones and two clones indicated with asterisk were selected as positive cell lines (Figure 5.11b).

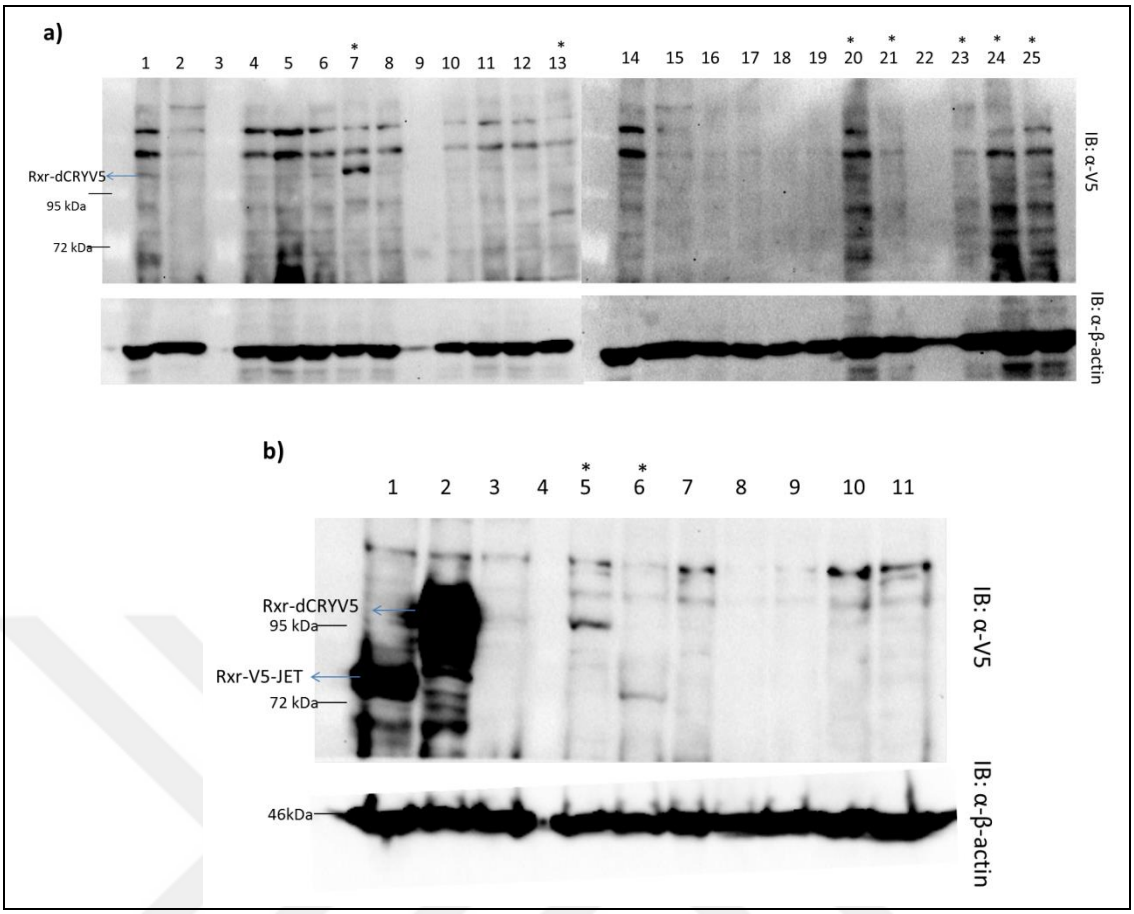

Figure 5.11: Western Blot analysis of Rxr-dCRY-JET clones. 8% gel was used. The expecting Rxr-V5-JET size ~80 kDa; Actin is internal control. a) Rxr-dCRY-JET 293T stable clones. Lane 1 and Lane 14: Rxr-dCRY 293T stable cells as negative control. Lane 2 and Lane 15: Transient pcDNA3.Rxr-V5-JET transfection at 293T cells as positive control. Lane 3: Protein ladder. Lane 4-5-6-7-8-9-10-11-12-13-16-17-18-19-20-21-22-23-24-25 represent Rxr-dCRY-JET clones respectively C1 to C20. b) Lane 1: pcDNA3.Rxr-V5-JET transfected into 293T cells. Lane 2: pcDNA3.Rxr-dCRYV5H transfected into 293T cells. Lane 3: pcDNA3 transfected into 293T cells. Lane 4: Protein ladder. Lane 5: Rxr-dCRY-JET C4 Lane 6: Rxr-dCRY-JET C10 Lane 7: Rxr-dCRY-JET C15 Lane 8: Rxr-dCRY-JET C16 Lane 9: Rxr-dCRY-JET C18 Lane 10: Rxr-dCRY-JET C19 Lane 11: Rxr-dCRY-JET C20.

As a result, Rxr-dCRY-JET C4 and C10 were selected as stable cell lines and kept maintenance for further experiments (Figure 5.11b, Lane 5-6).

5.3. Regulation of p21 mRNA Expression by Light Induction

We expected that light brings Rxr-dCRY and Rxr-JET into close proximity through dCRY-JET interaction and make a functional RXR transcription factor. We targeted control of p21 expression by using RXR transcription factor. In Rxr-dCRY-

JET 293T cell lines, we assumed that p21 expression was enhanced by light exposure.

To determine the p21 mRNA levels of both transiently transfected cells and stable cell lines, we first collected total RNA extracts from them, then synthesized cDNA's and analyzed them using qPCR with p21 and GAPDH primers. According to RT-qPCR result, transient both pcDNA3 and pcDNA3-Rxr-dCRYV5H:pcDNA3-Rxr-V5-JET co-transfected HEK293T cells had no p21 mRNA upregulation; on the other hand stable Rxr-dCRY-JET HEK293T C4 cells had little induction and Rxr-dCRY-JET HEK293T C10 cells had 16-fold induction of p21 mRNA upon 18 hr blue light treatment (0,02mW/cm^2) (Figure 5.12).

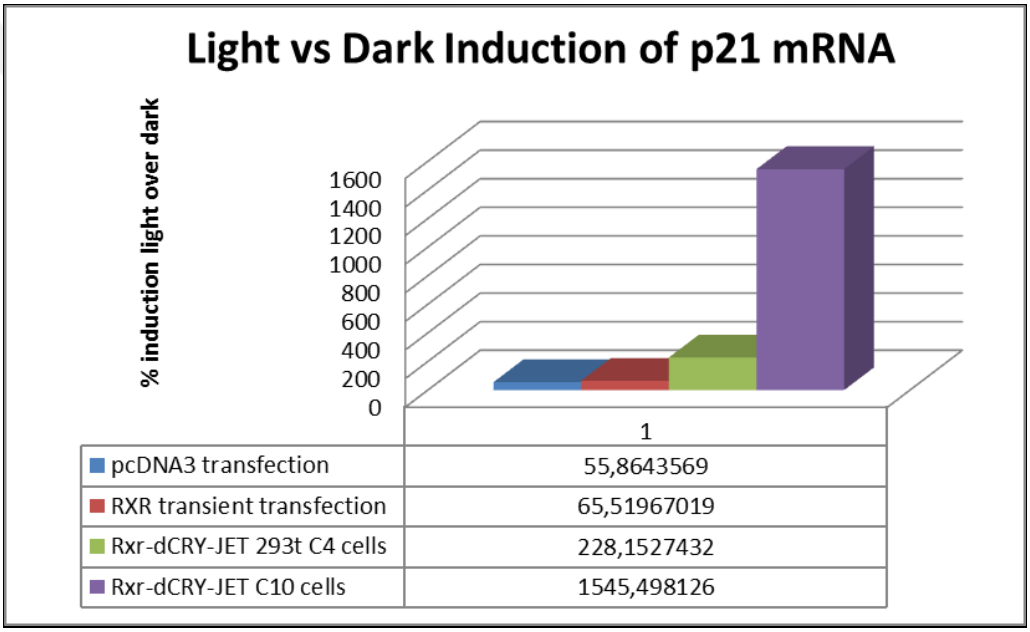

Figure 5.12: p21 mRNA levels of indicated cells. Fold change was calculated light samples over dark samples of the each cell. GAPDH was used for normalization.

It is suggested that transiently transfected cells overexpressed dCRY and JET in the both dark and light conditions; hence there was no light induction of expression in these cells.

- Subcloning of Rxr-dCRY-JET HEK293T C4 cells:

To get highly monoclonal population expressing both Rxr-dCRY –Rxr-JET, dilution step of Rxr-dCRY-JET C4 cells were repeated.

At 96-well plate, 9 wells containing one single cell population were transferred to 24-well plate and were expanded until reach high confluency, and then they were analyzed with western blot assay to assess Rxr-dCRY and Rxr-JET expression levels.

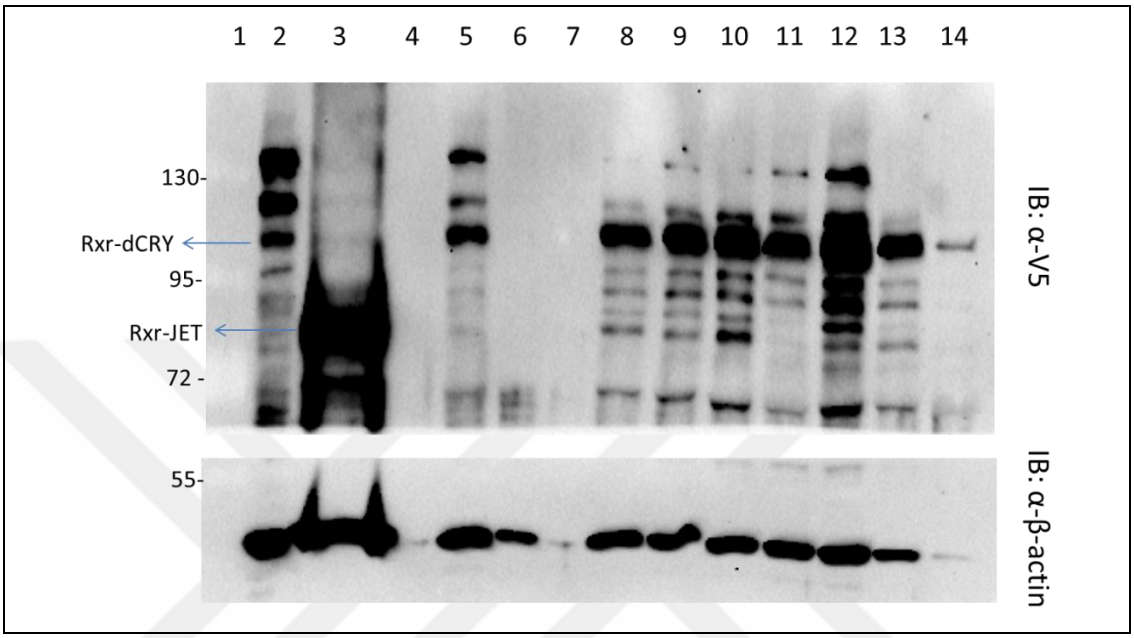

Figure 5.13: Western Blot analysis of Rxr-dCRY-JET clones. 8% SDS gel was used. Lane 1: Protein ladder. Lane 2: Rxr-dCRY HEK293T cells Lane 3: Rxr-JET transfected HEK293T cells Lane 4 and 7: empty. Lane 5-6-8-9-10-11-12-13-14: Rxr-dCRY-JET HEK293T C4 subclones C4.1 to C4.9.

Rxr-dCRY-JET HEK293T C4.5 (Figure 5.13 lane 10) stable cells expressed both Rxr-dCRY and Rxr-JET, this cell line was chosen for further experiments.

5.4. Regulation of p21 Protein Expression by Light Induction

To determine the p21 protein expression levels in the stable Rxr-dCRY-JET HEK293T clones upon light induction, western blot analysis was done after 18 hr 0, 02 mW/cm^2 blue light treatment. It was shown that endogenous p21 protein level is upregulated when cells were exposed blue light. Rxr-dCRY-HEK293T cells had no light induction of p21 level. However, in the presence of Rxr-JET, light dependent interaction between dCRY and JET induced p21 expression in the Rxr-dCRY-JET-HEK293T cells (Figure 5.14).

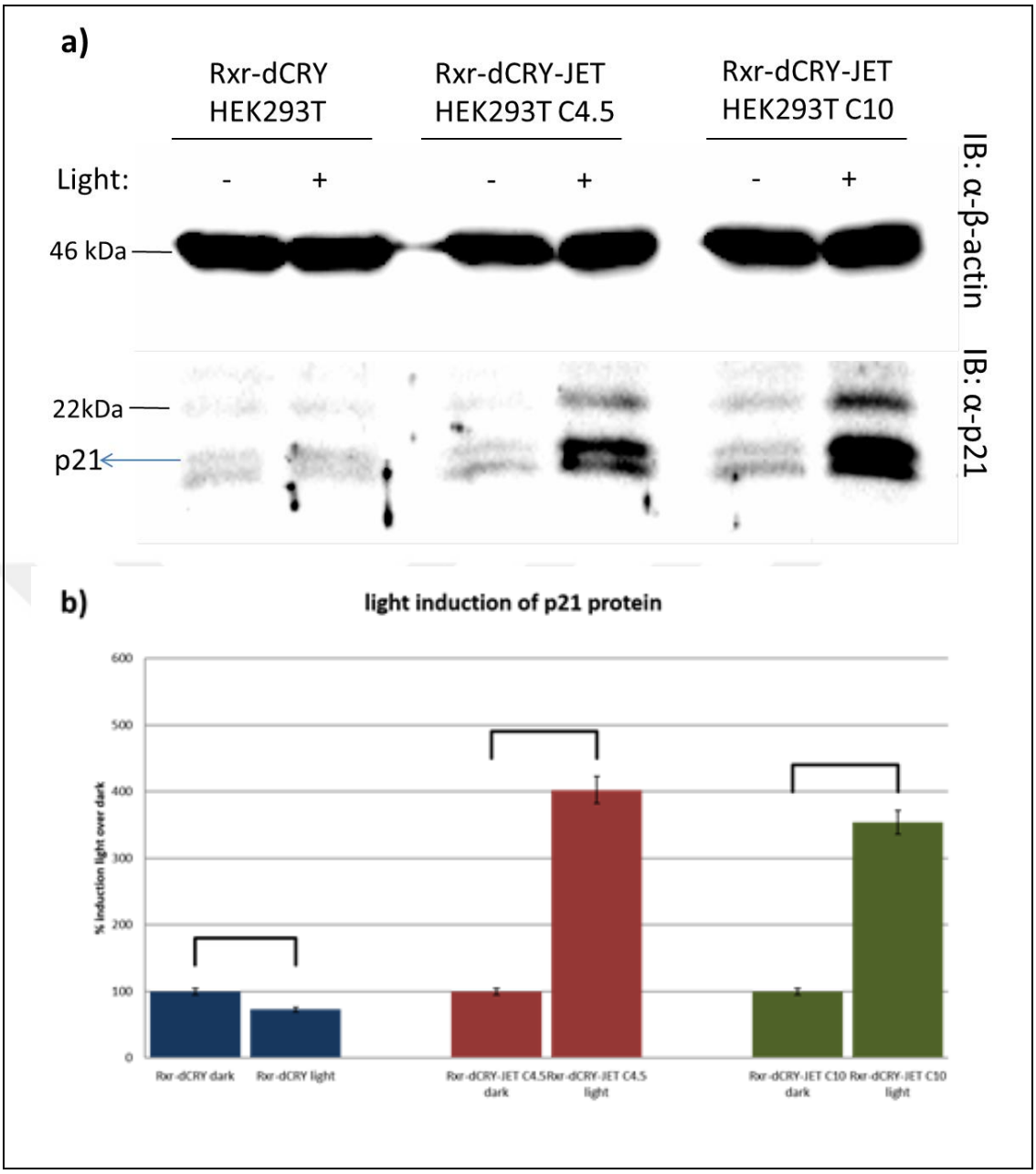

Figure 5.14: a) Western blot analysis of p21 expression at indicated cell lines. b) Graph of the Western Blot result quantification and it was calculated by using ImageLab Software (Bio-Rad). Actin levels were used for normalization. Dark blue bars, red bars, green bars indicate Rxr-dCRY-HEK293T, Rxr-dCRY-JET-HEK293T C4.5 and Rxr-dCRY-JET-HEK293T C10 cells, respectively.

Finally, time-dependent induction of p21 protein level was shown by immunoblotting up to 36 hours and p21 protein expression was enhanced upon light exposure time-dependently (Figure 5.15). Graphs showed the approximately 2 fold increased expression of p21 within the 24 hrs, 5 fold increased expression in the 36hrs light treatment (Figure 5.16).

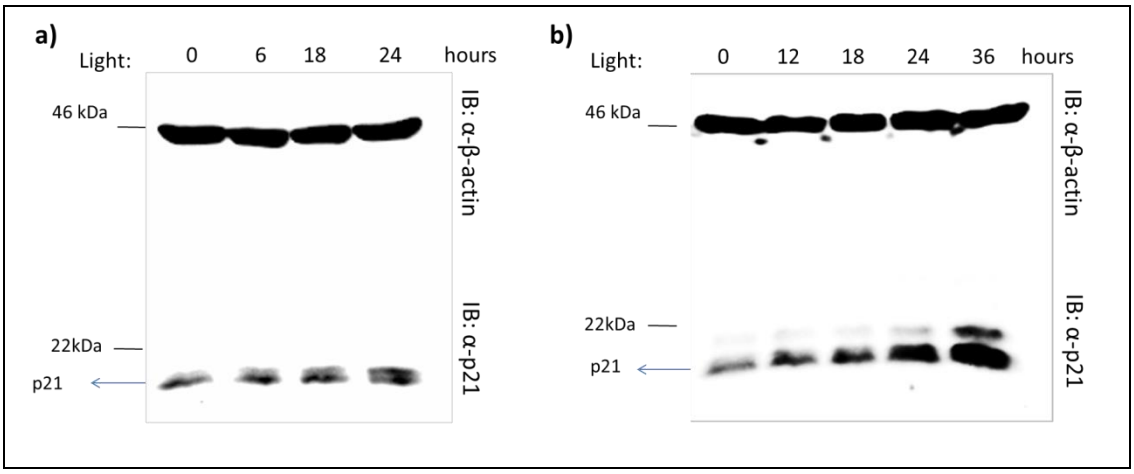

Figure 5.15: Time point induction of p21 protein expression at Rxr-dCRY-JET-HEK293T C4.5 cells. a) Up to 24 and b) 36 hrs light induction was shown.

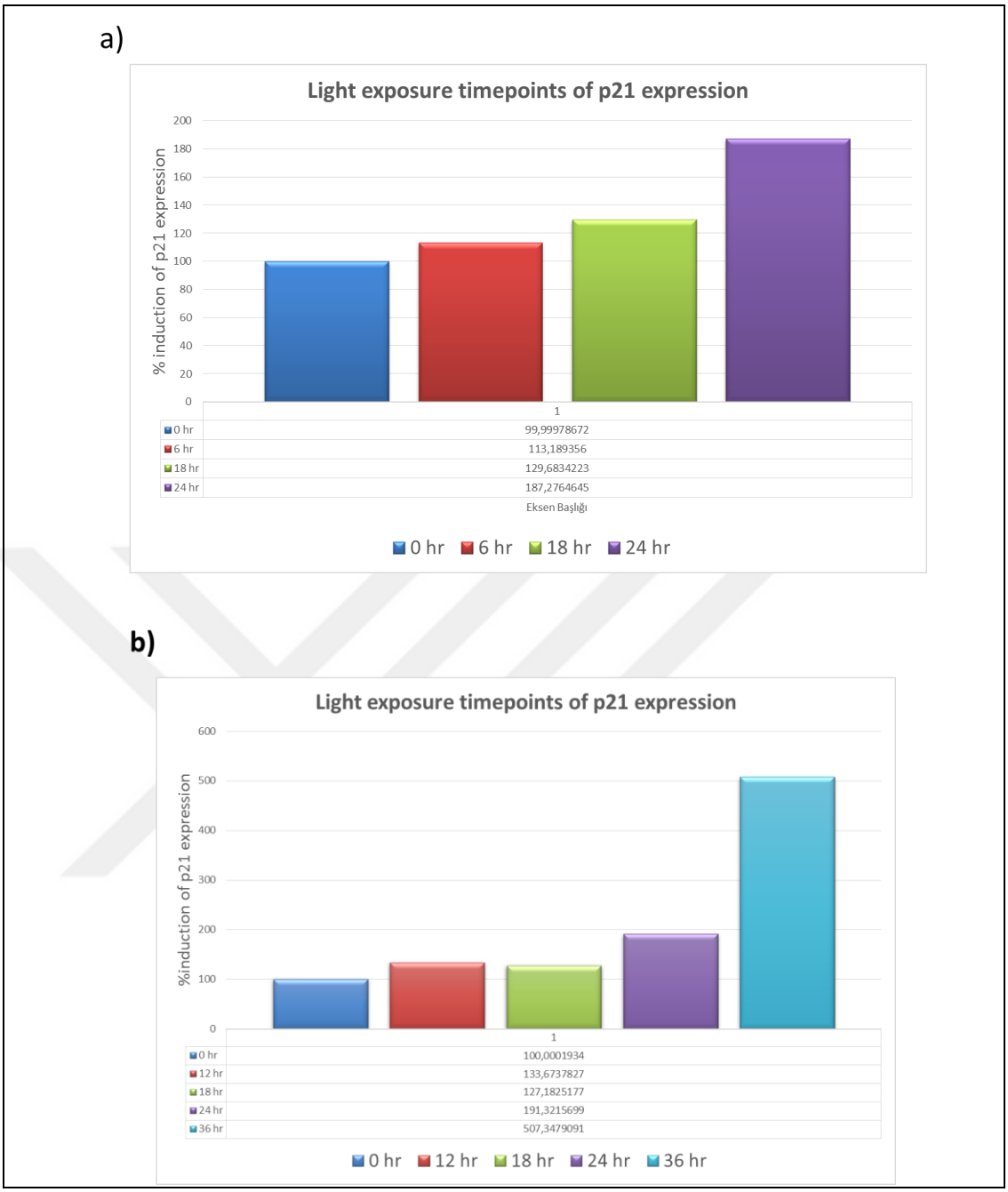

Figure 5.16: The quantification of Western Blot results in (Figure 5.15). a) Graph of 24 hrs. b) 36 hrs. light induction. It was calculated by using ImageLab Software (Bio-Rad). Actin levels were used for normalization.

6. DISCUSSION

The chemical-based gene regulation systems have played a critical role in the development of synthetic biology and have become routine tools in mammalian cell biology research [Clackson, 2000] [Lee and Mapp, 2010]. However, these systems require a chemical or physical pressure which give some stress even cause a shock or toxicity in the cells. Moreover resolution of regulation is so low that it takes hours. A new approach to the use of light to control specific signaling pathways in living cells has been developed which is called optogenetics. Therefore, the development of light-controlled devices is highly attracted and has recently received much attention [Muller and Weber, 2013].

The optogenetics technique has many advantages in comparison with classical gene regulation techniques that are less invasive, precise, easily-controllable and high time resolution. Optogenetic gene switch systems employ either the light-regulated recruitment of a transcriptional activation domain to a DNA-bound protein or use the light-triggered homo-dimerization of a photoreceptor for the reconstitution of a DNA-binding domain to guide an activation domain to the target gene [Tischer and Weiner, 2014]

Adoption of new light sensitive proteins into current optogenetic methods can improve upon the temporal tuning and sensitivity of these methods. In this thesis, an alternative method for blue light-inducible gene expression is designed by using circadian clock photoreceptor *Drosophila Cryptochrome* in mammalian cells. Circadian clock photoreceptors are photosensory proteins which reset the circadian clock after receiving the sun light early in the morning. Fortunately, mammalian cells and *Drosophila* cells use different systems as photoreceptor for circadian rhythm resetting [Bell-Pedersen et al., 2005]. Therefore, the transfer of *Drosophila CRY* does not affect any intrinsic pathway in mammalian cells because mammalian homolog of this protein is not light sensitive.

The developing this new tool is on the basis of *Drosophila Cryptochrome* binding its partner Jetlag protein under light condition (Figure 6.1). So far, only *Arabidopsis* Cryptochrome (AtCRY) was used as a tool to regulate oligomerization in mammalian cells [Ozkan-Dagliyan et al., 2013]. Even though it is a plant protein, AtCRY can make dimers under light conditions in mammalian cells as much

efficiently as it does in the plant cells [Conrad et al., 2014]. In a similar way, the light dependent interaction between CRY and JET was exploited in this study.

Here, it was critical that the degradation of *Drosophila* Cryptochrome protein in mammalian cells because previous studies showed that CRY is degraded in *Drosophila* cells in one hour by ubiquitin-dependent pathway, interestingly also degraded in mammalian cells [Ozturk et al., 2009]. This problem was overcome in this study by using very low intensity of light which causes very little degradation in the cells. The cells were exposed to black light 0, 02 $mWcm^{-2}$ intensity which was decided after measured the CRY degradation in the cells upon dose-dependent light intensity exposure (Figure 5.10). Alternatively, a degradation-resistant mutant form of *Drosophila Cryptochrome* could be used to prevent degradation. It is known that the mutation of Cysteine 402 residue to Alanine stops degradation of related CRY from Butterfly (*Danaus plexippus*) in *Drosophila* cells [Ozturk et al., 2008]. The mutation of this conversed residue in light sensitive CRY is expected also to make other CRY resistant to degradation.

In the first step of study, we showed the interaction between *Drosophila* CRY and JET in Human embryonic kidney 293T cells by using two-hybrid mammalian system. This system is based on one protein of interest fused to a DNA-binding domain and the other protein fused to a transcriptional activation domain. We used Check-Mate Two Hybrid Mammalian System which includes the pBIND vector contains the yeast GAL4 DNA-binding domain upstream of a multiple cloning region, and the pACT vector contains the herpes simplex virus VP16 activation domain upstream of a multiple cloning region and Dual-Luciferase™Reporter Assay System to detect interaction. GAL4 can activate gene expression only when it interacts with VP16 activation protein. Here, GAL4-VP16 hybrid protein induced gene expression through CRY-JET interaction under light. We confirmed the light dependent interaction induced gene expression via luciferase reporter assay (Figure 5.5).

When light exposure stops, interaction reverses within the 15 minutes. This system gains advantages over chemical treatments; therefore it can be studied a reporter protein metabolisms which either degradation or cellular localization time dependent etc. via millisecond light treatments. It was shown that 1 millisecond white light flash promotes cryptochrome degradation in *Drosophila* cells [Ozturk et al., 2011].

The abovementioned system describes the transfected a reporter gene expression regulation by using light. The endogenous gene expression regulation can be tested with different control elements which could be transcription factors, enzymes and any regulatory proteins. We chose Retinoid X Receptor (RxR) transcription factor to control its target cell cycle inhibitor protein p21 (Waf1/CIP1) expression. RXRa contains two signature domain of nuclear receptor family proteins, i.e. DNA-binding domain and ligand binding domain (LBD). It is a ligand-dependent transcription factor; in the absence of ligand RxR does not bind effectively DNA alone, in the presence of ligand, RXR homodimer binding to DNA is induced (Zhang et al 1994). In this study, we modelled the p21 transcriptional activation following RxRa dimerization upon light dependent interaction between JET and CRY to regulate endogenous gene expression. DNA binding domain of RxRa was fused to JET and CRY proteins without ligand binding domain. Under dark condition, truncated RxRa (DeltaRxRa) could not make dimer because of the absence of ligand binding domain; however when light induced CRY –JET interaction, two ΔRxRa could dimerize and activate p21 expression. Determination of p21 expression in both mRNA and protein level in the cells expressing our fusion proteins, RxR-dCRY and RxR-JET were performed under dark and light conditions by reverse transcriptase quantitative PCR (RT-qPCR) and western blotting, respectively. The expression levels were correlated with expecting results of our light dependent gene regulation system.

We stably expressed both RxR-CRY and RxR-JET in HEK293T cells via stable cell line generation method (as explained in the Methods Section). Transient transfection technique has limitations in terms of overexpression of interacting proteins, vector uptake limiting to cycling cells and non-homogenous expression in the cells. In contrast to transient expression, stable expression allows long term, stable and reproducible, expression of the gene. Hence, we generated RxR-dCRY-Rxr-JET-HEK293T stable cells, and then these cells were exposed to black light 0, 02mWcm^{-2} light intensity up to 36 hrs. According to immunoblotting results, we found that endogenous p21 was activated by light in RxR-dCRY-RxR-JET expressing cells (Figure 5.15) by using our system.

Eventually, we showed that *Drosophila Cryptochrome* can be used as an optogenetic tool in mammalian cells. The light dependent JET-CRY interaction system can be used to manipulate the gene expression or protein function. However,

we only tested endogenous transcriptional control in our system with targeting p21 expression regulation by RxRa homodimerization. It can be also examined either the control of any other transcriptional regulation of a gene or any other cellular events coupled to our system by light. Next, we will study endoplasmic reticulum stress pathway regulation by light. IRE1alpha activation through dimerization by light is expected to mediate Xbp1 mRNA splicing [Back et al., 2006]. This model will be tested by our system in the further experiments.

In *Drosophila cells*, the light-induced CRY-JET interaction initiates a signaling pathway within minutes and interaction is reversed within ~15min when light off. Therefore, time resolution in minutes for controlling cellular events can be realized. Some signaling pathways are responsive to timing of input; therefore control of different cellular signaling pathways by special design from minutes to days can be possible with our system.

To sum up, we generated a new tool to regulate the endogenous gene expression and protein function in mammalian cells. This system offers an alternative to classical gene regulation systems and it expands photoregulation strategies which are used in the current optogenetic systems. This study may find large application area for research and biotechnological purposes, because it is proven to be practicable.

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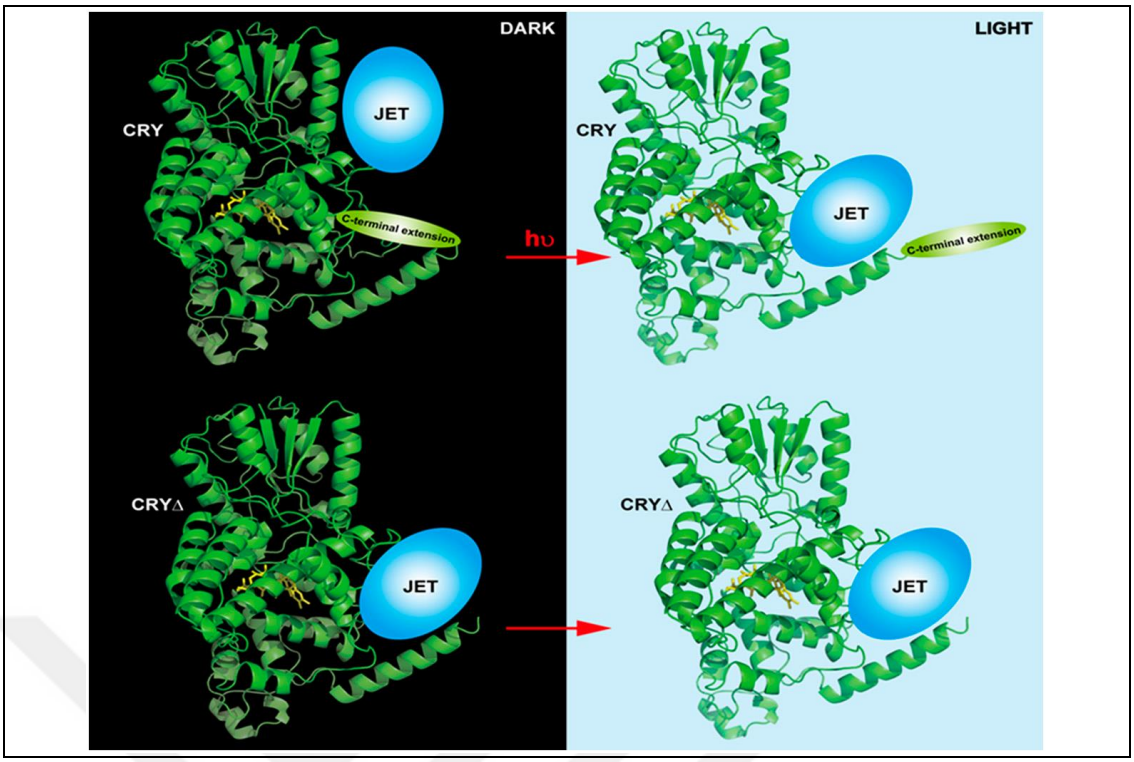

Figure 6.1: Model of *Drosophila* Cryptochrome conformational change and interaction with JETLAG under dark or light.

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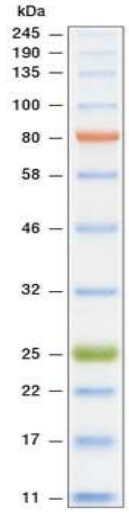
BIOGRAPHY

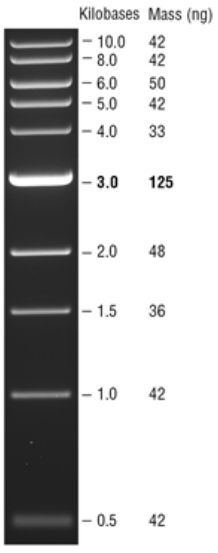
Gözde ÖZÇELİK was born in Kütahya, Turkey, October 20, 1992. She received her B. Sc. Degree in Molecular Biology and Genetics Department at Gebze Institute of Technology in 2014. She joined the MSc. Program in Gebze Technical University, Graduate School of Natural and Applied Science, Molecular Biology and Genetics Department and then she graduated with MSc degree in 2016. She worked for 2 years under the supervision of Assoc. Prof. Dr. Nuri OZTURK.

APPENDICES

Appendix A: Protein and DNA Markers



Protein Ladder
(Color Prestained Protein Standard)
P7712S



DNA Molecular Weight Marker
(1kb DNA Ladder)
N3232S

Figure A1.1: Protein Ladder and DNA Molecular Weight Marker.

Appendix B: Copyright Permission

Table B1.1: Copyright permission to used materials.

Figure No.:	License Number	License Date
Figure 2.1 [Tischer and Weiner, 2014]	3893031012861	20.06.16
Figure 2.2 [Pudasaini et al., 2015]	License is granted at no charge	-
Figure 2.3 [Che et al., 2015]	License is granted at no charge	-
Figure 2.4 [Toettcher et al., 2011]	3893041344332	20.06.16
Figure 2.5 [Zhou et al., 2012]	3893050041693	20.06.16
Figure 2.6 [Bacchus et al., 2013]	License is granted at no charge	-
Figure 2.9a [Zoltowski et al., 2011]	3893031313574	20.06.16
Figure 2.12a [Peschel et al., 2009]	3893050538605	20.06.16