

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

**MAPPING THE LIGHT-DEPENDENT INTERACTION SITES
ON DROSOPHILA BIOLOGICAL CLOCK PHOTORECEPTOR**

FERAYE HATİCE CANBAZ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
ASSOC. PROF. DR. NURİ ÖZTÜRK

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DROSOPHILA BİYOLOJİK SAAT
FOTORESEPTÖRÜNDE IŞIĞA BAĞIMLI
ETKİLEŞİM BÖLGELERİNİN
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FERAYE HATİCE CANBAZ
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MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. NURİ ÖZTÜRK

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SUMMARY

The main photoreceptor responsible for circadian clock resetting in *Drosophila* is Cryptochrome (dCRY) protein. After exposure to light, dCRY initiates a signaling cascade which results in rapid removal of Timeless (TIM) and Period (PER) proteins from the core clock. In the core clock, CLOCK and CYCLE proteins transactivate TIM and PER expressions, of which products then suppress the CLOCK/CYCLE activity to generate an oscillatory expression pattern in CLOCK/CYCLE complex controlled genes. Light activates dCRY by relaxing its C-terminus, which is bended over the FAD binding region under dark condition. TIM, JET and BRWD3 proteins bind to dCRY to form the photoreceptor complex after light opens C-terminus of dCRY. On this photoreceptor JET and BRWD3 ubiquitylate TIM and dCRY and lead to their degradation respectively. Degradation of dCRY ensures that the photoreceptor is removed to prevent a continuous resetting. The degradation of TIM also leads to PER degradation which resets the circadian clock by releasing the inhibition of CLOCK/CYCLE activity. We aimed to discover the binding/interaction sites of JET and TIM on dCRY protein. Point or deletion mutations generally prevents proper folding of dCRY on its light sensitive co-factor FAD, therefore we used partial trypsinization to detect probable interaction sites of dCRY for JET and TIM, under dark and light conditions. JET and TIM block certain trypsin attack sites via attaching its docking site on light induced dCRY. Conserved fragments were detected by immunoblot and SDS-PAGE/protein staining techniques. With the guidance of these data, we have mapped approximate light-dependent binding sites of JET and TIM on dCRY.

Key Words: Circadian Clock, *Drosophila* Cryptochrome, Conformational Change, Protein-Protein Interactions, Partial Trypsinization.

ÖZET

Cryptochrome (dCRY) proteini, *Drosophila*'da sirkadiyen saat resetlenmesinden sorumlu başlıca fotoreseptördür. Işığa maruz kaldıktan sonra dCRY; TIMELESS (TIM) ve PERIOD (PER) proteinlerinin merkezi saatten hızlıca uzaklaştırılmasıyla sonuçlanan bir sinyal yolağı başlatır. Merkez saatte, CLOCK ve CYCLE proteinleri TIM ve PER ekspresyonlarını aktive eder ve daha sonra ekspresyon ürünleri CLOCK/CYCLE aktivitesini durdurarak CLOCK/CYCLE kompleksiyle kontrol edilen genlerin ekspresyonunda osilasyon paterni oluşmasını sağlar. Işık, karanlık durumda FAD bağlanma bölgesi üzerine kapanmış karboksil ucunun (C-ucu) açılmasını sağlayarak dCRY'yı aktive eder. TIM, JETLAG ve BRWD3 proteinleri, ışıqla birlikte dCRY'ın C-ucu açıldıktan sonra fotoreseptör kompleksini oluşturmak için dCRY'a bağlanır. Bu fotoreseptörde, JET ve BRDW3, TIM ve CRY'ı ubiquitinleyerek sırası ile degrade olmalarını sağlar. Sürekli resetlenmeyi önlemek için fotoreseptörün ortadan kaldırılması CRY degradasyonu ile olmaktadır. TIM degradasyonu ayrıca PER degradasyonunu sağlar ve bu da CLOCK/CYCLE üzerindeki inhibasyonu kaldırarak sirkadiyen saati resetler. Biz JET ve TIM'in dCRY proteinindeki bağlanma / etkileşim yerlerini keşfetmeyi amaçladık. Nokta veya delesyon mutasyonları, genellikle, ışığa duyarlı kofaktör FAD üzerinde dCRY'ın uygun şekilde katlanmasını önler, bu nedenle biz de karanlık ve ışık altında JET ve TIM için dCRY'ın muhtemel etkileşim bölgelerini tespit etmek için kısmi tripsinizasyon kullandık. JET ve TIM, ışıqla indüklenmiş dCRY üzerinde birleşme bölgelerine bağlanarak belirli tripsin atak noktalarını bloke eder. Korunmuş fragmanlar immünoblot ve SDS-PAGE / protein boyama teknikleri ile tespit edildi. Bu verilerin ışığında, dCRY üzerinde JET ve TIM'in ışık bağımlı yaklaşık bağlanma bölgelerini haritaladık.

Anahtar Kelimeler: Biyolojik Saat, *Drosophila* Cryptochrome, fotoreseptör, Protein-Protein Etkileşimi, Kısmi Tripsinizasyon.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
µg	: Microgram
µl	: microliters
mW/cm ²	: 1 milliwatt per centimeter square
APS	: Ammonium persulfate
CRY	: Cryptochrome
dCRY	: <i>Drosophila</i> Cryptochrome
CRY ΔC	: CRY Delta C (Cryptochrome without the last 20 a.a)
Clk	: Clock gene
Cyc	: Cycle gene
FAD	: Flavin Adenine Dinucleotide
JET	: JETLAG protein
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tim	: Timeless gene
TIM	: Timeless protein
TTFL	: Transcription Translation Feedback Loop

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1. INTRODUCTION

Circadian clock is a crucial mechanism for most organisms to adjust their biological activities to geological (solar) clock which consists of 24hr periods. Circadian clock is a genetically encoded molecular mechanism and this genetic-based system has to be adjusted to geological clock in order to enhance the survival capacity of the organism [Sancar A., 2004].

The functional biological clock consists of a core clock and a photoreceptor system. Mammalian and fruit fly (*Drosophila*) molecular core clocks are analogous [Ozturk N. *et al.*, 2007]. On both, the core clock is composed of a Transcription-Translation Feedback Loop (TTFL) model that has positive and negative arms [Ueda H. R., 2007]. The factors of positive arm transactivate the genes on the negative arm and, in the course of time, translated negative arm factors inhibit positive arm factors. Thus, they block themselves indirectly. Hereby, this activation-inhibition loop forms a circular pattern [Bell-Petersen D. *et al.*, 2005].

The mammalian cells and *Drosophila* cells use different systems as photoreceptor even though they have similar core clock mechanisms. Cryptochrome (dCRY) is the sole or main photoreceptor of the circadian clock responsible for the resetting of the clock in *Drosophila* [Ozturk N., 2017]. Mammalian Cryptochrome proteins are not light sensitive and instead of Cryptochrome-based photoreceptor a melanopsin-based circadian clock photoreceptor functions in mammals.

From the genetic aspect, *Drosophila*, as a model organism, is easier to work than mammalian model organisms and this makes it the holder of the best identified non-image forming photoreceptor so far. Yet there are many uncharted spots of light triggered reactions on *Drosophila* photoreceptor system.

1.1. The Purpose, Contribution and Content of Thesis

Interaction domains of dCRY for Jetlag (JET), Timeless (TIM) and Brwd3 (also known as Ramshackle) proteins are not known. This present work focuses on light dependent interactions between dCRY and JET and as well as TIM using purified proteins. Priority was given to revealing TIM and JET interaction domains rather than Brwd3, because these proteins contribute to resetting mechanism directly.

Point or deletion mutations of dCRY may not work well for the identification of domains, since any simple point mutation may have dramatic effect on protein

functioning because they might directly affect the folding capacity which results in the fall of flavin co-factor (FAD) which renders dCRY protein insensitive to light. In order to overcome this problem, we will use partial trypsinization technique to detect the probable interaction domains of dCRY for JET and TIM proteins, under dark and light conditions [Ozturk N *et al.*, 2011; Czarna A. *et al.*, 2013]. Profiling approach is that JET (and/or TIM) blocks the certain trypsin attack sites during the attachment of its docking site on blue-light induced dCRY protein, which is followed by SDS-PAGE and protein probing/staining techniques for detection. Hence, these blocked trypsin attack sites on certain region of dCRY may give clues about the interaction sites of dCRY to JET and TIM.

If the regions responsible from JET and TIM binding is clearly revealed, mutations, those are made in order to investigate further featuring of dCRY, such as clock resetting mechanism investigations, can be designed without disrupting 3D structure of dCRY. We can construct such a dCRY mutant that cannot reset the circadian clock while still able to sense blue-light. Namely, retaining a more functional dCRY model in order to find out how light resets the circadian clock.

2. LITERATURE REVIEW

2.1. Circadian Clock

Circadian clock is a molecular timekeeper that oscillates in a constant profile in synchrony with geological clock. This synchrony is crucial for the organism to adapt to daily environmental changings. The most important cyclic event is the 24h-lasting fluctuation in solar light intensity during the day. Most of organism from complex eukaryotes to prokaryotes, including cyanobacteria [Ishiura M., 2000] has an endogenous clock which is adjusted to this 24h solar clock.

The functional biological clock is generated by a functional the core clock and a photoreceptor mechanism. While the core clock is a genetic system which oscillates within a period of 24 hours, photoreceptors reset the circadian clock early in the morning thanks to the sun light as an input signal thereby retain the internal circadian clock and geological clock in the same phase [Partch C. L. et al., 2014].

Synchronizing the endogenous core clock to the solar clock is achieved by a process called resetting or entrainment. Photoreceptor molecules manage this resetting process by receiving solar light and by conveying this signal to the core clock system for the initiation of transcriptional and translational alterations.

The establishment and schematization of molecular core-clock system in *Drosophila* was started with the discovery of the Period (per) gene locus by Ronald Konopka and Seymour Benzer [Konopka R., 1971]. Seymour Benzer (1921–2007) and his PhD student Ron Konopka were working on the fruit fly in the early 1970s. They performed chemical mutagenesis on *Drosophila* and screened for flies with abnormal circadian behavior. They obtained a few fly strains with abnormal endogenous eclosion periods. One strain had a longer period (29 h), another had a shorter period (19 h) and a third strain did not show any rhythmic eclosion. All the three fly strains had a mutation in the same gene locus, located on the X-chromosome. This locus was then called period [Konopka R., 1971]. Since the discovery of the Period genes in *Drosophila*, the main mechanism of the circadian clock nearly all model organisms in been revealed [Bell-Pedersen D. et. al., 2005]. Most of these clock genes are driven by a transcriptional-translational feedback loop (TTFL) which is consist of a consecutive repeat of transcriptional activation and

repression of a subset of genes in a 24 hour period [Dunlap J.C., 1999]. In fruit fly (*Drosophila melanogaster*) TTFL model, transcription factors Clock (CLK) and Cycle (CYC) activate the transcription of negative arm genes, Period (PER) and Timeless (TIM) as well as the circadian clock- regulated genes (CCG) [Sancar A., 2008]. After their translation process, PER and TIM migrate into the nucleus and inhibit CLK and CYC activity. In the end, this activation and inhibition creates a cyclic expression pattern [Bell-Pedersen D. *et al.*, 2005]. The resulting cycle is regulated by approximately 24 hours after the post-translational modifications [Reppert S.M., 2002; Gallego M., 2007].

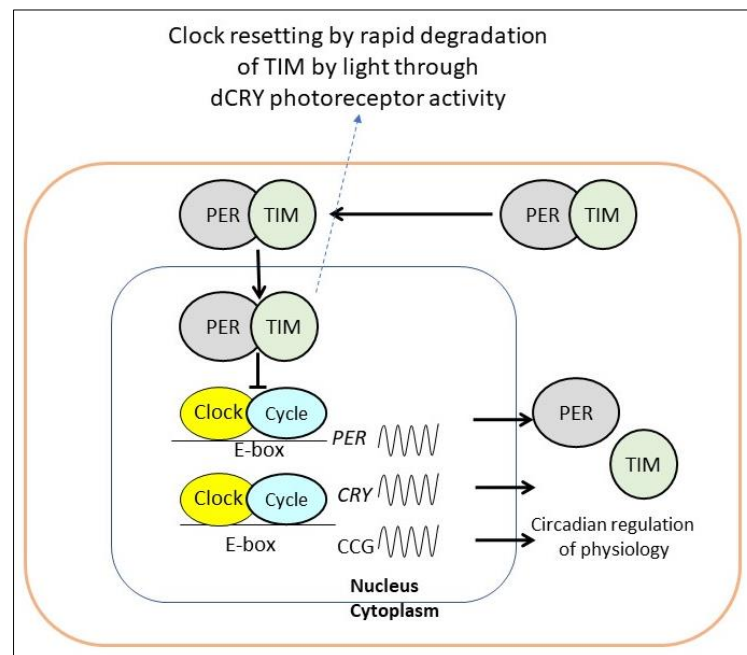


Figure 2.1: Organization of the molecular clock in fruit fly (*Drosophila*) cells.

2.2. Clock Resetting in *Drosophila*

Endogenous clock is adjusted to 24hr by sunlight at daybreak with a process called resetting [Sancar A., 2008]. Light is received by photoreceptor molecules and these molecules transmit the resetting signal to the core clock. Photoreceptor molecules work in this process by receiving blue light from the daylight spectrum.

Drosophila Cryptochrome (dCRY) is the main photoreceptor manages the circadian clock resetting in *Drosophila* [Emery P. *et al*, 1998]. Light activated dCRY initiates a signaling cascade which results in the rapid removal of Timeless (TIM)

and Period (PER) proteins from the core clock. In the core clock, CLOCK and CYCLE proteins transactivate TIM and PER expressions, which suppress the CLOCK/CYCLE activity [Peschel N., 2011]. This activation and suppression generates an oscillating expression pattern on the genes which are controlled by CLOCK/CYCLE complex. Light activates dCRY by relaxing its C-terminus, which is bended over the FAD binding region under dark conditions [Levy C. *et al.* 2013]. TIM, JET and BRWD3 bind to dCRY to form the photoreceptor complex after light opens C-terminus of dCRY. On this photoreceptor JET and BRWD3 ubiquitylate TIM and dCRY and yield to degradation respectively. Degradation of dCRY ensures that the photoreceptor is removed to prevent a continuous resetting. The degradation of TIM also leads to PER degradation which resets the circadian clock by releasing the inhibition of CLOCK/CYCLE activity [Ozturk N. *et al.* 2011; Ozturk N. *et al.* 2014].

Light-induced dCRY starts a signaling cascade ending in the rapid removal of TIM and PER proteins from an oscillatory core clock. Light signal turns dCRY cofactor FAD into lit (active) state and dCRY opens its C-terminal tail, which is bended on itself in dark [Bouly J. *et al.*, 2007]. This conformational change provides docking sites for JET and TIM proteins on dCRY. Brwd3 also binds to dCRY with a slower kinetic rate. In case of JET and TIM, these proteins come in close proximity and JET ubiquitylates TIM protein. Since Period (PER) protein is not stable without TIM, PER is also degraded immediately after TIM degradation [Peschel N., 2009]. TIM-PER heterodimer removal resets the circadian clock daily and initiates a new cycle. Later on, Brwd3 leads dCRY degradation through ubiquitination. In this manner, light is received once a day by dCRY which means that clock is reset once in a 24 hour period.

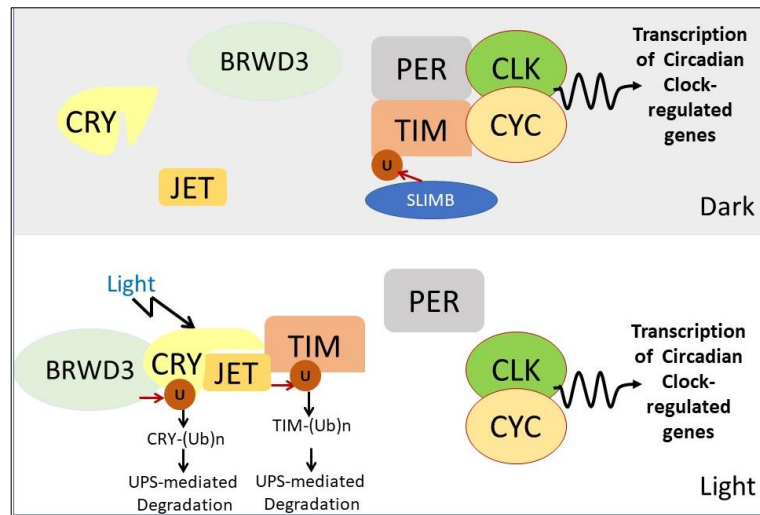


Figure 2.2. Photoreceptor complex that resets the circadian clock in *Drosophila*. U, ubiquitin; UPS, ubiquitin-proteasome system.

2.3. Structure of *Drosophila* Cryptochrome (dCRY)

Drosophila Cryptochrome (dCRY) is a photolyase-like blue light sensitive photoreceptor with a Flavin cofactor (FAD), which is embedded into the core region of the protein that absorbs the light and triggers the conformational changes on dCRY [Zoltowski, 2011; Ozturk N. *et al* 2011]. *Drosophila* Cryptochrome (dCRY) belongs to the Cryptochrome/Photolyase family of photoreceptors [Levy C. *et al.* 2013; Czarna A., 2013]. Photolyases are ancient proteins, responsible from repairing the UV-damaged lesions in the DNA. UV-light exposure causes the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6–4) photoproducts on the DNA. Photolyases also utilize FAD as their cofactor and use blue light into solar light spectrum to invert those photoproducts to pyrimidine-pyrimidine dinucleotides [Brettel K. *et al.*, 2010; Ozturk N. 2017].

Cryptochromes are composed of two domains, the N-terminal PHR (Photolyase-Homologous Region) domain and the C-terminal domain of various lengths. The PHR domain binds non-covalently to the chromophore flavin adenine dinucleotide (FAD). Like photolyases, cryptochromes are also activated by blue light due to their FAD cofactors, but they do not repair damaged DNA. Nevertheless, they have acquired C-terminal and N-terminal extensions that bring them signal transduction property. Both photolyase and cryptochrome proteins are found in all species of three

kingdoms of life, which provide incontrovertible prominence to survival capacity of an organism [Brettel K. et al., 2010; Müller, 2009; Falciatore, 2005].

Cryptochromes have different missions in different organisms. In plants, they regulate development and growth, in some animals including the fruit fly *Drosophila melanogaster* work as photoreceptors in biological clock resetting process, and in mammals despite they have lost their photoreceptor property, they are essential components for the biological clock [Vaidya A. T. et al., 2013].

The FAD cofactor is surrounded by an alpha helical domain of dCRY. On the ground state, dCRY has FAD in its oxidized state (FADox). Reduction of dCRY in response to blue light yields to FAD•-, an anionic semiquinone (ASQ) [Song, 2007; Ozturk, N., 2008; Ozturk *et al.*, 2014]. In the end, the C-terminal tail of dCRY, which is composed of an alpha-helix, changes its conformation. This conformational change enables dCRY to transmit this light signal to the core clock elements [Vaidya A. T. et al., 2013]. This photoreduction during light exposure has been suggested as the action mechanism of dCRY.

The FAD cofactor is photo-reduced by an electron transfer process through the three evolutionarily-preserved tryptophan residues named Tryptophan triad on dCRY. The crystal structure of dCRY was solved in 2011 and it was found that the 3 tryptophan that make up the triad are Trp-342, Trp-397, and Trp-420, respectively [Zoltowski B. D. et al., 2011; Levy C. et al. 2013; Ozturk, 2014]. There are also a number of loops surround the C-terminal tail. Those are the protrusion loop (Phe288 to Ala306), the phosphate binding loop (Glu246 to Met266), the C-terminal lid (Ser426 to Pro440) and the electron-rich sulfur loop, which contains Met331 and Cys337 [Czarna A., 2013].

High resolution crystal structure of dCRY revealed that a Trp residue (Trp-536) in the C-terminal extension of the dCRY in the proximity of FAD. This amino acid could function as an alternative electron donor to FAD *in vivo* in Trp mutants [Ozturk N. et al, 2014]. But it was shown that W536F, and the W397F/W536F mutants were equally susceptible to photoinduced proteolysis. The Trp-536 residue does not contribute to light-induced photo-signaling [Ozturk N. et al, 2014].

On both oxidized and reduced states of dCRY permit photo-transduction process that suggests photo-reduction does not run photocycle [Ozturk N. et al, 2014]. In addition, both oxidized and reduced forms of dCRY are able to change its conformation at similar rate. dCRY (FAD-) and dCRY (FADox) are equally

effective photoreceptors for light induced conformational changes. Moreover, both forms can interact with JET and TIM proteins in the same rate under light exposure. These outcomes suggest that excitation of oxidized/ reduced FAD *in vivo* causes a conformational change that increases the JET affinity [Ozturk N. et al, 2014]. It can be concluded that light induced dCRY dependent TIM proteolysis is depend on photosensitive ability of dCRY [Ozturk N. et al, 2014].

2.4 Light dependent interactions of *Drosophila* photoreceptor

JETLAG (JET) protein is the E3 ligase and responsible for the ubiquitination and proteolysis of Timeless (TIM). dCRY binds to TIM and JET in a light triggered manner; in this way, brings TIM and JET into close proximity for degradation of TIM that yields to the circadian clock resetting. CRY also bind to Brwd3 and this bindings allows the degradation of itself at a slower rate after TIM degradation, thus the clock resets only once in a day [Ozturk N. et al., 2013].

Timeless (TIM) protein hosts a 10-residue long region that resembles the C-terminus extension of dCRY. This region of Tim is called “C-terminal tail-like” (CTL). It has been reported that an artificially made CTL like region can interact with light induce dCRY [Dissel S. et al., 2004]. This indicates that light induced C-terminus opening allows TIM-dCRY interaction. In dark, C-terminus blocks the interaction regions of dCRY [Vaidya A. T. et al., 2013].

Light induced proteolysis of dTIM and dCRY is specific and depend on dCRY photosensory function. The W397F mutation had no effect on dTIM proteolysis [Ozturk N. et al., 2014].

JET and dCRY interaction was visualized on yeast colonies by substrate color change via galactosidase gene expression. Constitutively active mutant CRY Δ C was used as a positive control. *In vitro* illustration of JET and dCRY interaction was also shown. Sepharose bead bound to GST-JET incubated with FH-CRY and FH-CRY Δ C at 25°C/12min under blue light (1 mW/cm²) or dark. CRY Δ C which binds JET strongly even in the dark was also positive control in there. Wild type dCRY is another positive control that interacts with JET weakly at dark conditions, yet strongly interacts with JET at light [Ozturk N. et al., 2011].

Light triggers a conformational change in the C-terminus of dCRY which is revealed by partial trypsinization. This technique enables the comparison of trypsin-cut fragments of both light-treated wild-type and CryDelta (CRY Δ C) which is the C-terminal deletion mutant. It was known that deletion of 20 amino acid in C-terminal gives dCRY a constitutively active phenotype so this suggest that light cause conformational change which affects the its interactions [Dissel, S. *et al.* 2004]. It was confirmed by partial proteolysis. In addition, it is demonstrated that light enhanced the interaction between JET and dCRY. However CryDelta (CRY Δ C) binds JETLAG strongly even in the dark. In the end, light-induced conformational change in the C-terminus of dCRY allows JET interaction necessary for clock resetting [Ozturk N. *et al.*, 2011; Ozturk N. *et al.*, 2013].

3. MATERIALS

3.1. Kits, Enzymes, Reagents and Equipment

Table 3.1: List of kits, enzymes and reagents.

Protein Molecular Weight Marker	P7712S, New England Biolabs, USA
DNA Ladder	N3232S, New England Biolabs (NEB), USA
DNA Loading Dye	B7021S, New England Biolabs (NEB), USA
Trypsin-ultra™, Mass Spectrometry Grade	P8101S, New England Biolabs (NEB), USA

Table 3.2: List of equipment.

Agarose Gel Electrophoresis	1704406, Bio-Rad, USA
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, Bio-Rad, USA
Incubator	Thermo-Scientific, USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad, USA
Blue light lamp	MLP 58 Multiple ray lamp, UVP, Canada
Power Supply	Bio-Rad, USA
TransBlot Transfer System	1704155, Bio-Rad, USA
Shaker	Standard Analog shaker, VWR, USA
Vortex	Scientific Industries, USA
Water Bath	Wisd, Germany
Inverted Microscope	(Nikon, Eclipse, E100)
Laminar Flow Cabinet	Hera-Safe Class II Cabinet, Thermo Scientific, USA
Microplate Reader	Varioskan Flash, Thermo Scientific, USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad, USA

3.2. Buffers and Solutions and Antibodies

Table 3.3: 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
10X PBS	BE17-517Q, Lonza, Belgium
Coomassie Blue Staining Solution	0.1% Coomassie Blue R250 (Sigma) 10% acetic acid, 50% methanol
Destaining Solution	10% acetic acid, 50% methanol
LB Broth Medium	LBP03, Caisson Labs, USA
LB Agar	LBP04, Caisson Labs, USA
Silver Nitrate Solution	0.1 % AgNO ₃ 1.5 % 37 % Formaldehyde
Fixation Solution	10% Acetic Acid

Table 3.3: Table of Continuation.

Developing Solution	%3 Na ₂ CO ₃ 1.5 % 37 % Formaldehyde Before use: 20% Sodium Thiosulfate Solution
Sodium Thiosulfate Solution	10% Na ₂ S ₂ O ₃
Acryl:Bis Solution	40% Acrylamide:N,N'-Methylenebisacrylamide 29:1 solution (Sigma Cat No: 01708-500ML)

Table 3.4: List of Antibodies.

Name	Dilution	Source
Anti-V5	1:2000	V8012, Sigma-Aldrich
Anti-mouse, HRP	1:3000	7076, Cell Signaling Technology

Table 3.5: List of SDS-PAGE Gel Recipe

8% separating gel	5 ml	10 ml	15 ml
<i>ddH₂O</i>	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
10% separating gel	5 ml	10 ml	15 ml
<i>ddH₂O</i>	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	0.005	0.01	0.015

Table 3.5:Table of Continuation.

12% separating gel	5 ml	10 ml	15 ml	
<i>ddH₂O</i>	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	
15% separating gel	5 ml	10 ml	15 ml	
<i>ddH₂O</i>	1.775	3.55	5.325	
40% Acryl:Bis	1.875	3.75	5.625	
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	0.005	0.01	0.015	
5% Stacking Gel	2 ml	4 ml	6 ml	8 ml
<i>ddH₂O</i>	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. Plasmids

Constructs:

- pAc5.1-dCRY-V5/HisA [generated as follows: HisV5-dCRY cDNA was amplified from pAc5.1.V5.dCRY [Ozturk N. et al., 2011].
- pGEXT4T1-His-JETLAG (JETLAG). [Ozturk N. et al., 2014].

- pAc5.1_sTIM-FHis (TIMELESS) [Ozturk N. et al., 2014].
- pFast-Bac-His-V5-dCRY.

This plasmid was generated as follows: HisV5-dCRY cDNA was amplified from pAc5.1.V5.dCRY [Ozturk N. et al., 2011] using oligos Forward: AGCGAATTCGCCACCATGCACCACCACCACCACGGTAAGCCTATCCC TAAC and Reverse: GCTCTAGATCAAACCACCACGTCGGC, and digested with EcoRI and XbaI restriction enzymes and cloned into pFast-Bac1 plasmid.

3.4. Baculoviruses

Baculoviruses were prepared using the Gibco BRL Bac-to-Bac® baculovirus expression system (Invitrogen). Briefly, pFastBac1 based plasmids containing target coding sequences were transformed into *Escherichia coli* DH10Bac, and recombinant bacmids were isolated from 1 mL of bacterial cultures grown from colonies of transformants. For recombinant virus generation, Sf21 cells plates were transfected with 1-2 µg of bacmids using 6 µL of Cellfectin reagent (Invitrogen) in 6-well plates, and parental virus was collected after 72 hours. After three more 72-hour virus amplification steps, the fourth passage (P4) or P5 high titer stock was obtained and used to infect Sf21 cells for large-scale expression of protein.

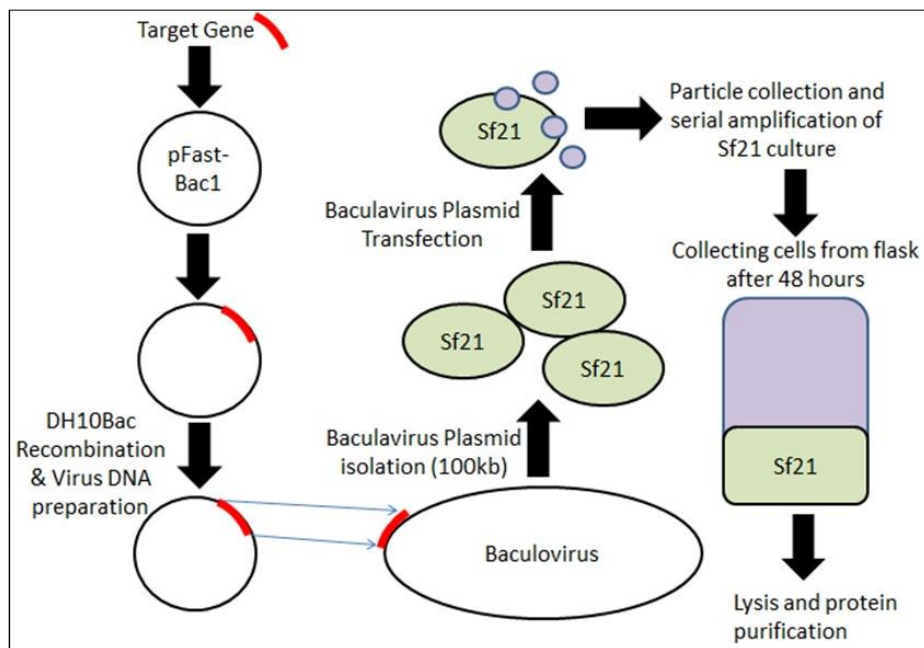


Figure 3.1: Baculovirus preparation schematization.

4. METHODS

4.1. Recombinant Protein Isolation

pAc5.1.dCRY-V5HisA was obtained from Prof. Aziz Sancar (University of North Carolina at Chapel Hill) [Ozturk N. et al., 2011]. Coding sequence for dCRY-V5His or HisV5-dCRY was transferred to the commercially available Bac-to-Bac Baculovirus gene expression system (Invitrogen) and used to prepare insect viruses. For this aim, pFast-Bac-dCRY-V5H (or pFast-Bac-HisV5-dCRY) vector was transformed into *Escherichia coli* DH10Bac strain, and the recombinant virus DNA at the end of the recombination were manually isolated using Alkaline Lysis Method [Birnboim H.C., 1979]. Isolated virus DNA was transfect into Sf21 insect cells to obtain baculoviruses. At 72 hours following transfection, viruses were collected from the cell culture medium and P1 virus stock was stored at 4°C. These viruses were then added to a new Sf21 in T25 cell culture flasks cell at 1: 100 (volume: volume) ratio, and after 72 hours, new viruses were collected as P2 viruses. This process was repeated until P4 was obtained and 20 ml of P4 viruses were stored at 4 °C until the date of infection. Sf21 cells at 80% confluency in ten of 150 mm cell culture dishes, were infected with P4 virus at 1:100 (volume: volume) ratio. After 48 hours of infection, the cells were scraped and collected in 50 ml conical tube, and then the cell pellets were stored at -80°C until purification procedure. These cell pellets were lysed by dissolving in 30 ml lysis buffer (100 mM NaCl, 50 mM Tris pH 7.5), and by sonicating 10 times of 10- second stroke and 10-second resting cycles on ice. The lysates were cleaned by spinning at 4°C and 25,000 g for 90 minutes, and the supernatant was taken in a conical tube and 30 mM imidazole at final concentration was added to prevent non-specific binding to Ni-NTA beads. Five hundred microliter of lysis buffered calibrated Ni-NTA beads were added to the lysate. A two hour incubation at 4°C was performed to allow His-tagged dCRY proteins bind to Ni-NTA-beads. Subsequently, the unbound portions of the 30 mM imidazole-containing 30 mM imidazole were removed and the proteins from the beads were eluted using 300 mM imidazole (in lysis buffer). Eluted protein samples was dialyzed against dialysis buffer containing 50% glycerol, 100 mM NaCl, 50 mM Tris pH 7.5) for 4 hours and samples were stored at -20 °C. Flag-His-TIMELESS (FH-sTIM) was expressed using bac-to-bac baculovirus expression system (invitrogen), and

recombinant protein was purified using Ni-NTA beads as described for His-V5-dCRY purification.

GST-His-JETLAG was expressed from the pGex4T1-h-JET vector in *Escherichia coli* BL21 strain. This plasmid was transferred into the BL21 cells, and grown in 1 liter Luria-Bertani medium in the presence of ampicillin antibiotic (100 µg/ml). When cell density reached O.D. 0.6, 0.3 mM of IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the culture in order to induce the expression of recombinant protein for 5 hours. At the end of the induction process, the cells were pelleted and stored at -80 °C until purification. For purification, cells were dissolved in 30 ml lysis buffer, and cracked by sonication (10 times of 10-second stroke and 10-second rest on ice). Lysate was cleared by centrifugation at 21,000 g for 90 minutes. Supernatant was transferred into a new conical tube and 400 ml of Sepharose 4B beads were added into supernatant. Beads in cleared lysate were incubated for 2 hours to allow GST-His-JET bind to the beads. Beads were washed with lysis buffer for four times to remove unbound proteins and GST-His-JET was eluted and dialyzed using 40 mM reduced Glutathione from these beads, dialyzed against dialysis buffer, and kept -20 °C.

4.2. Protein Purity Determination

In order to confirm the purity, the isolated proteins were visualized by coomassie staining after separation by SDS-PAGE application. For quantitation, Bovine Serum Albumin (BSA) at varying amounts was loaded as reference and used to calculate the concentration of purified proteins.

4.3. Preparation of SDS-PAGE Gel

10 ml of separating gel (Table 3.5) was poured into beakers placed in a falcon tube and placed in a gel casting system. The 2-propanol was poured on top of the gel to prevent bubbles and to enhance the polymerization. After the gel was solidified, the stacking gel was poured on top of the separating gel with materials stated on table 3.3 and the combs were inserted into the gel immediately after stacking gel was poured and shaken gently, if necessary, to remove any bubbles. After polymerization

of the gel (about 15 minutes), the comb was removed and the gel was get wetted with *ddH₂O* and stored at 4°C for further use or placed in a vertical electrophoresis system filled with 1 × running buffer.

4.4. SDS-PAGE Gel Electrophoresis

Samples in 1x SDS-Loading buffer were incubated in a heat block at 95°C for 10 minutes, and then spun down at 5,000 g for 10 seconds.

The SDS-PAGE was performed at 80 V until proteins reach to the separating gel, then the voltage was increased to 120-150 V. Electrophoresis was stopped when bromophenol blue dye reached end of the gel.

4.5. Western Blotting

Proteins from SDS-PAGE was blotted to nitrocellulose membranes using a semi-wet transfer apparatus (Biorad Trans-Blot® Turbo™ Transfer System Cat No: 1704150). After gel electrophoresis, 4 blotting papers and 1 nitrocellulose membrane with the same sizes of SDS PAGE gel were cut. The blotting paper and the nitrocellulose membrane were soaked into 1x transfer buffer and then a transfer sandwich was made, SDS-PAGE gel being in the middle. The components were assembled on the cassette in the order as follows: Top (-) Cassette electrode (cathode)-Two Blotting papers -Gel-Blotting Membrane-Two blotting papers-Bottom (+) cassette electrode (Anode) (Figure 4.1). A roller was used to remove any air bubbles trapped between the layers. Blotting (transfer of proteins from SDS-polyacrylamide gel to nitrocellulose) was performed at 12 Volts for 90 minutes.

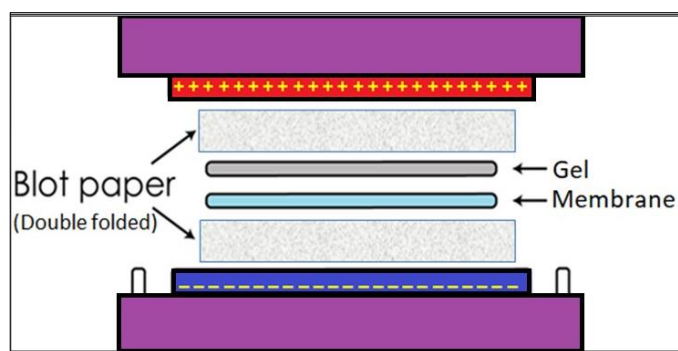


Figure 4.1: Transfer sandwich set-up.

4.6. Antibody Incubation

After the transfer step, the membrane was soaked in 5% blocking solution for 1 hour at room temperature after transferring process. The blocking solution contains 5% non-fat dry milk in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20 (TBS-T) (weight/volume). The primary antibody (anti-V5 antibody) was diluted at 1:5000 in the blocking solution, the membrane was incubated with primary antibody overnight at 4°C. The membrane was washed four times (5, 15, 5, and 5 minutes) with 1X TBST and then incubated with secondary antibody (anti-mouse HRP) in the blocking solution at 1:5000 dilution.

4.7. Gel Imaging

After the incubating the membrane with the secondary antibody for 1 hour at room temperature, the membrane was washed four times with TBS-T (5, 15, 5, and 5 minutes) to remove the unbound antibodies. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation (Western Blotting Detection Kit Advansta Cat: K-12043-D10 or Signal Fire™ ECL Reagent, Cell Signaling Cat: 6883S substrates are used).

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).

4.8. Coomassie Blue Staining

The gels were stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% double distilled water for one hour. Gels were then destained to remove background staining in order to visualize protein bands. For destaining, gels were washed in a destaining solution (consisting of 10% acetic acid, 50% methanol, and 40% double distilled water) for times with 1 hour incubation for each wash. Images of gels were captured using an imaging system (Biorad, Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545 is used).

4.9. Partial Trypsinization

The working principle of the experiment is shown in figure 4.2. Trypsin is a protease that cleaves peptides after certain amino acids (Arg or Lys). As exemplified herein (Figure 4.2a), specific regions of dCRY are present in different conformation under dark and light, in the dark there is a peptide fragment between trypsin and arginine, which prevents breakage after arginine in that conformation. When the reaction products are run in SDS-PAGE, formation of peptides of different lengths indicates that both samples are in different conformation. When JET is added to the medium, a different fragment pattern will be produced according to the control because it interacts with CRY protein and blocks some Trypsine attack sites (Figure 4.2b). After the analysis of these different cropping products by SDS PAGE and then by immunoblot, the length of the different fragments is calculated and the primary structure of the JET protein is calculated and the 3-dimensional structure of the CRY protein can be determined.

For trypsin titration, 5 μg of dCRY-V5His and His-V5-dCRY were aliquoted into tubes containing 1 $\times$ PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) pH 7.4 in a total volume of 50 μL , and reactions were initiated with the addition of sequencing grade trypsin (Promega) at final trypsin:protein ratios of interest. Tubes were exposed to blue light at 0.5 mW cm^{-2} without (light) or with (dark) aluminum foil covers. The blue light source was one F15T8-BLB Black Light (General Electric) with emission maximum at 366 nm. Reactions were stopped with 15 μL of 5 $\times$ SDS sample buffer [250 Tris-Cl mM, pH 6.8, β -Mercaptoethanol (5%), Bromophenol blue (0.02%), Glycerol (30%), SDS (Sodium dodecyl sulfate) (10%)]. Then, 15 μL of each reaction was resolved by 8 to 15 % SDS-PAGE gels, and then proteins were transferred onto a nitrocellulose immunoblotting membrane.

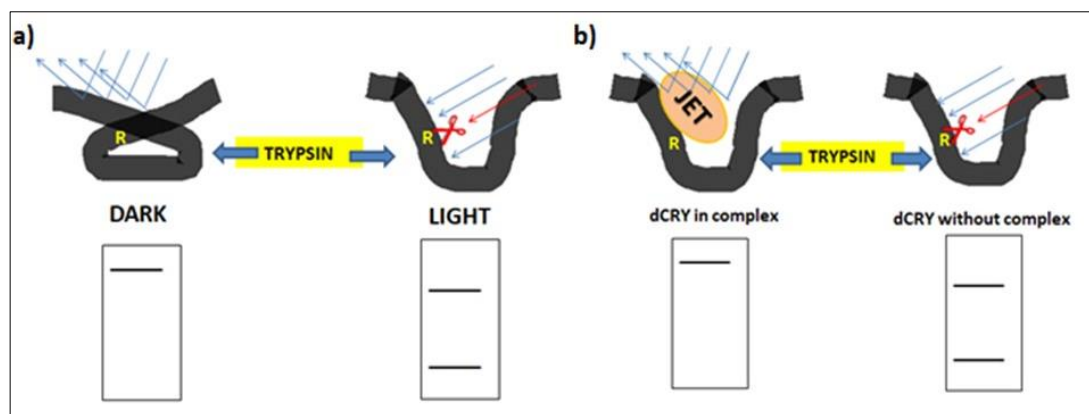


Figure 4.2: Partial trypsinization mechanism. a) Partial trypsinization of dCRY under dark and light. b) Partial trypsinization of dCRY in complex/ without complex. R, Arginine.

4.9.1. Partial Trypsinization for 30 Seconds under Blue Light

At this stage, the ratio of trypsin and dCRY protein was increased and the incubation time was shortened. The light here is used for 30 seconds for the formation of different fragments under light. This incubation time was manually assessed as the shortest reproducible time interval for the addition of trypsin. The reaction is stopped with 15 μ L of $5 \times$ SDS sample buffer. Tryptic attack points were detected on the recombinant protein according to the sizes of the fragments obtained.

4.10. Silver Staining Protocol

After completing the separation of samples in SDS-PAGE, SDS polyacrylamide gels were removed from the glass plates and put it in a plastic tray (the tray washed with a small amount of detergent and double distilled water and air dried before the procedure). The gel was washed with double distilled water for 2 minutes in order to get rid of any remnant from SDS gels. After washing, gels were fixed with 10 % acetic acid (fixation buffer) for 1 hour. After fixation, gels were washed with double distilled water for 2 minutes. This step was repeated 3 times, until the fixation buffer was totally removed. Fixed gels were then incubated with AgNO_3 solution in dark for 30 minutes. After staining with silver nitrate, gels were washed with double distilled water for 30 seconds three times and then developed in developing buffer (6% sodium carbonate, 0.02% formaldehyde, 0.0004% sodium thiosulfate) until the bands get visible. Development of the staining was stopped with the addition of fixation

buffer when the visibility of bands and contrast of gel were sufficient for interpretation of the image. Finally, gels were washed with double distilled water. The images of the gels were taken via imager (BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545 is used).

5. RESULTS

5.1. Analysis of Expression of Proteins

Recombinant dCRY-V5His (V5-6xHis tags at the C-terminus), HisV5-dCRY (6xHis-V5 tags at the N-terminus) and Timeless-Flag-6xHis (sTIM-FH) were expressed in Sf21 cells following Bac-to-Bac insect cell expression system protocol as supplied by the manufacturer (Invitrogen). The expression from Sf21 cells infected with their corresponding baculoviruses was analyzed by immunoblotting using anti-V5 or anti-FLAG antibodies (Sigma). (Figure 5.1. A).

GST-H-JET expression was analyzed in *Escherichia coli* strain BL21 following the induction with 0.3 mM IPTG for 3 hours. Cells were lysed in 1x SDS Sample buffer, and run in SDS-PAGE. Overexpression was confirmed with coomassie staining (Figure 5.1. B).

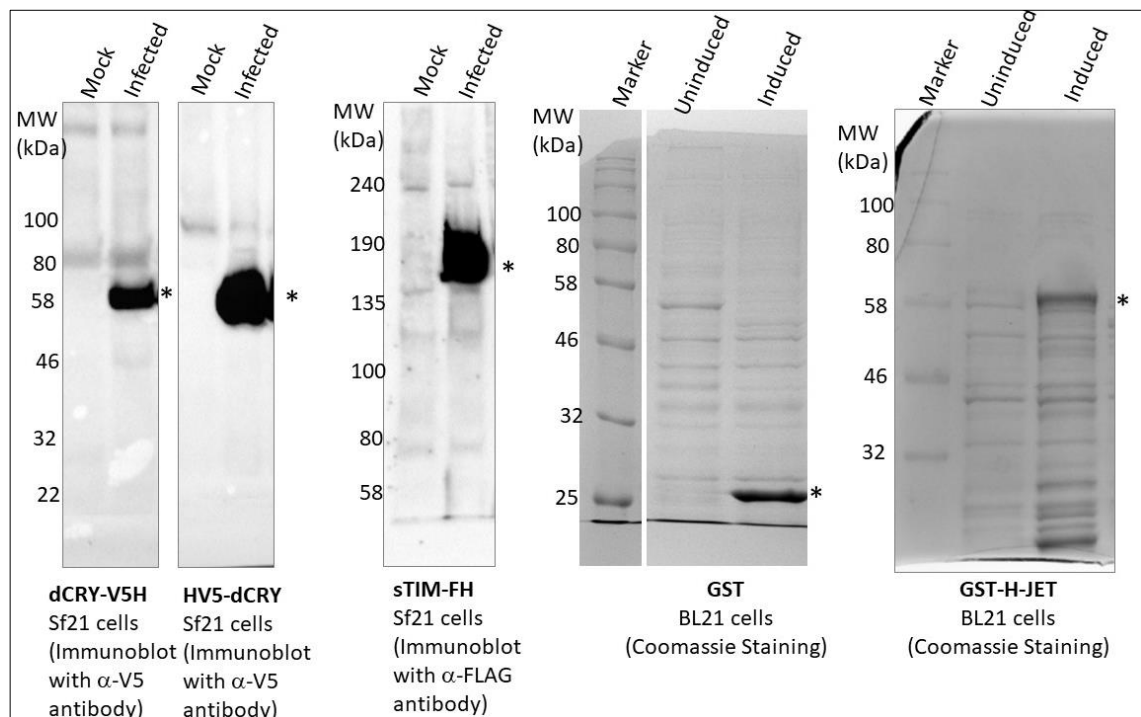


Figure 5.1: Analysis of expressions of recombinant proteins used in this study. A) The expression of dCRYV5H (Cryptochrome-V5H) protein in Sf21 cells B) The expression of GST-H-JET construct.

5.2. Purification of Recombinant Proteins

In this study, proteins were purified from either Sf21insect cells or *Escherichia coli* strain BL21 cells by His or GST affinity purification. In both cases, cells from 500 ml of infected-Sf21 culture or 1 liter of BL21 culture (induced with 0.3 mM IPTG for 5 hours) were lysed in 30 ml of 1x Lysis buffer containing 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 0.5% Tween-20, and 0.1% NP-40 on ice. Cells were cracked using sonication with 10 times of 10 second-strokes and 10-second resting cycles. Then lysates were cleared by centrifugation at 21000 g for 90 minutes, and clear supernatant were transferred into conical tubes. Then 25 mM imidazole were added into insect cell lysates (to be purified with His affinity) in order to reduced non-specific binding to beads. For 500 µl of Protino® Ni-NTA Agarose or Protino Glutathione Agarose 4B (MACHEREY-NAGEL GmbH, Germany) which were equilibrated with lysis buffer were added into cleared lysates from Sf21 cells or BL21 cells, respectively. Lysates and beads were incubated for 4 hours, and unbound fractions were washed 4 times with excess lysis buffer. For washing Ni-NTA beads, lysis buffer contained 25 mM imidazole. Proteins were eluted with a buffer of 50 mM Tris.Cl pH 7.5, and 100 mM NaCl containing 300 mM imidazole (for His affinity purification) or 40 mM reduced glutathione (for GST affinity purification) for 2-hour incubation. Eluted proteins were dialyzed again a dialysis buffer containing 50 mM Tris.Cl pH 7.5, 100 mM NaCl, 50% glycerol to remove excess imidazole or glutathione. Samples in dialysis buffer were kept -20°C where they can be stored without freezing. Appropriate amounts (1-5 µl) of purified proteins were analysis with SDS-PAGE followed by coomassie staining or immunoblot (Figure 5.2).

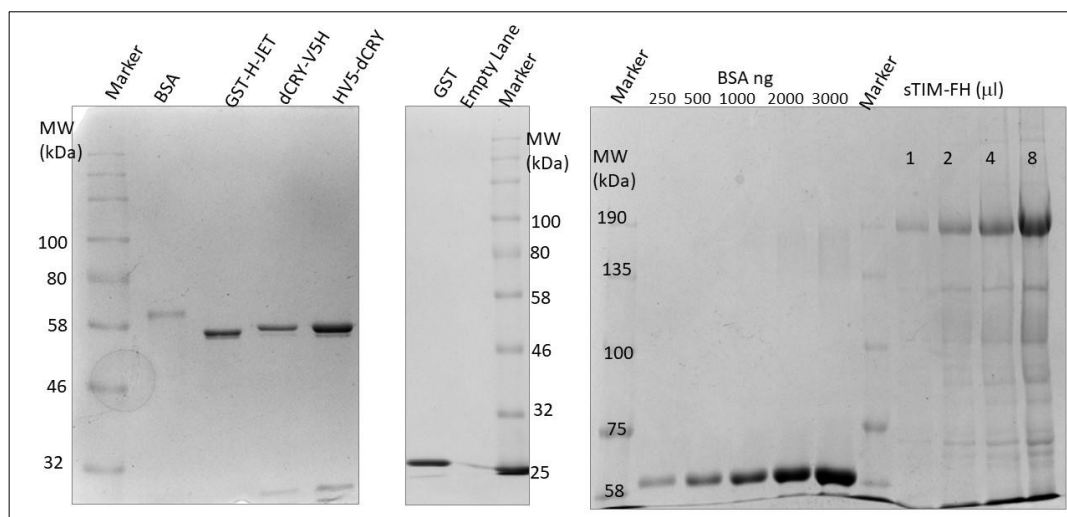


Figure 5.2: Analysis of purified proteins. Samples from purified proteins were analyzed with SDS-PAGE followed by a coomassie staining.

5.3. Optimization of Partial Trypsinization Reaction

Before starting partial trypsinization reactions all proteins from different purification batches were analyzed with coomassie staining (Figure 5.3). BSA at different amounts were also loaded in the same gel in order to calculate the concentration of purified proteins. After confirming that all proteins are purified to homogeneity, they were diluted with 1x PBS for partial trypsinization assay.

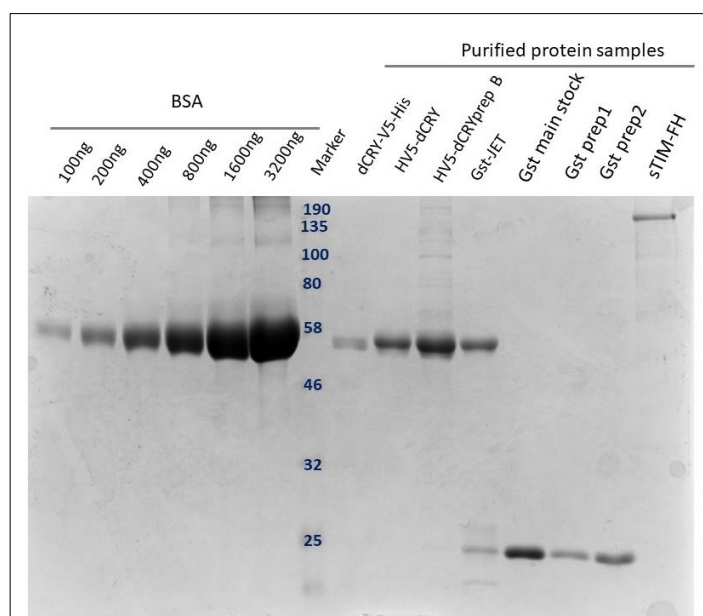


Figure 5.3: Coomassie blue staining for the determination of homogeneity and amounts of purified proteins.

Firstly, changes in the light-dependent 3D structure of dCRY which is analyzed by partial trypsinization indicate that purified protein are function or at least they response to light and undergo conformation change. To see these changes, at least 20 different ratios of Trypsin to dCRY (substrate) were tried in partial trypsinization experiments. On the Figure 5.4., there are 4 ratios of trypsin to protein, that are 1:100, 1:400, 1:800, 1:1600, respectively. Fragments representing structural changes were detected by immunoblotting the V5 tag at the C-terminus of the Cryptochrome protein (Figure 5.4). Undigested recombinant dCRY runs around 60 kDa, while partial trypsinization reaction produces fragment from 60 to a few kDa. In order to confirm that these fragmentation is a repeatable we repeated this assay at least more than three times and compared the patterns to previously published data [Ozturk N. et al., 2011]. A fragment of about 17 kDa appears only in the light-activated Cryptochrome protein indicated that purified protein undergoes light-dependent conformational change as depicted with red circles on figure (Figure 5.4).

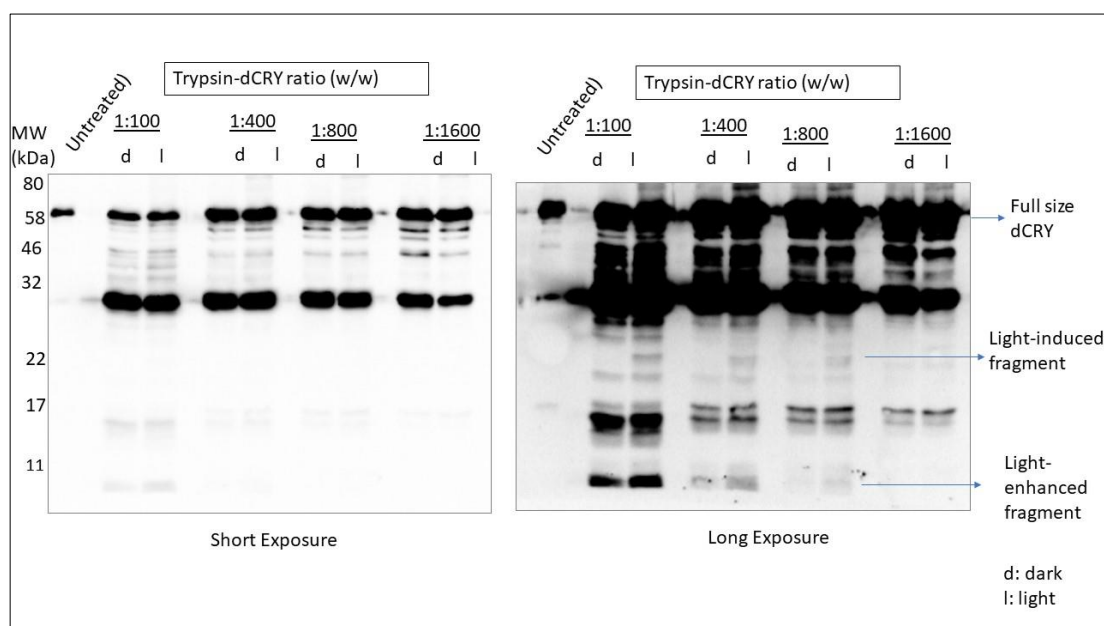


Figure 5.4: Detection of light-dependent structural changes in Cryptochrome with partial trypsinization.

5.4. Demonstrating the Changes in the C-terminus of dCRY by Optimizing the Partial Trypsinization Conditions

As the first process, dose and time optimization of partial trypsinization experiment were performed in order to determine the light induced change in C-

terminus position. The proteins with the His and V5 tags in the N- or C-terminus were treated with trypsin for 10 minutes at dark (D) and light (L, with the light density of 0.5 mW/cm^2) conditions and blotted with V5 antibody to detect different fragmentation at different ends. Both HV5-dCRY and dCRY-V5H displayed similar conformation change patterns indicating that both recombinant proteins were equally functional. Some of the fragments were observed in only light-treated samples while others were observed more intensely in light-treated samples, and therefore they were named induced or enhanced fragments, respectively (Figure 5.5).

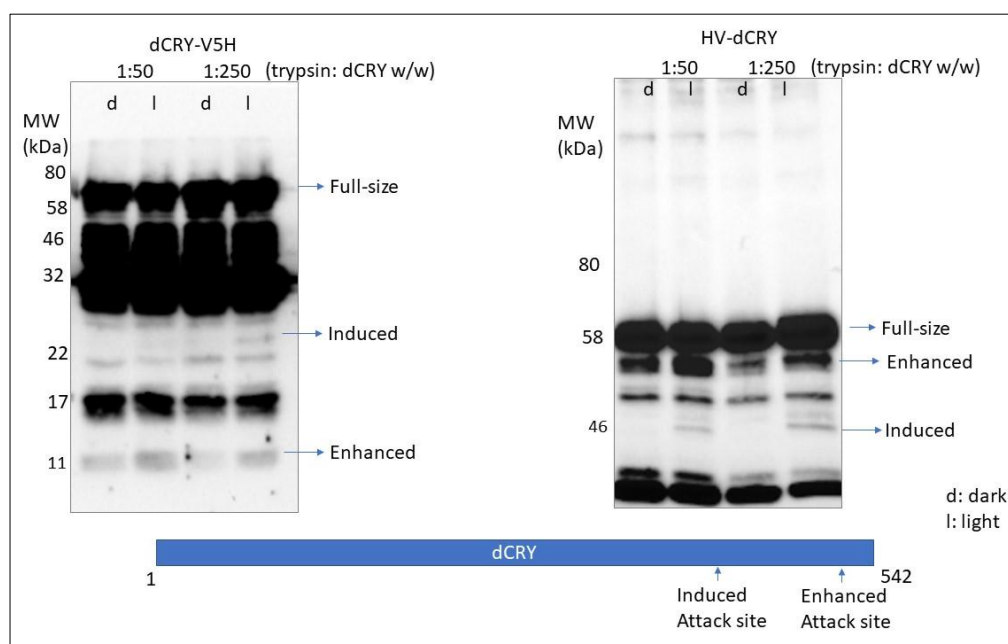


Figure 5.5: Optimizing of partial trypsinization conditions to show changes in C-terminus. Conditions: Dark (d) and Light (l), with the light density of 0.5 mW/cm^2 and two different trypsin: protein ratios as 1:50 and 1:1250 (weight/weight).

5.5. Detection of the Earliest Structural Change Zone by Partial Trypsinization at High Dose

In order to understand the early conformational change in dCRY, we used higher trypsin ratio to substrate (dCRY) but shorter light treatment durations. Manually, we could obtain repeatably results with a 30-second trypsinization reaction under light- and dark-conditions. Within the first 30 seconds, the tagged purified proteins were divided into fragments with partial trypsinization. Point here was to catch the structural region that the first change of FAD was affected. On the Figure 5.6.A, the V5 at the C-terminus was detected and analysis of 45the fragments

according to molecular sizes indicated that earliest conformational change was close to Q311. In the case of the sample blotted from the N-terminus on the right side, a fragmentation was obtained showing that it was also cut in the region close to H378. Three dimensional crystal structure of dCRY was already solved, and the amino acids which are in contact with FAD are already known (Figure 5.6). Two of them (Q311 6-4 photolyase, H378 is theoretically important for structural change of dCRY) gave rise to the idea that they can participate in detecting or relaying light-induced conformational change in FAD to protein part of dCRY.

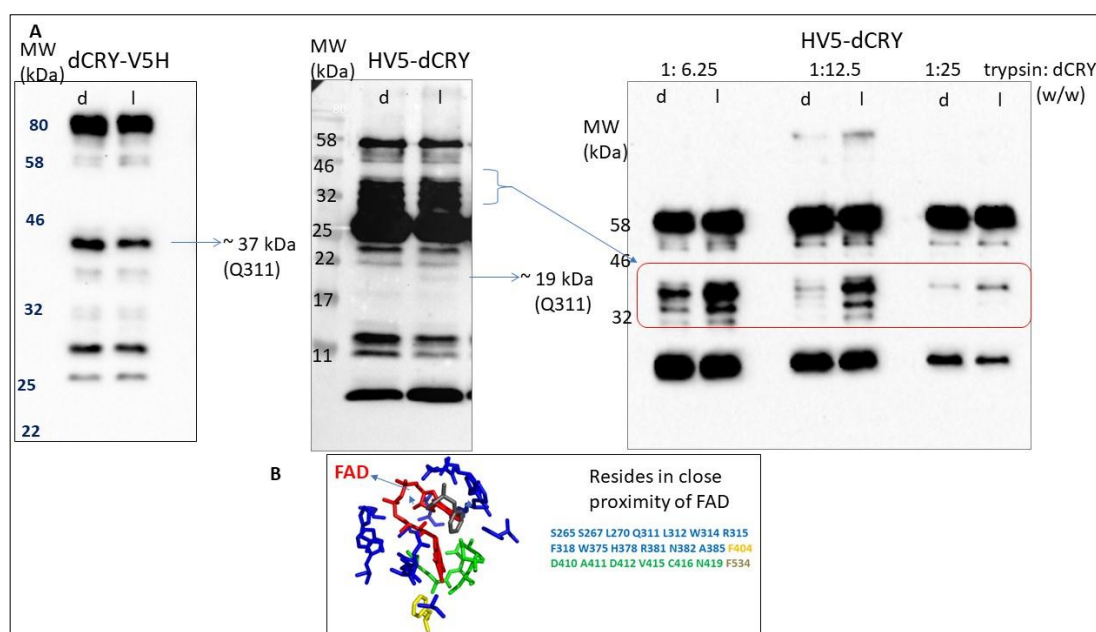


Figure 5.6: Detection of the earliest structural change zone with partial trypsinization at high dose. A) Western blot images of HV5-dCRY and dCRY-V5H trypsinization fragments B) Schematization of critical amino acids on dCRY that may have a role in cofactor interaction.

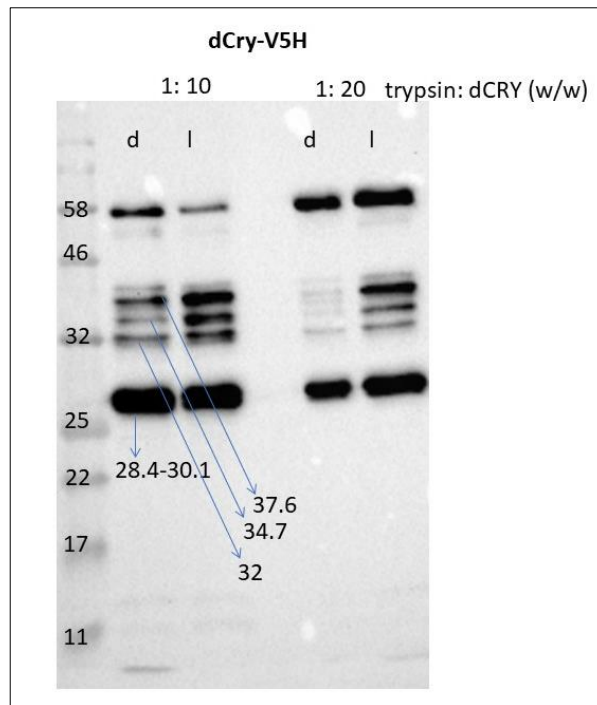


Figure 5.7: Partial trypsinization of dCRY-V5His under blue light (30second)

As seen on figure 5.7., some fragments between 25 and 38 kDa were induced with a 30-second light exposure. Light-dependent conformational changes during the first 30 second allowed trypsin access to the areas normally hidden in dark. In order to understand which parts or zones of dCRY undergo structural changes, molecular weights of these light induced fragments were calculated by molecular weight calculator tool of Image Lab 6.0 (BIORAD). Molecular weights of all bands on figure 5.7. were calculated and results can be seen in Figure 5.8.

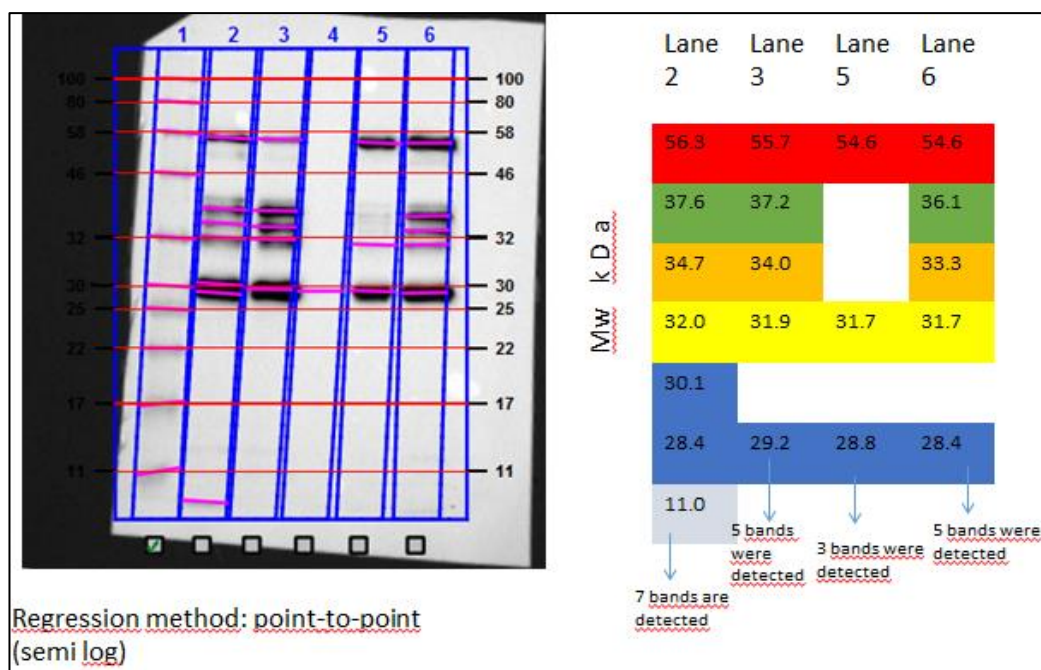


Figure 5.8: Molecular weight calculation of fragments from dCry-V5H partial trypsinization under blue light (30second).

A fully fragmentation of recombinant proteins were calculated from primary structure of them and given on Table 5.1. This fragmentation products were used to estimate the approximate sizes of partial trypsinization fragments.

Table 5.1: Molecular weight calculation of fragments from dCry-V5His partial trypsinization under blue light.

Lane 2 dCry-V5-His (dark) 1:2.5	Lane 3 dCry-V5-His (light) 1:2.5	Lane 5 dCry-V5-His (dark) 1:5	Lane 6 dCry-V5-His (light) 1:5
56.3 kDa	55.7 kDa	54.6 kDa	54.6 kDa
37.6 kDa	37.2 kDa	-	36.1 kDa
34.7 kDa	34 kDa	-	33.3 kDa
32 kDa	31.9 kDa	31.7 kDa	31.7 kDa
30.1 kDa	-	-	-
28.4 kDa	29.2 kDa	28.8 kDa	28.4 kDa
11 kDa	-	-	-
7 bands were detected	5 bands were detected	3 bands were detected	5 bands were detected

Table 5. 2: Table of resulting peptide sequence from partial trypsinization reaction of dCRY-V5H.

Position of cleavage site	Resulting peptide sequence	Peptide length [aa]	Peptide mass [Da]
4	MATR	4	477.579
12	GANVIWFR	8	962.119
16	HGLR	4	481.555
50	LHDNPALLAALADKDQGIALIPVFIFD GESAGTK	34	3522.015
56	NVGYNR	6	721.771
58	MR	2	305.395
78	FLLDSLQDIDDQLQAATDGR	20	2234.404
80	GR	2	231.255
93	LLVFEGEPAYIFR	13	1553.821
94	R	1	174.203
100	LHEQVR	6	780.882
103	LHR	3	424.503
117	ICIEQDCEPIWNER	14	1747.958
122	DESIR	5	618.644
126	SLCR	4	477.579
135	ELNIDFVEK	9	1106.241
179	VSHTLWDPQLVIETNGGIPPLTYQMF LHTVQIIGLPPRPTADAR	44	4894.669
194	LEDATFVELDPEFCR	15	1783.970
197	SLK	3	346.427
220	LFEQLPTPEHFNVDYGDNMGFLAK	23	2668.019
224	INWR	4	587.679
237	GGETQALLLLDER	13	1414.578
239	LK	2	259.349
247	VEQHAFER	8	1015.094
264	GFYLPNQALPNIHDSFK	17	1911.147
271	SMSAHLR	7	800.931
278	FGCLSVR	7	780.940
279	R	1	174.203
289	FYWSVHDLFK	10	1341.532
294	NVQLR	5	628.729
298	ACVR	4	447.553

Table 5. 2: Table of continuation.

315	GVQMTGGAHITGQLIWR	17	1825.118
330	EYFYTMSVNNPNYDR	15	1913.048
354	MEGNDICLSIPWAKPNENLLQSWR	24	2815.214
368	LGQTGFPLIDGAMR	14	1475.726
381	QLLAEGWLHHTLR	13	1573.818
390	NTVATFLTR	9	1022.169
406	GGLWQSWEHGLQHFLK	16	1923.164
430	YLLDADWSVCAGNWMWVSSSAFER	24	2794.108
445	LLDSSLVTCPVAK	15	1529.855
446	R	1	174.203
455	LDPDGTIYK	9	1021.135
466	QYVPELMNVPK	11	1317.566
474	EFVHEPWR	8	1099.214
494	MSAEQQEQYECLIGVHYPER	20	2410.658
503	IIDLMAVK	9	989.238
504	R	1	174.203
510	NMLAMK	6	706.917
513	SLR	3	374.440
532	NSLITPPPHCRPSNEEEVR	19	2175.404
549	QFFWLADV VWRPLESR	17	2148.495
568	GPFEGKPIPNLLGLDSTR	19	2008.305
576	TGHHHHHH	8	999.019

5.6. The Effect of GST-JET and sTIM-FH on the Trypsinization Pattern of dCRY and Detection of the Interaction Regions

The theoretical location of the interaction region between Cryptochrome and JETLAG can be estimated from previous publications [Ozturk N. et al., 2011]. The C-terminal of dCRY blocks binding or docking site for JET in the dark, opening the C-terminal by light expose this hidden docking site which allow JET bind to dCRY. dCRY Δ C, a dCRY mutant without the last twenty amino acids, which is a continuously active form of dCRY, binds strongly to the JET even in the dark, and the dark and light structure of dCRY Δ C resembles the structure of the wild dCRY in light which described in figure 2.2 [Ozturk N. et al., 2011]. For this reason, when

JET and dCRY are mixed, some fragments may be invisible in some regions when trypsin attack sites on dCRY are blocked by bound JET. At the same time, some fragment that is not visible may become visible. JET, for example, prevents one V5 from being truncated, allowing one fragment to become visible with the immunoblot. GST-JET (5 μ g) and GST (10 μ g) were mixed with dCRY (5 μ g) in separate reactions. In addition, the samples were visualized by silver staining (Figure 5.9a) for the possibility that these fragments could not be detected with V5 tag and then compared to immunoblotting, the JET binding site was predicted (Figure 5.9b). According to these results, it is interpreted that the light induced fragment, indicated with a red box on figure 5.9 a, lies away from the C-terminus with a distance of 27 kDa. This fragment corresponds exactly to the trypsin attack point before 289 amino acids. The fact that this fragment cannot be seen with the V5 tag indicates that the fragment is close to the N-terminal region. Since dCRY is masked by JET in the presence of light, hence the trypsin access is restricted, this fragment is not visible. When the data obtained are positioned in the Cryptochrome crystal structure, the model that was created by the previous works [Ozturk N. et al., 2011] is supported.

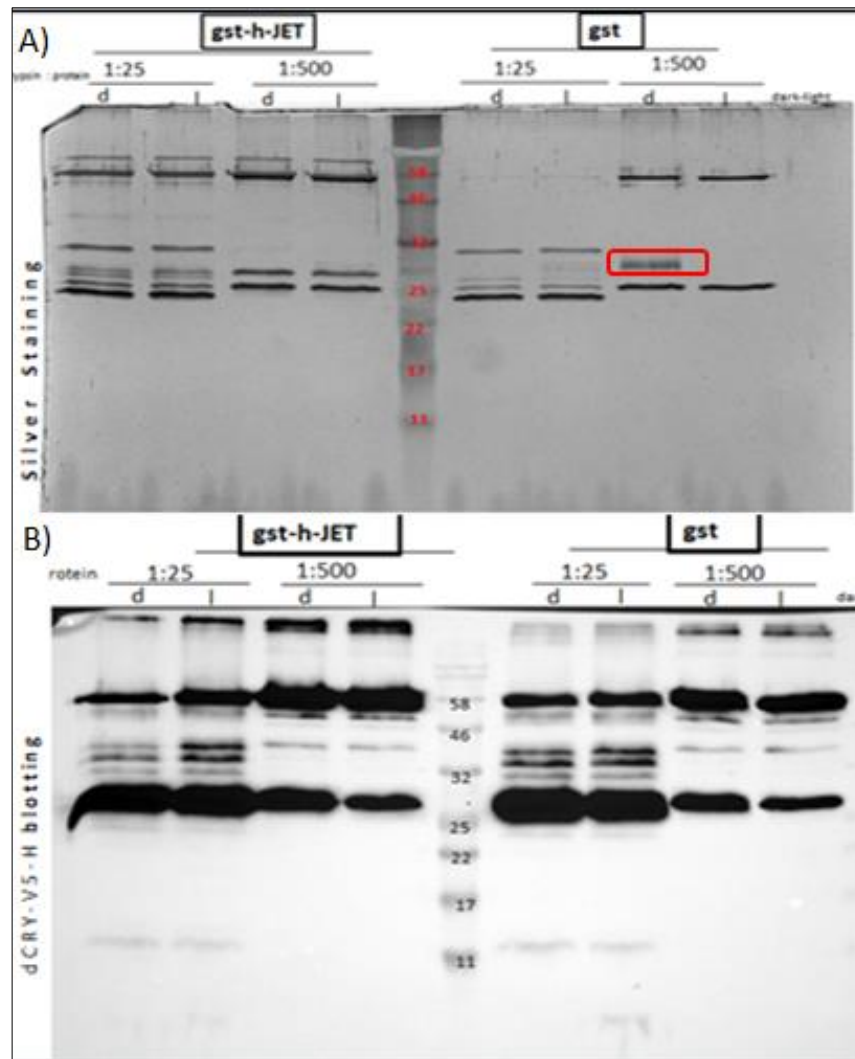


Figure 5.9: Determination of the binding site of GST-H-JET protein to light induced dCRY by partial trypsinization.a) Silver Staining image of partial trypsinization with JET/GST b) WB image of partial trypsinization with JET/GST.

In the dark this region is masked by the C-terminal end. On figure 5.10, trypsinization patterns of dCRY with sTIM-FH content and GST-JET content were detected with a similar fragmentation pattern on immunoblot result. These patterns may due to both TIM and JETLAG proteins have binding sites in immediate vicinity. This is a crucial status because TIM protein needs to JET for ubiquitination. Light induced dCRY aids this process.

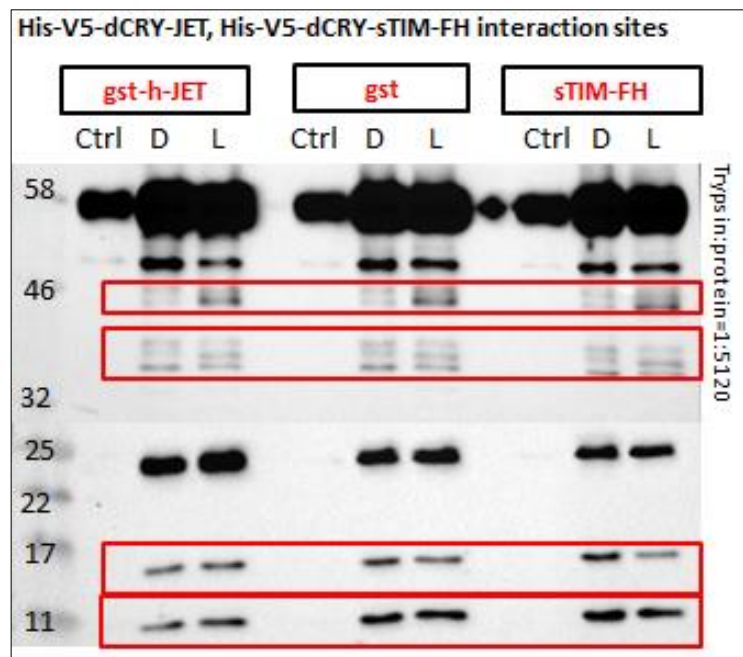


Figure 5.10: Determination of the binding site of sTIM-FH protein to light induced dCRY by partial trypsinization.

6. DISCUSSION

The resetting mechanism of the circadian clock in fruit fly is well studied. However, the molecular interaction sites are not known for photoreceptor molecule dCRY. First of all, the interaction domains of *Drosophila* Cryptochrome for Jetlag and Timeless are not known. The same is also true for Cryptochrome and Brwd3 interaction sites. In this thesis study, light-dependent interactions between Cryptochrome and Jetlag (as well as Timeless) using purified proteins were studied. It was not possible to identify the interaction domains of Cryptochrome using point or deletions mutations because even simple point mutations in Cryptochrome might cause the unfolding of proper structure (where FAD cannot bind to Cryptochrome protein) and renders recombinant protein insensitive to light.

As a solution, partial trypsinization technique was used to detect the interaction sites of Cryptochrome for Jetlag and Timeless proteins under dark and light conditions. When Jetlag (and/or Timeless) binds to Cryptochrome, they blocks trypsin attack sites on a certain region of Cryptochrome which can be detected by immunoblotting or SDS-PAGE/protein staining techniques. With the help of these immunoblot and SDS-PAGE/protein staining data of partially trypsinized (fragmented) dCRY, it can be possible to identify the binding sites of Jetlag and Timeless on light induced Cryptochrome and to form a more complete model how light resets the circadian clock without awaiting the expensive and time-consuming crystallographic identification of Cryptochrome-Jetlag-Timeless complex. Therefore, the partial proteolysis (trypsin-cut) patterns obtained in this thesis were analyzed to calculate the size of the fragments that are masked (the bands that are dense or decreasing).

Detection of fragments by immunoblot technique after partial trypsinization has some technical limitations or drawbacks due to the fact that trypsin cut sites close to the tag may prevent detect crucial fragments by immunoblotting. For solving this problem, silver staining method was also performed each set of partial trypsinization products. Results of immunoblots and silver staining were comparatively used to interpret or estimate the binding sites. The same issue was also valid for SDS-PAGE gel percentages. It was a necessity to scan each fragment on immunoblot and SDS-PAGE gel staining. On lower percentage of SDS-PAGE gels,

it was quite possible to miss some light sensitive key fragments. Thus, optimum gel percentage was 15% for most of the experiments. Especially while working on HV5-dCRY and dCRY-V5H, different percentages of SDS-PAGE gels had to be used due to the fact that approximately 5-6 kDa of the N-terminus was lost during trypsinization reaction which makes difficult the interpretation of the results (Figure 5.5).

Flavin Adenine Dinucleotide, which is non-covalently bound to the dCRY protein, undergoes to a structural change after it detects light (from 250 nm to 500 nm). This structural change is theoretically associated with an electron jump between the nucleotide and isoalloxazine rings of FAD [Liu et al. 2013]. The thesis topic interested in is the fact that the 'buckled butterfly' structure of the FAD cofactor with about 30° bending in the isoalloxazine caused a change in the protein part of dCRY at the end of this small conformational (structural) change. This results in the JET being connected to the dCRY under the light and by connecting the TIM protein at the same time renders TIM to undergo ubiquitination by JET, leading to rapid circadian resetting in the morning.

The first part of this thesis was to identify the critical amino acids which senses the light-induced conformational changes in the FAD cofactor and relay this structural distortions to the (apo)-protein part of dCRY photoreceptor. For this purpose, partial trypsinization reaction was first performed for 10 minutes to confirm previously published data [Ozturk N. et al., 2011] and establish a working protocol. Then, short trypsinization reactions were set-up, and the experiences of lasted minimum 30-second partial trypsinization reaction with higher trypsin-to-dCRY ratio gave reproducible patterns. Point here was to catch the structural region that is affected by the structural change of FAD. On the Figure 5.6.a, the V5 tag at the C-terminus was detected and the fragment was found to be close to Q311. In the case of the sample blotted from the N-terminus on the right side, a fragmentation was obtained showing that it was cut in the region close to H378. At this point, it was not possible which residue is the earlier sensor of the structural change of FAD. It is hypothesized that follow-up studies with site-directed mutagenesis of dCRY may help with understanding with which of them are really critical for the relaying of conformational change in FAD to the C-terminus which results in the relaxation of C-terminal end and opening of docking sites of JET and/or TIM proteins. At the same time, mutants of dCRY should be purified from Sf21 cells and analyzed for the

presence of stoichiometric FAD to exclude possibility that a non-specific effect of those mutations render the protein unfolded or misfolded.

The second part of this thesis was to identify approximate docking sites JET (and/or TIM) on dCRY protein. After dCRY and JET or dCRY and GST were mixed, the products were analyzed by immunoblotting and silver staining after trypsinization. On figure 6.1, the fragment, highlighted by the red circle, is induced by light at the end of the digestion is at a C-terminal distance of 27 kDa. Since the fragment enclosed in the circle cannot be detected in the presence of GST-H-JET, it was thought that the trypsin episode was blocked by JET. This fragment corresponds to the exact attack point of the trypsin prior to the first 289 amino acids from C-terminus. This fragment is invisible with the V5 tag, indicating that it is close to the N-terminal region of the protein. In the presence of GST-JET, this fragment is not visible because the region exposed by the light is masked by JET. This obtained Cryptochrome crystal structure is supported by the model created in a previous publication [Ozturk N. et al., 2011] (Figure 6.1). We could not obtain a clear docking site of TIM, and this indicates that either more optimization or new approaches are required for finding docking site of TIM on dCRY. Overall this thesis identified two residues as candidates for sensing the structural changes in FAD and relay them to protein part of dCRY photoreceptor. These residues Q311 and H378 were actually suggested theoretically as important residues in the action mechanism of photolyases or cryptochromes [Wijaya I. M.. et al., 2014; Ganguly A. et al, 2016]. However, more molecular studies are required in order to determine exactly which of them are the real sensor or, which of them are the most critical for the early changes induced by the conformational changes in FAD co-factor.

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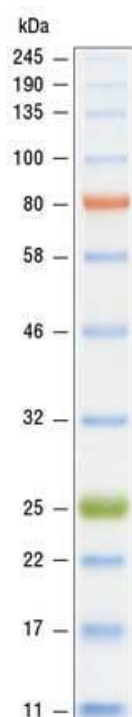
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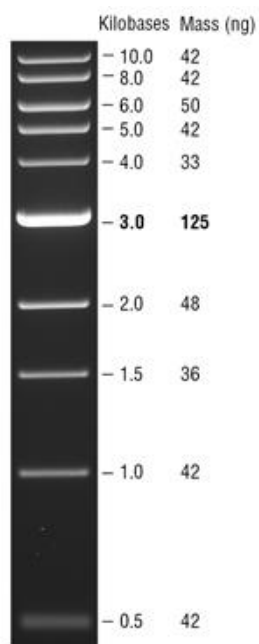
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APPENDICES

Appendix A: Protein and DNA Markers



Protein Ladder
(Color Prestained Protein Standard) P7712S



DNA Molecular Weight Marker
(1kb DNA Ladder) N3232S