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MOLECULAR CHARACTERIZATION OF INTERACTIONS IN *DROSOPHILA* CIRCADIAN PHOTORECEPTOR

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A THESIS OF DOCTORATE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

DROSOPHILA SİRKADYAN FOTORESEPTÖRÜNDEKİ ETKİLEŞİMLERİN MOLEKÜLER KARAKTERİZASYONU

GÖZDE ÖZÇELİK

DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMAN: PROF. DR. NURİ ÖZTÜRK

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JURY

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APPROVAL

Gebze Technical University Graduate Administration Board

…..…/…..…/..…… date and ……/…….. numbered decision.

SIGNATURE/SEAL

Dedication / İthaf

To my family,
my friends and Gods
of Light..

ABSTRACT

The circadian clock is found in organisms from cyanobacteria to humans and is a genetically programmed mechanism that regulates daily rhythms in biological systems. Functionally, the clock consists of an endogenous core clock and a photoreceptor. At the molecular level, the clock is generated by a transcription translation feedback loop (TTFL). The period of endogenous clock is close to, but not exactly, 24 hours, therefore it should be synchronized to the solar clock. Exposure to sunlight in the morning synchronizes the endogenous clock precisely to the daily geological clock via photoreceptors in a process called resetting. In mammals, Cryptochrome proteins play a role in TTFL but not photoreception, whereas Drosophila Cryptochrome (DmCry) plays a central role in the light-dependent clock resetting in *Drosophila*. Since *Drosophila* has been used as a model organism for circadian clock research and DmCRY has been suggested to be a magnetoreceptor, understanding the mechanism of photoreception is crucial. Although the main mechanism of clock resetting by DmCRY is known, its interactions have not been fully investigated, mostly due to technical limitations.

In this thesis, we decided to investigate the regulatory mechanisms of the circadian clock in *Drosophila* by focusing on light-induced interactions of the DmCry photoreceptor.

We specifically aimed to unravel the light-dependent interactions of DmCry and the molecular mechanisms of resetting by identifying unknown proteins within the photoreceptor complex. Proximity labeling techniques, specifically using TurboID and APEX2 enzymes, were employed to explore light-dependent and independent interactions. Given the rapid degradation of DmCry after light exposure, TurboID or APEX2 enzymes fused to DmCry were used in S2 cells to identify proteins in proximity under dark and light conditions, providing insights into the circadian clock's intricate regulatory network. We identified several novel interactions and confirmed some of them using the co-immunoprecipitation technique. Applying the proximity labeling technique coupled to mass spectrometry to identify short-lived interactions opens a new avenue that can also be applied to other short-lived interactions which might be affected by magnetic field changes.

Keywords: APEX2, Cryptochrome, Photoreceptor, Proteomics, TurboID

ÖZET

Sirkadiyen saat, siyanobakteriden insana kadar neredeyse tüm organizmalarda bulunur ve biyolojik sistemlerdeki günlük ritimleri düzenleyen, genetik olarak programlanmış bir mekanizmadır. İşlevsel olarak saat, endojen bir çekirdek saatten ve bir fotoreseptörden oluşur. Moleküler düzeyde saat, bir transkripsiyon translasyon geri bildirim döngüsü (TTFL) tarafından üretilir. Endojen saatin periyodu tam olarak olmasa da 24 saate yakındır, bu nedenle güneş saatiyle senkronize olması gerekir. Sabah güneş ışığına maruz kalma, sıfırlama adı verilen bir işlemle endojen saati, fotoreseptörler aracılığıyla tam olarak günlük jeolojik saatle senkronize eder. Memelilerde, Kriptokrom proteinleri TTFL'de bir rol oynar ve fotoresepsiyonda rol oynamazken, Drosophila Kriptokromu (DmCry) proteini ise *Drosophila*'da saatin ışığa bağımlı olarak sıfırlanmasında hayati bir rol oynar. *Drosophila*, sirkadiyen saat araştırmaları için model bir organizma olarak kullanıldığından ve DmCRY bir magnetoreseptör olarak öne sürüldüğünden dolayı, fotoresepsiyon mekanizmasının anlaşılması çok önemlidir. DmCRY tarafından saatin sıfırlanmasının ana mekanizması bilinmesine rağmen, çoğunlukla teknik sınırlamalar nedeniyle etkileşimleri tam olarak araştırılmamıştır.

Bu tezde, DmCry fotoreseptörünün ışığa bağımlı etkileşimlerine odaklanarak *Drosophila'*daki sirkadiyen saatin düzenleyici mekanizmalarını araştırmaya karar verdik.

Özellikle fotoreseptör kompleksi içindeki bilinmeyen proteinleri tanımlayarak DmCry'nin ışığa bağımlı etkileşimlerini ve sıfırlamanın moleküler mekanizmalarını çözmeyi amaçladık. Işığa bağımlı ve bağımsız etkileşimleri araştırmak için TurboID ve APEX2 enzimlerini kullanan yakınlığa bağımlı etiketleme tekniği sonrası adaylar kütle spektrometre analizi ile kimliklendirildi. Işığa maruz kaldıktan sonra DmCry'nin hızlı yıkılımı göz önüne alındığında, DmCry'ye kaynaştırılan TurboID veya APEX2 enzimleri, S2 hücrelerinde karanlık ve aydınlık koşullar altında yakındaki proteinleri tanımlamak için kullanıldı ve sirkadiyen saatin karmaşık düzenleyici ağına dair içgörüler sağlandı. Yeni tanımlanan bazı etkileşimleri birlikte immünopresipitasyon tekniğini ile de doğruladık. Kısa ömürlü etkileşimlerin yakınlığa bağımlı etiketleme ardından kütle spektrometre ile tanımlanması, manyetik alan değişikliklerinden etkilenebilecek diğer kısa ömürlü etkileşimlere de uygulanabilecek yeni bir metodoloji ortaya koydu.

Anahtar Kelimeler: APEX2, Kriptokrom, Fotoreseptör, Proteomiks, TurboID

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	:	microgram
µL	:	microliter
Ago	:	Argonaute
APEX2	:	engineered peroxidase
APS		Ammonium persulfate
BioID	:	*Escherichia coli* BirA R118G enzyme
BioID	:	proximity-dependent biotin identification
BMAL1	:	brain and muscle arnt-like 1
Brm	:	Brahma
ccgs	:	circadian clock-regulated genes
CKII	:	Casein kinase II
Clock	:	circadian locomotor output cycles kaput
co-IP	:	co-immunoprecipitation
Cry	:	cryptochrome
DBT	:	doubletime
DmCry	:	*Drosophila* Cryptochrome
E-box	:	enhancer box
EDTA	:	Ethylenediaminetetraaceticacid
ERM	:	Ezrin, Radixin, Moesin
FAD	:	flavin adenine dinucleotide
FBS	:	Fetal Bovine Serum
FDR	:	False-discovery rates
FAR1	:	FARRED IMPAIRED RESPONSE1
FHY3	:	FAR-RED ELONGATED HYPOCOTYL3
H_2O_2	:	hydrogen peroxide
HRP	:	horse radish peroxidase
InaD	:	Inactivation no afterpotential D
INES	:	Individual Node Exceptions Strategy
Inos	:	myo-inositol-phosphate synthase
Jet	:	Jetlag
kDa	:	kilodalton
Khc	:	Kinesin heavy chain
KPM	:	KeyPathwayMiner
LB	:	Luria Bertani
min	:	minute
Moe	:	Moesin
MS	:	Mass Spectrometry
mW	:	milliwatt
MW	:	molecular weight
MT	:	miniTurbo
NAD	:	nicotinamide adenine dinucleotide
NaCl	:	Sodium chloride
NinaC	:	Neither inactivation nor afterpotential protein C
nm	:	nanometer
NOP58	:	Nucleolar protein 58
PBS	:	phosphate buffer saline

PCA	:	principal component analysis
PCR	:	Polymerase chain reaction
PDFR	:	PDF neuropeptide receptor
PEI	:	polyethyleneimine
Per	:	period
PPI	:	protein–protein interactions
RdgA	:	retinal degeneration A
RIPA	:	radio-immunoprecipitation assay
RNAi	:	RNA interference
SAINT	:	significance analysis of INTeractome
SDS	:	sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	:	short interfering RNA
TBS	:	Tris Buffered Saline
TBS-T	:	Tris buffered Saline with Tween 20
TEMED	:	Tetramethylethylenediamine
TTFL	:	Transcription translation feedback loop
TID	:	TurboID
Tim	:	timeless
UV	:	ultraviolet
Y2H	:	yeast two-hybrid

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1. INTRODUCTION

Circadian clock is the mechanism that controls the biological events in almost all organisms from cyanobacteria to human in 24-hour (hr) periods in synchrony with the geological clock. Circadian clock is a genetically encoded molecular mechanism, and it will continue to oscillate, even though not exactly, around 24-hr periods even without a signal from solar clock [Reppert and Weaver 2002]. Endogenous clock is synchronized to 24 hr by sunlight early in the morning in a process called resetting. Light is received by photoreceptor molecules and resets the circadian clock [Sancar 2008].

The functional biological clock consists of a core clock and a photoreceptor system. Mammalian cells and *Drosophila* cells use different photoreceptor systems even though they have similar core clock systems. In both organisms' circadian core clock is generated by a transcription-translation feedback loop (TTFL) which consists of negative and positive arms [Ueda et al, 2007]. Once positive arm factors activate expression of genes in the negative arm, the translated products of the negative factors inhibit the activity of positive factors. This activation and inhibition create a cyclical pattern of expression [Bell-Pedersen et al, 2005].

The biggest difference between mammalian and *Drosophila* molecular clock lies in the resetting mechanism. Cryptochrome (Cry) molecule is the main photoreceptor of the circadian clock responsible for the resetting of the clock in *Drosophila* [Ozturk N, 2017]. On the other hand, mammalian Cryptochrome proteins are not light sensitive and mammals use melanopsin-based photoreceptor system which is expressed only in the very low number of retinal ganglion cells [Do MT, Yau KW, 2010].

The *Drosophila* Cryptochrome is a non-image forming photoreceptor which was studied in the most detailed way so far. Because, as a model organism, *Drosophila* is easier and faster to work genetically compared to mammalian organisms. The basic principle of how light signal resets the biological clock in *Drosophila* photoreceptor is explained with a well-defined model. According to this model, light is detected by Cryptochrome cofactor (flavin) and induces conformational change on C-terminus of Cry which allows interaction with Jetlag and Timeless proteins. This interaction initiates the degradation of Timeless after its ubiquitination by Jetlag and resets the

circadian clock. However, there are still major gaps in the mechanism to explain light triggered reactions of *Drosophila* photoreceptor system.

1.1 The Aim of the Thesis

This thesis aims to elucidate the light-dependent interactions of DmCry and uncover the molecular mechanism of resetting by identifying unknown proteins within the photoreceptor complex. Previous studies have employed yeast two-hybrid (Y2H) and mass-spectrometry (MS)-coupled co-immunoprecipitation (co-IP) to discover interactors for circadian clock photoreceptors [Peschel et al, 2009 and Ozturk et al, 2013] An attempt to identify new molecules involved in light-dependent resetting through a genome-wide RNAi screen in the S2 cell culture system failed due to the nonspecific effects of high-throughput RNAi screening on other pathways [Sathyanarayanan et al, 2008] Y2H, although prone to false-positive results, remains a promising method for identifying transient interactions directly. Co-IP faces challenges in capturing weak/transient interactions of photoreceptors, given the rapid light-induced proteasomal degradation of DmCry within an hour [Ozturk et al, 2011, Ozturk et al, 2014].

Proximity labeling, particularly using BioID and APEX2 systems, has emerged as an alternative to classical co-IP combined with MS. BioID relies on proximity-dependent cellular biotinylation using a promiscuous bacterial biotin ligase (*E. coli* BirA R118G, BioID), while APEX2 utilizes an engineered soybean ascorbate peroxidase for proximity labeling [Roux et al, 2012, Rhee et al, 2013]. Both methods involve lysing cells in a harsh buffer, isolating biotinylated proteins on streptavidin-coated beads, and identifying them through MS analysis. However, candidates identified by these methods may represent indirect or vicinal non-interacting proteins in addition to direct interactors.

In this thesis, we applied proximity-dependent biotin labeling to identify light-dependent and independent interactions in the *Drosophila* photoreceptor system. Given the rapid degradation of DmCry after light exposure, we opted for a complementary approach using the fastest enzymes, TurboID or APEX2, fused to DmCry in S2 cells to identify proteins in proximity under both dark and light conditions.

2. LITERATURE SUMMARY

2.1. *Drosophila* Circadian Clock

The fruit fly, *Drosophila*, exhibits various easily measurable circadian behavior patterns, with notable mention of locomotor activity, displaying two peaks during the day—one in the morning and another in the evening—which persist under constant conditions due to its crepuscular nature. Konopka and Benzer, using mutagens to generate circadian rhythm abnormalities, identified three mutants—one arrhythmic, one with a shorter period (19 h), and another with a longer period (28 h)—it was a milestone discovery for the exploration of molecular mechanisms behind circadian rhythms [Konopka and Benzer 1971]. In the beginning of 1980s, the first clock gene period (per) was identified when it was cloned and rescued *Drosophila* period by Jeffery C. Hall and Michael Rosbash from Brandeis University and Michael Young at Rockefeller University [Bargiello and Young 1984, Reddy et al, 1984, Zehring et al, 1984].

The existing model (Fig. 2.1.) postulates that the helix-loop-helix transcription factors Clock (Clk) and Cycle (Cyc) form heterodimers binding to E-Box sequences (CACGTG) in the fly genome around midday [Rutila et al, 1998, Allada et al, 1998]. The core of the circadian clock involves the activation of *per* and *tim* genes. Subsequently, per and tim mRNA increases in the evening/night, leading to the accumulation of Per and Tim in the cytoplasm, primarily after dark due to Tim's light sensitivity and its stabilizing effect on Per. Without Tim's protection, Per protein undergoes phosphorylation by the Doubletime (Dbt) kinase [Price et al, 1998], followed by ubiquitination by the F-Box protein Slimb, and subsequent degradation in the proteasome [Grima et al, 2002]. Period's degradation is counteracted by protein phosphatase 2A (PP2A) [Sathyanarayanan et al, 2004]. Tim and Per then enter the nucleus either individually or as a heterodimer, allowing Per-associated Dbt to also enter the nucleus. This transport is facilitated through phosphorylated Per by Casein Kinase 2 (CK2) [Lin et al, 2002]. Within the nucleus, the Per-Dbt-Tim complex accumulates and binds to Clk/Cyc dimers through a Per-Clk interaction. This interaction hyperphosphorylates the clock, preventing Clk/Cyc dimers from DNA binding and inhibiting the transcription of *tim* and *per* [Lee et al, 1999]. Period's

inhibition of its own transcription establishes a negative feedback loop, resulting in cyclic expression of Period and Timeless protein and mRNA.

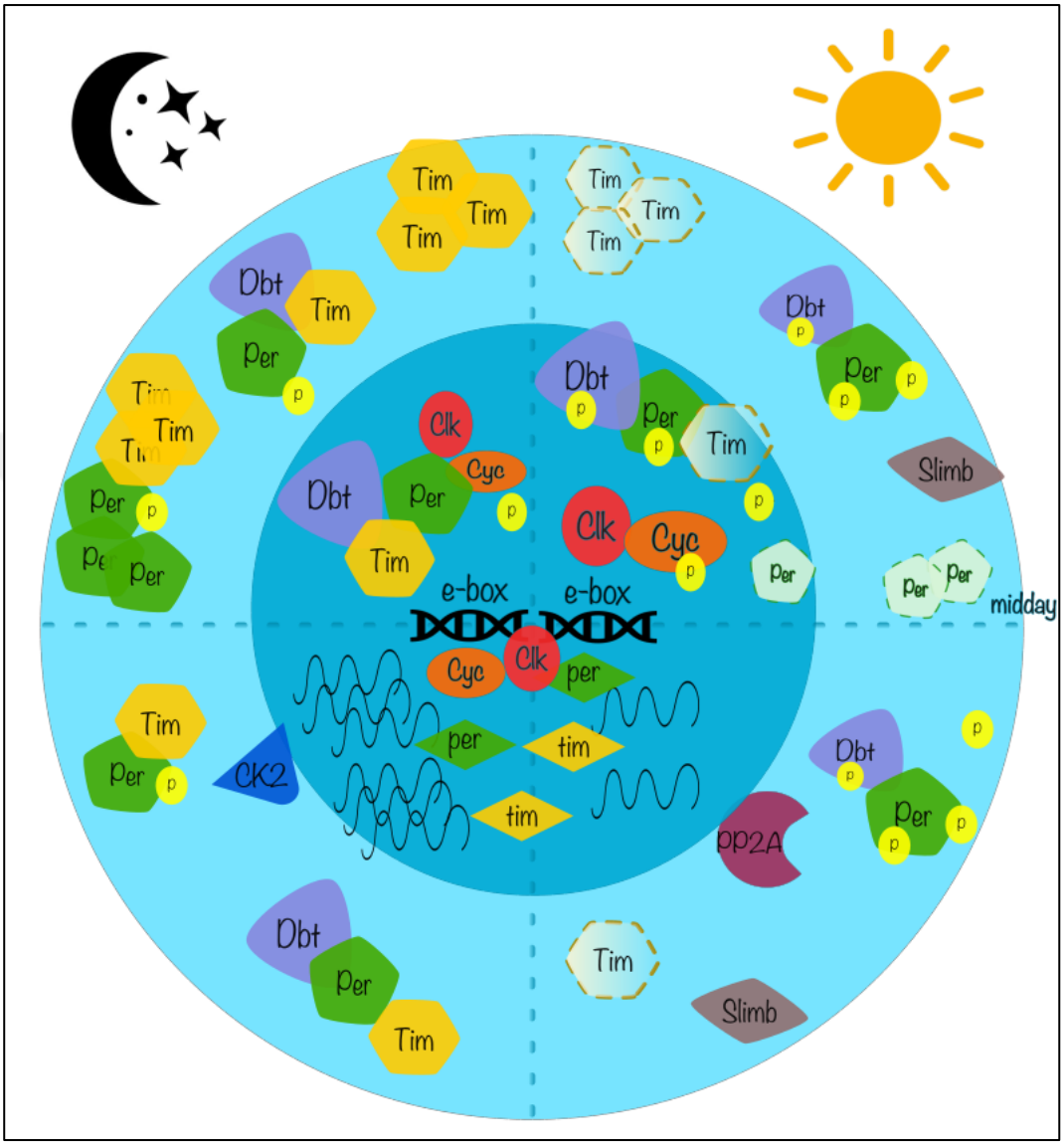

Figure 2.1: Molecular mechanisms of the *Drosophila* circadian clock. The figure displays a cell at the different times of a day. Each quarter of the cell roughly represents six hours of the circadian day, while the right side (0–12) is in light and the left side (12–24) in darkness. P indicates phosphate/phosphorylation. Dashed lines represent degraded protein. Figure is adapted from N. Peschel, C. Helfrich-Förster / FEBS Letters 585 (2011).

In summary, the Clock (Clk) and Cycle (Cyc) transcription factors in the positive arm of the *Drosophila* TTFL activate the negative arm genes Period (Per) and Timeless (Tim), as well as the circadian clock-regulated genes (ccg), and after translation of per and tim mRNAs, these proteins migrate to the nucleus to inhibit the activity of Clk and

Cyc transcription factors through protein-protein interaction [Sehgal et al, 1994, Emery et al, 1998, Stanewsky et al, 1998]. The resulting oscillation is adjusted to approximately 24 hours by post-translational modifications.

The coordinated actions of clock-controlled genes (ccgs) and their associated genes/networks contribute to the circadian regulation of tissue metabolism [Hardin 2011]. The collective function of circadian clocks in various tissues and organs forms the organism's endogenous circadian clock. In mammals, the synchronization of peripheral organ clocks is regulated by a master clock, which, in turn, aligns itself with the geophysical or solar clock through the photoreceptor system [Damulewicz and Mazzotta 2020]. Unlike mammals, peripheral tissues in *Drosophila* and zebrafish are directly sensitive to light and temperature, allowing for their in vitro synchronization. Light, as zeitgeber, resets the master clock which then synchronizes peripheral clocks in other organs via the circadian photoreceptor of the fly [Plautz et al, 1997].

2.2. Role of Cryptochrome as Circadian Photoreceptor

The photoreceptor molecules absorb light and transmit the resetting signal to the core clock. In *Drosophila*, Cry is a member of the cryptochrome/photolyase family (CPF) of flavoprotein protein serves as a photoreceptor which is sensitive to blue light [Partch and Sancar 2005]. Unlike *Drosophila*, mammalian Cryptochrome proteins lack light sensitivity, and mammals rely on a melanopsin-based circadian clock photoreceptor. Plants also use Cryptochrome proteins as a photoreceptor, and photoreception is mainly dependent on the DmCry in *Drosophila*.

The structural change in DmCry exposes protein–protein interaction (PPI) sites on its surface and relaxes its C-terminus which was initially bended over the FAD binding region in darkness [Levy et al, 2013]. This allows the E3 ligase Jet and its substrate Tim protein to bind to DmCry only after light exposure, leading to the rapid degradation of Tim protein [Koh et al, 2006]. Notably, Per protein is also swiftly degraded in the absence of Tim. Light rapidly removes negative arm components from the TTFL, causing a rapid increase in Clk and Cyc proteins, activating the expression of *ccgs* and marking the reset of the circadian clock. Following the degradation of Tim, DmCry undergoes degradation as well, preventing the reset of the circadian clock until newly synthesized Tim and DmCry proteins follow a circadian pattern. [Foley and

Emery 2020]. The degradation of DmCry ensures removal to prevent continuous resetting (Figure 2.2).

Cry serves as the primary light sensor for the insect circadian clock, utilizing a photoinduced FAD via electron transfer processed through three conserved tryptophan residues named the Tryptophan triad. The crystal structure of DmCry reveals the triad's components as Trp-342, Trp-397, and Trp-420, along with additional loops such as the protrusion loop, phosphate binding loop, the C-terminal lid, and electron-rich sulfur loop [Zoltowski et al, 2011, Czarna et al, 2013]. In the ground state, DmCry has FAD in its oxidized state (FADox). In response to blue light, FAD is reduced to FAD•-, an anionic semiquinone (ASQ). The C-terminal tail undergoes a conformational change, enabling DmCry to transmit the light signal to the core clock elements. Both oxidized and reduced forms of DmCry can change their conformation at a similar rate, promoting interaction with Tim. The light-induced DmCry-dependent Tim proteolysis depends on the photosensitive ability of DmCry [Ozturk et al, 2014].

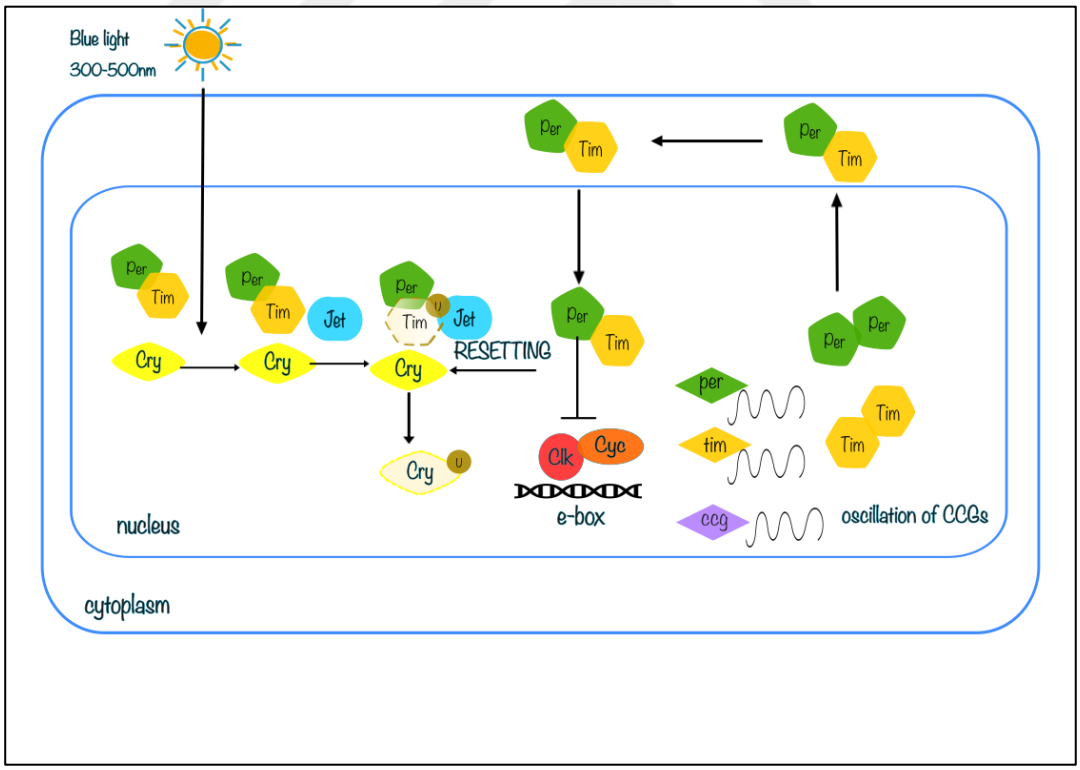

Figure 2.2: Schematic of photoreceptor mechanism and circadian clock of *Drosophila melanogaster*. Figure is adapted from N. Ozturk the FEBS Journal 289 (2022).

2.3 Proximity-dependent Biotinylation

Studying the light-dependent reactions of DmCry is crucial for comprehending circadian photoentrainment. Circadian clock photoreceptor interactors have been identified using various methods, including yeast two-hybrid (Y2H) systems, mass-spectrometry coupled co-immunoprecipitation (co-IP), and large-scale genetic screening assays [Ozturk 2022, Kavakli 2022]. Most photoreceptor-protein interactions for the circadian clock have been uncovered through Y2H systems. For instance, the identification of DmCry's E3 ligase BRWD3 began with a Y2H assay and was further validated by RNAi-mediated inhibition of light-dependent DmCry degradation [Ozturk et al, 2013]. Additionally, mass-spectrometry analysis post co-IP of DmCry interactors in fly heads revealed light-dependent interactions with various proteins [Mazzotta et al, 2013]. While these classical approaches are promising, especially with in vivo settings, the challenge lies in that these interactions are expected to be weak and/or transient.

Recently, proximity-dependent labeling (PL) methods have been developed and utilized for mapping protein interactomes in living cells. Examples of such techniques include ascorbate peroxidase (APEX) [Rhee et al, 2013] and proximity-dependent biotin identification (BioID) [Roux et al, 2012]. In general, proximity labeling relies on enzymes that convert a substrate into a reactive radical that covalently tags neighboring proteins with biotin. To achieve regional protein labeling, the enzymes are usually fused with a protein of interest. The introduction of enzymes with diverse substrates and kinetics can be used to address the challenges associated with short-lived or weak interactions. In vivo proximity-dependent labeling primarily employs two main types of enzymes: biotin ligases (such as BirA*, BioID2, miniTurbo, and TurboID) [Roux et al, 2012, Kim et al, 2016, Branon et al, 2018] and peroxidases (including APEX, APEX2, and HRP) [Rhee et al, 2013, Lam et al, 2015].

The BioID method is based on the proximity-dependent biotinylation of cellular proteins. This is achieved by using a bacterial biotin ligase, *Escherichia coli* BirA R118G (referred to as BioID), fused to a bait protein of interest, facilitating the biotinylation of neighboring proteins within living cells. After labeling, cells are lysed, and streptavidin-coated beads are employed to selectively isolate biotinylated proteins for subsequent MS analysis (Figure 2.3). In mammalian cells, the BioID enzyme (~35

kDa) needs 16–24 hours for full proximity-dependent labeling following biotin substrate supply [Roux et al, 2012]. Indeed later, a smaller and more selective version, BioID2 (~25 kDa), was derived from *Aquifex aeolicus*, exhibiting improved targeting by fusion proteins and requiring less biotin supplementation [Kim et al, 2016]. Despite these enhancements, BioID2 still requires a relatively long 16-hour incubation, posing challenges for studying short-lived interactions.

As proximity labeling techniques continue to evolve, faster-acting engineered versions of the BioID enzyme, namely TurboID and MiniTurbo, were developed by being active in 10 minutes and 1 hour, respectively [Branon et al, 2018]. Given that most interactions in the photoreceptor complex occur within 1 hour, TurboID and MiniTurbo-based BioID methods hold significant promise. Another available option is APEX2, an engineered soybean ascorbate peroxidase, for proximity-dependent labeling [Lam et al, 2015]. In this approach, APEX2, fused to a protein of interest, labels nearby proteins with biotin-phenol upon the addition of H_2O_2 to the cell culture medium. The biotinylated proteins can then be affinity-purified for identification via mass spectrometry. Notably, the labeling with biotin-phenol occurs in just 1 minute, providing temporal control over the labeling process. However, it's important to consider that candidates identified through BioID, or APEX2-mediated proximity labeling may include indirect interactors or vicinal proteins that do not physically interact with the fusion protein.

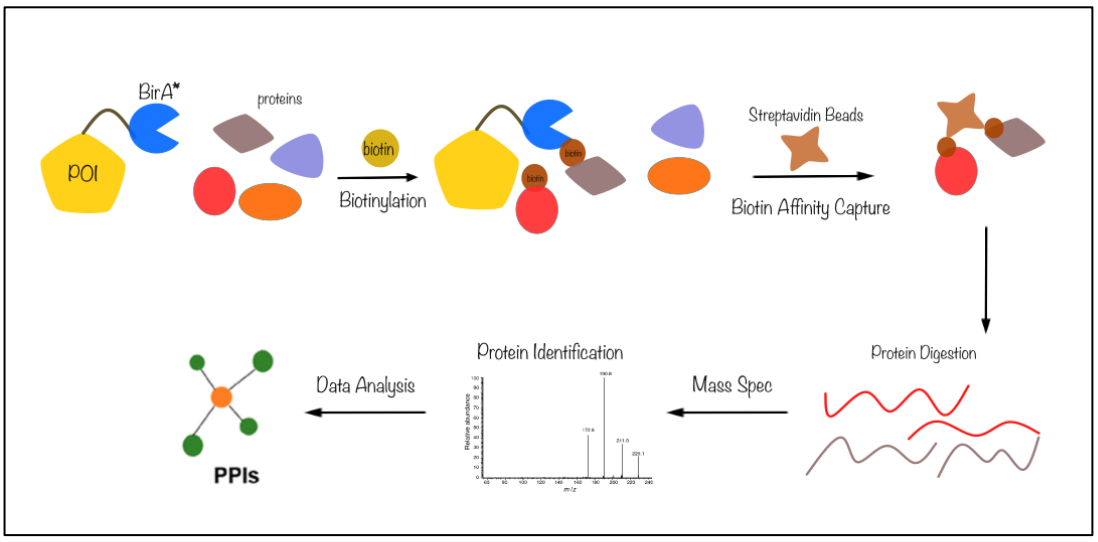

Figure 2.3: BioID proximity labeling method. Figure is adapted from Roux et al. 2012.

Utilizing TurboID, MiniTurbo, or APEX2 can expedite the identification of PPIs involving DmCry or other photoreceptors under various conditions such as light and dark in living cells. The BioID technique has the advantage of recording a history of PPIs, allowing, for example, the fusion of TurboID to DmCry to label proteins in proximity from the resting state to the degradation of DmCry, providing enough time for whole process labeling.

To reveal comprehensive models for Cry reaction mechanism in photoreception and potentially in magnetoreception, we aimed to understand light-dependent reactions of DmCry and discover novel PPIs. This thesis answers the question of how cell lines can be used to dissect the light- dependent reaction of CRYs. Given the rapid degradation of DmCry following light exposure, we chose a complementary approach using the fastest enzymes. In this study, we used either TurboID or APEX2 to DmCry in S2 cells and identified the proteins proximal to DmCry under both dark and light conditions.

3. MATERIALS

3.1. Plasmids, Oligos and Cloning Reagents

Table 3.1: List of plasmids.

Name of Plasmid	Source/Provider	Cat No.
Inducible Caspex expression	Addgene	#97421
3xHA-TurboID-NLS_pcDNA3	Addgene	#107171
3xHA-miniTurbo-NLS_pcDNA3	Addgene	#107172
pAc5.1/V5HisA	Thermofisher Scientific	V411020
pCoHygro/ DES-Inducible Kit	Thermofisher Scientific	K5150-01
pCoBlast/ DES-Inducible Kit	Thermofisher Scientific	K4110-01
pAc5.1-DmCry-V5His	Zhu et al, 2005	
pFast-FLAG-His-DmCry	Ozturk et al, 2009	
pAc5.1-Tim-V5HisA	Peschel et al, 2009	
pAc5.1-V5-Jet	Ozturk et al, 2010	

Table 3.2: Primers used to clone the selected interactors into pAc5.1-V5-3XHA plasmid.

Gene Name	Uniprot ID	Forward Oligo (5'→3')	Reverse Oligo (5'→3')
CG1785 (NOP53)	Q9W3C2	ATCGAATTCGCCACCATGACGGCCGTAGAGCCAG	TATCTTGATATTGGCAGTAG
CG8414 (NOL9)	A1ZA92	ATCGGTACCGCCACCATGCTTGACGAACATATTC	ATCGCGGCCGCGTTTCGTTGATTCGCTGGTA
Fax	Q95RI5	ATCGAATTCGCCACCATGGCAAGCGAAGTGGCCCA	CTTTGTTTCCTCCTTCTCTTTG

Table 3.2: Continued.

AcCoAS	Q9VP61	ATCGAATTCGCCACCATGCCAGCGGAAAAATCAAT	ATCGCGGCCGCCTTGGCCTCCACTGGCCG
Hsc70-3	P29844	ATCGAATTCGCCACCATGAAGTTATGCATATTACTG	ATCGCGGCCGCCAGCTCGTCCTTGAGATCG
Fkbp59	Q9VL78	ATCGGTACCGCCACCATGCCGGAAGGGAATAAAATC	ATCGCGGCCGCGATCATGATTATATTGTCGC
Ahcy	Q27580	ATCGAATTCGCCACCATGTCGAAGCCCAGCTACAA	ATCGCGGCCGCGTACCGGTAATGGTCGGGCT
CkIIalpha	P08181	ATCGAATTCGCCACCATGACACTTCCTAGTGCGGC	ATCGCGGCCGCTTGCTGATTATTGGGATTC

Table 3.3: Primers used for cloning.

Name	**Sequence (5' →3')**	**Construct generated**
APEX2_Forward	TGACGCGTGGAAAGTCTTACCCAACTGTG	pAc5.1-DmCry-V5-APEX2
APEX2_Reverse	GCTGAGCTCTTAGTTTAAACTCGGCATCAGCAAACCCAA	pAc5.1-DmCry-V5-APEX2
V5-APEX2_Forward	AAACTCGAGTTGCCACCATGGGTAAGCCTATCCCTAAC	pAc5.1-V5-APEX2
V5-APEX2_Reverse	GCTGAGCTCTTAGTTTAAACTCGGCATCAGCAAACCCAA	pAc5.1-V5-APEX2
3xFLAG_CDS_Forward	ATCGAATTCGCCACCATGGACTACAAAGACC	pAc5.1-3xFLAG-DmCry
3xFLAG_CDS_Reverse	CTGGCTAGCCTTGTCATCGTCATCCT	pAc5.1-3xFLAG-DmCry
3xHA_CDS_Forward	TAGACCGGTATGTACCCGTAT	pAc5.1-3xHA-V5
3xHA_CDS_Reverse	CATGAGCTCTCATGCGTAGTCTGGGACGTCATAGGGATACCCGGCATAGT	pAc5.1-3xHA-V5

Table 3.4: List of commercial enzymes used for cloning.

Name of Enzyme	Source/Provider	Cat No.
T4 polynucleotide kinase	Thermofisher Scientific	EK0031
T4 DNA ligase (1U/μL)	Invitrogen	15224017
Q5 DNA polymerase	New England Biolabs, USA	M0491S
Pfx DNA Polymerase	Thermofisher Scientific	11708039

Restriction enzymes are commercially available from Thermo Fischer or NEB provided with appropriate 10x reaction buffers in different units in 20 or 50 μL.

Table 3.5: List of kits and reagents used for molecular cloning.

Name of Material	Source/Provider	Cat No.
PCR clean-up and gel extraction Kit	Macherey Nagel, Germany	740609.50
LB Broth Medium	Caisson Labs	LBP03
LB Agar	Caisson Labs	LBP04
Ampicillin	Sigma	59349
Nucleospin Plasmid Kit	Macherey Nagel, Germany	740588.50
DNA Ladder and DNA Loading Dye	New England Biolabs, USA	N3232S and B7021S

3.2. Cell Culture Solutions and Supplements

The cell lines derived from *Drosophila* S2 cells were maintained in Schneider's *Drosophila* medium PAN-Biotech cat no: P04-91500 containing 10% heat inactivated FBS Thermofisher Scientific, cat no: 26140079 and penicillin- streptomycin solution Thermofisher Scientific, cat no: 15140130 at the final 50 U/mL-50 mg/mL concentration, respectively. For stable cell line generation, antibiotic selection was done either with 50 μg/mL of Hygromycin B, Thermofisher Scientific, cat no: 10687010, or 5 μg/mL blasticidin, Thermofisher Scientific cat no: R21001. For transfection, Polyethyleneimine (PEI), Sigma, cat no: 408727 was used. Biotin from

Bio Basic Inc, cat no: BB0078, or biotin tyramide from Iris Biotech, Cat No: LS-3500, was added to cell lines.

3.3. Buffers and Chemicals

Table 3.6: List of general lab chemicals.

Name of Chemical	Source/Provider	Cat No.
Protein Molecular Size Marker	Thermofisher Scientific	26616
NP-40 Lysis Buffer	Thermofisher Scientific	FNN0021
Protease Inhibitor Cocktail	Sigma	S8830
Streptavidin-coated Magnetic Beads	New England Biolabs, USA	S1420S
Dynabeads magnetic beads	Thermofisher Scientific	10003D
MS-grade Trypsin	Sigma	T1426
Bovine Serum Albumin	Sigma	A2153
Skim milk non-fat powder	Biochop	SKI400
Tween-20	Sigma	P2287
Beta-Mercaptoethanol	Applichem	A1108
WesternBright Sirius HRP substrate	Advansta	K-12043-D20
TRIS	Fisher Scientific	AC228030051
Sodium Dodecyl Sulfate	Fisher Scientific	BP166
NaCl	Fisher Scientific	S271
Glycine	Fisher Scientific	G45
KCl	Fisher Scientific	BP366
40% Ac-BisAcrilamide	Biorad	161-0146
TEMED	Applichem	A1148
Bromophenol Blue	Fisher Scientific	BP115
EDTA	Fisher Scientific	BP118

Table 3.7: List and recipe of buffers.

Name of Buffer	Recipe
RIPA Buffer	▪ 50 mM Tris-HCl pH 8.0, ▪ 150 mM NaCl, ▪ 0.1% SDS, ▪ 0.5% sodium deoxycholate, ▪ 1% Triton X-100
1X PBS	▪ 137mM NaCl ▪ 2.7mM KCl, ▪ 4.3mM Na_2HPO_4, ▪ 1.47mM KH_2PO_4
4X Laemmli Buffer	▪ 250mM Tris-Cl (pH: 6.8), ▪ 8% SDS, ▪ 40% Glycerol, ▪ 20% β-mercaptoethanol, ▪ 0.008% Bromophenol Blue
Tris-EDTA Buffer	▪ 100 mM NaCl, ▪ 10 mM Tris-HCl (pH 7.5), ▪ 1 mM EDTA
10X TBS	▪ 200 mM Tris-Cl (pH: 7.6), ▪ 1.5 M NaCl
10X SDS Running Buffer	▪ 250 mM Tris-Base (pH: 8.3), ▪ 1.90 M Glycine, ▪ 1% SDS

SDS-PAGE Gels were prepared by following the instructions at Table 3.8.

Table 3.8: Stacking and separating gel recipes.

10% separating gel	8 ml	5% Stacking Gel	2 ml
ddH_2O	3.8mL	ddH_2O	1.46mL
40% Acryl:Bis	2mL	40% Acryl:Bis	250µL
1.5 M Tris pH8.8	2mL	1 M Tris pH6.8	250µL
10% SDS	80µL	10% SDS	20µL
10% APS	80µL	10% APS	20µL
TEMED	8µL	TEMED	2µL

3.4. Antibodies

Table 3.9: List of antibodies.

Name of Antibody	Source/Provider	Cat No.	Dilution
Anti-FLAG	Sigma	F1804	1:3000
Anti-HA	Cell Signaling Technology	C29F4	1:3000
Anti-V5 antibody	Thermofisher scientific	R96025	1:5000
Control IgG	Cell Signaling Technology	7076S	1:5000
HRP-conjugated streptavidin	Abcam	ab7403	1:10000
Anti-histone H3	Abcam	1791	1:2500
Anti-Beta Actin	Cell Signaling Technology	4967	1:1000
Anti-mouse HRP	Cell Signaling Technology	7076S	1:6000
Anti-rabbit HRP	Thermo Fisher Scientific	31460	1:5000

3.5. Equipment

Insect Incubator (Velp), Laminar flow (Hera –Safe, KS-12) and Inverted Microscope (Optech IB) were equipment for cell culture. White light (Led Desk Lamp-TT-DL13, Taotronics), 366-nm F15T8-BLB black lights from General Electric, Fisherbrand Traceable Dual Display Light Meter (Fisher Scientific, cat no: 06-662-64) and UVX-36 UVP UVX Radiometer (Analytik Jena US) were used for light experiments. Trans-blot Turbo Transfer System (Bio-Rad Trans-blot Turbo Transfer System, Serial No: 690BR011760), DynaMag-2 magnetic rack (Thermo Fischer Scientific, USA, cat no: 12321D), Chemiluminescence Imaging System (ChemiDoc XRS+ System, Bio-Rad) were western blot system. Mass Spectrometry equipment from KUPAM were refered in Methods section.

4. METHODS

4.1. Molecular Cloning

Biotinylation system constructs are listed below:

The APEX2 coding sequence was amplified from Inducible Caspex expression plasmid and inserted into pAc5.1-DmCry-V5His digested with MluI-SacI restriction enzymes. 3xHA-TurboID-NLS_pcDNA3 was digested with NotI, filled to get blunt ends, and then digested with EcoRI. The TurboID coding fragment was ligated into linearized pAc5.1/V5HisA digested with EcoRI in the presence of T4 polynucleotide kinase. NotI-XbaI fragment from 3xHA-TurboID-NLS_pcDNA3 was inserted into same site of pAc5.1-DmCry-V5HisA. This construct was then digested with NotI, filled to get blunt ends and re-ligated to correct the frameshift between DmCry and TurboID coding sequences. EcoRI fragment from this new construct was cloned into the EcoRI site of pAc5.1/V5HisA to leave the nuclear localization sequence out. The miniTurbo was cloned into pAc5.1 / V5HisA vector in same way as TurboID. 3xHA-miniTurbo-NLS_pCDNA3 was inserted into same site of pAc5.1-DmCry-V5HisA.

The constructs expressing proteins of interest are listed below:

The FLAG-His-DmCry coding fragment was then inserted into EcoRI-PmeI site of pAc5.1/V5HisA from pFast-FLAG-His-DmCry construct, which was previously described in Ozturk et al, 2009, was digested with HindIII, filled to get blunt ends, and then digested with EcoRI. The FLAG tag was then replaced with a 3xFLAG coding sequence to improve the detection of the tagged recombinant protein [Ozturk et al, 2009].

pAc5.1-V5-JET and pAc5.1-TIM-V5HisA constructs were previously described in Ozturk et al, 2011. pAc5.1-TIM-V5HisA construct encodes the shorter (by 23 N-terminal amino acids) and more light-sensitive form of Tim (called s-Tim) according to Peschel et al, 2009 [Peschel et al, 2009, Ozturk et al, 2011, Ozturk et al, 2014].

The constructs used for validation of interactions are explained below:

The interactors were cloned into pAc5.1-3xHA-V5/Q5ed plasmid after amplifying coding sequences from S2 cell line cDNA. This plasmid was generated via 3xHA coding sequence inserted into AgeI-SacI site of pAc5.1/V5HisA plasmid. This plasmid

was then digested with XhoI-XbaI, filled to get blunt ends, and then re-ligated in the presence of T4 polynucleotide kinase to put 3xHA and V5 tags into the same frame.

To digest plasmids with restriction enzymes, each mixture was set up in 50 µL reaction volume: 1-2 µg DNA, 2 µL 10x restriction enzyme buffer, 1 µL restriction enzyme(s), ddH_2O to a final volume of 50 µL. The reaction was performed at 37°C for 1-2 hours. To isolate the double digested vector, it was run in agarose gel and then was extracted from gel by using gel and PCR clean-up kit according to manufacturer's instructions.

All ligation reactions were performed with 1 µL T4 ligase in 10 µL reaction volume with a final concentration of 1X T4 ligation buffer at 4ºC overnight.

4.1.1 PCR Amplification

The PCR reaction master mix utilized Platinum Pfx DNA Polymerase for one sample was as follows: 5 µL 10X Pfx buffer; 1 µL $MgSO_4$ (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; 200 ng template DNA; with ddH_2O added to achieve a final volume of 50 µL. The PCR reaction proceeded with an initial denaturation at 94°C for 5 minutes, followed by 33 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 68°C for 1.5 minutes each. A final elongation step at 68°C for 5 minutes concluded the PCR reaction.

4.1.2 Transformation to Competent Bacteria

Escherichia coli DH5α competent cells were used for transformations. Competent cells were retrieved from the -80°C freezer and thawed on ice. Five microliters of ligated plasmids were added to the competent cells and incubated for 30 minutes on ice before a heat shock at 42°C for 1 minute. The samples were then cooled on ice for 1.5 minutes, approximately 700 µL of Luria broth (LB) was added to the tubes, and they were incubated in a shaker at 37°C for 1 hour to allow sufficient time for the expression of the antibiotic resistance gene. Two hundred microliters of the samples were spread on ampicillin-containing LB agar plates and incubated overnight. Single colonies were picked using a micropipette tip and inoculated into 5 mL of ampicillin-containing LB medium, followed by overnight incubation for plasmid isolation.

4.1.3 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.

4.2. Cell Culture

4.2.1. Cell Maintenance

S2 cells were cultured at 27°C under 5% CO2 in Schneider's *Drosophila* medium supplemented with 100U/mL penicillin & 100µg/mL streptomycin, and 10% heat inactivated fetal bovine serum. S2 cells do not completely adhere to surfaces, making it difficult to rinse the cells. To passage them, cells were resuspended in the conditioned medium and centrifuged at 2500 x g for 2 to 3 minutes. The medium was then discarded, and fresh medium was added before the cells were replated.

4.2.2. Generation of Stable Cell Lines

A stable cell line expressing S2-3xFLAG-DmCry was established through a co-transfection process involving the plasmids pAc5.1-3xFLAG-DmCry and pCoHygro, using a 40:1 molar ratio. To select for transfected cells, 50 µg/mL of Hygromycin B was applied. For each well within a 6-well plate during the transfection procedure, 1 µg of the plasmids was dissolved in 75 µL Tris-EDTA (TE) buffer. Subsequently, 3 µL of 1 mg/mL PEI was introduced into the solution, and the mixture was gently pipetted up and down three times. After a 15-minute incubation at room temperature, this transfection mixture was added to S2 cells cultured in complete Schneider's medium.

Following 24 hours post-transfection, the cells were seeded into 96-well plates using serial dilution in Schneider's medium supplemented with hygromycin. The selection process involved replenishing the medium with hygromycin every 3 days for a period

of 15 days. 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. The colonies were inspected for a clonal appearance ~1 week after plating (rounded colonies radiating from a central point). Single-cell-derived clones were subsequently selected and expanded, and their expression of S2-3xFLAG-DmCry was verified via Western blotting.

Stable cell lines expressing fusion biotin ligase-gene of interest proteins are explained below:

S2 cell lines expressing V5-APEX, DmCry-V5-APEX, 3xHA-TID-V5His, and DmCry-3xHA-TID-V5His, 3xHA-MT, 3xHA-MT-13x-V5-DmCry were established through co-transfection of the respective plasmids along with pCoHygro. Single-cell-derived clones were subsequently obtained through a serial dilution technique followed by selection using hygromycin, following the same protocol as previously described above.

4.3. Light-dependent Degradation Assay

S2 cells cultured in complete medium were exposed to two different light sources for the activation of the DmCry protein. White light, provided by the Led Desk Lamp-TT-DL13 from Taotronics, and 366-nm F15T8-BLB black lights from General Electric were utilized. Light intensity was quantified using the Fisherbrand Traceable Dual Display Light Meter for white light and the UVX-36 UVP UVX Radiometer for black light. It's worth noting that the black light was applied through glass to filter out UV light. The activation of DmCry was assessed by monitoring the light-dependent degradation (proteolysis) of DmCry, with normalization to actin or Histone H3 levels for accurate measurement.

4.4. Biotin Labeling in Cells

Stable cell lines, S2-APEX, S2-DmCry-APEX, S2-TID, and S2-DmCry-TID, were plated in cell culture dishes based on the experimental setup. For proximity labeling of proteins in S2-TID or S2-DmCry-TID cells, D-Biotin was added into media at a final concentration of 500 µM. The labeling process was stopped by transferring the cells to ice and subjecting them to three consecutive washes with ice-cold 1X PBS.

Following the last wash, cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

In the case of proximity labeling for APEX2, Biotin-tyramide which is biotin-phenol the substrate of ascorbate peroxidase was used. The biotin substrate was directly added to the cell culture medium of S2-APEX or S2-DmCry-APEX cells at a final concentration of 500 mM. The medium was gently swirled until the precipitate dissolved. After a 30-minute incubation at 25°C with biotin-tyramide, hydrogen peroxide was introduced at a final concentration of 1 mM to induce biotinylation. Following a one-minute incubation with gentle swirling, the cells were centrifuged, and the resulting cell pellets were subjected to three washes with ice-cold PBS. Subsequently, the cell pellets were snap-frozen in liquid nitrogen and stored until further processing. Proximity labeling of proteins in S2-MT and S2-MT-DmCry cells was done as described for TID cells.

4.5. Immunoblotting

The cell lysate samples were diluted to 1X Laemmli sample buffer, while the samples attached to beads were resuspended in 1.5X Laemmli buffer. Subsequently, all samples underwent separation via SDS-PAGE and were then transferred onto a nitrocellulose membrane. To prepare the samples, they were briefly incubated at 95°C for 10 minutes, followed by a quick spin-down. Each well of the gel was loaded with the respective samples, and 1X SDS Running Buffer was used for electrophoresis. The gel was run at 80 V until the proteins entered the separating gel, after which the voltage was increased to a range of 120-150V. For the semi-dry transfer process, the Trans-blot Turbo Transfer System was employed. Four blotting papers and one nitrocellulose membrane, matching the dimensions of the SDS-PAGE gel, were prepared. These were first incubated with 1X transfer buffer, and then a transfer sandwich was constructed. To assemble the blotting sandwich, the components were arranged in the following order on the cassette: Top (-) Cassette electrode (cathode), two blotting papers, the gel, blotting membrane, two more blotting papers, and the Bottom (+) cassette electrode (Anode). A roller was used to ensure there were no trapped air bubbles between the layers. The transfer took place at a constant voltage of 12V for 60 minutes in the Trans-blot transfer system.

After completing the transfer process, the membrane was placed in a container filled with blocking solution (5% dry milk in TBS-T) for 1 hour at room temperature. Following this, the primary antibody was prepared by diluting it in 5% blocking solution as per the manufacturer's datasheet recommendations. The membrane was then incubated with this primary antibody overnight at 4°C. Subsequently, the membrane underwent a series of washes, consisting of four rounds of washing with 1X TBS-T (5 minutes - 15 minutes - 5 minutes - 5 minutes, respectively). Following these washes, the membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody, which had been diluted in TBS-T according to the manufacturer's datasheet, for 1 hour at room temperature. At the conclusion of the secondary antibody incubation, the membrane was once again subjected to four washes with TBS-T. Finally, the chemiluminescent substrate was applied to the membrane following the manufacturer's recommendations. The images were captured using the ChemiDoc XRS+ system.

Biotinylated proteins were also resolved by SDS-PAGE and transferred to nitrocellulose membrane using a semi-dry transfer apparatus. Following the transfer, the membranes were blocked with a blocking solution composed of 5% bovine serum albumin in 1X PBS with 0.1% Tween-20 for overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated streptavidin at a 1:10000 dilution in the blocking solution during 1hr at room temperature. After removing any unbound antibodies from the membrane, chemiluminescence was developed and the images were captured as described above.

4.6. Co-immunoprecipitation (co-IP)

To pull down 3xFLAG-DmCry and its interactors, S2-3xFLAG-DmCry/Interactor-3xHA cells were cultured in 100-mm plates. The presence of 3xFLAG-DmCry in complexes, pulled down by anti-HA antibody-coated magnetic beads under white light or in the dark, was analyzed by immunoblotting. Interactors were probed with an anti-HA tag antibody, while 3xFLAG-DmCry was detected using an anti-FLAG antibody. S2-3xFLAG-DmCry cells expressing interactors were collected in 1X PBS, washed, and lysed in NP-40 lysis buffer with protease inhibitors. After centrifugation, supernatants were transferred to new tubes. Dynabeads magnetic beads were equilibrated and incubated with anti-HA antibody or IgG. The lysate was divided into

two tubes, one processed under light conditions (Light sample, L) and the other in the dark (Dark sample, D). These samples were incubated with anti-HA/magnetic bead or control IgG/magnetic bead complexes overnight. For Jet and Tim samples, anti-V5 antibody or control IgG was used. Beads were collected, washed, and dissolved in Laemmli-SDS loading buffer. Following separation with SDS-PAGE, 5% of input and 30% of precipitated complexes were analyzed by immunoblotting.

4.7. Streptavidin Pulldown for Mass Spectrometry

Streptavidin pulldown protocol was adapted from Branon et al, 2018 and Kim et al, 2016. Cell pellet was lysed in RIPA lysis buffer according to volume of pellets by gentle pipetting and incubating on ice 10-15 min. Then cell lysate was sonicated for 6 sessions with 10 pulses using a Branson Sonifier 250 at 30% duty cycle and an output level of 3. The tube was sat on ice for 20 sec between each session to prevent overheating. Lysate was clarified by centrifugation at 13,000 rpm for 15 min at 4°C. During the centrifugation step, streptavidin beads were washed twice with 0,5X Lysis Buffer on magna rack. Before adding cell lysates to the beads, 2% volume of samples was taken for Western blot confirmation. Cleared cell lysates were bound to equilibrated beads and rotated end to end overnight at 4ºC. Next day, the beads are subsequently washed; once with 1mL of RIPA lysis buffer, once with 1mL of 1 M KCl, once with 1 mL of 0.1 M Na_2CO_3, once with 1 mL of 2 M urea in 10 mM Tris-HCl (pH 8.0). The beads can be stored at 2M urea at 4°C if necessary. The beads were then resuspended in 1 mL fresh RIPA lysis buffer, transferred to a new Eppendorf tube, and shipped to Koç University Proteomics Facility on ice for further processing and preparation for LC-MS/MS analysis.

4.8. Mass Spectrometry

4.8.1. Mass Spectrometry Analysis to Identify Biotinylated Proteins

To identify the biotinylated proteins by mass spectrometry, on-bead tryptic digestion was performed for each sample at the Koç University Proteomics Facility (KUPAM). The complexes of protein and beads underwent a series of steps. Initially, they were washed with 8 M urea in a solution of 0.1 M Tris-HCl pH 8.5, followed by reduction

with 100 mM DTT in the urea solution at 56°C for 45 minutes, and subsequent alkylation with 50 mM IAA at room temperature for 30 minutes. To eliminate urea, the beads were washed with an ammonium bicarbonate solution (50 mM NH_4CO_3). For tryptic digestion, 600 µg of protein and 4 µg of trypsin (Sigma, cat no: T1426) were added to the beads, and the mixture was incubated overnight at 37°C. The digestion reaction was terminated by treating the samples with 10% formic acid. Subsequently, peptides were desalted and eluted using C18 columns. The eluted samples were then dried in a speed vacuum system (Thermo Fisher Scientific, cat no: SPD1010) and subjected to analysis using a C18 nanoflow reversed-phase Dionex Ultimate 3000RSL liquid chromatography (Thermo Scientific, LC) system configured with a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, MS/MS). The data sets were searched against the *Drosophila* UNI-PROT database (UP00000083_2021).

4.8.2. Mass Spectrometry Data Analysis and Evaluation

Raw files were analyzed by database engine Thermo Proteome Discoverer versions 1.4 and 2.3 using Sequest HT and Mascot for peptide identification. Carbamidomethylation of cysteine and acetylation (protein N-termini) and oxidation of methionine were chosen as a fixed modification and variable modifications, respectively. Less than two missed cleavages were filtered for the tryptic peptides. The precursor mass tolerance was arranged to 10 ppm, and peptide-protein FDRs were set to 0.01.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038052.

To evaluate the Mass Spectrometry results, the SAINT express version 3.6.3 statistical tool [Teo et al, 2014] was used via default parameters and background contaminants. SAINT express is a widely used method for scoring and visualizing protein interactions derived from affinity purification mass spectrometry (AP-MS) data. The tool needs experimental data of test protein with appropriate controls (e.g., bait-only, empty vector) which is in a suitable format, such as a matrix of spectral counts or other quantitative measures. SAINT express will perform a statistical analysis of the interactions, generating a Bayesian false discovery rate (BFDR) and an interaction score for each bait-prey pair. It interprets data by prioritizing interactions based on the

BFDR and interaction score which is called Saint Score and generates lists of high-confidence interactions for further investigation. The scoring system aims to assess the likelihood that a given prey protein is a true interaction partner with the bait protein. In the context of the SAINT express tool, a Saint score of 1 typically indicates the highest level of confidence in a protein-protein interaction.

Principal component analysis (PCA) was performed using the R ggfortify package as quality control step of dataset. The heatmap graph is generated via pheatmap package.

4.8.3. PPI Network Analysis

Utilizing the SAINT express analysis, a PPI network was constructed by incorporating significantly interacting proteins with DmCry. This network was then divided into two subnetworks, designating high-confidence proteins under dark (66 proteins) and light (83 proteins) conditions. The BIOGRID 4.4.214 PPI database was employed for subnetwork generation [Oughtred R et al, 2021]. Subsequently, the entire interactome network from the BIOGRID database was retrieved, forming a new network with *Drosophila melanogaster* as both the source and target organisms. Employing proteins with a Saint Score greater than or equal to 0.95, 20 subnetworks were generated using the KeyPathwayMiner (KPM) tool [List M et al, 2016] on Cytoscape [Shannon P et al, 2003]. KPM, utilizing Greedy as the search algorithm and INES (Individual Node Exceptions Strategy) as the search strategy, created subnetworks enriched with differentially active nodes. Key parameters, such as K (number of allowed inactive nodes) and L (maximum case exceptions for a particular node), were specified for optimal connectivity. Network analysis identified bottleneck proteins based on betweenness centrality values. Cytoscape 3.7.2. facilitated network visualization [Shannon P et al, 2003], representing proteins with circle and triangle nodes. Circle nodes denote proteins from the SAINT express analysis [Teo et al, 2014], while triangle nodes represent proteins added to the network. The Compound Spring Embedded algorithm was applied for protein clustering.

4.9. Statistical Analysis

Statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software, California, USA). The statistical significance of differences between light

treatment groups with or without substrate was validated with One-way Anova. The significance levels were determined as follows: $p>0.05$ (ns, non-significant), $p\leq0.05$ (*), $p\leq0.01$ (**), $p \leq 0.001$ (***).

5. RESULTS

The main purpose of the thesis is to understand the mechanism of light-dependent interactions of DmCry protein by revealing protein interactions in photoreceptor complex under light and dark conditions. For this purpose, we applied proximity-dependent biotin labeling technique to reveal protein-protein interactions. In our study, we used two different biotin ligases; TurboID and MiniTurbo which are mutant forms of *E. coli* BirA enzyme, and APEX2 which is an engineered soybean ascorbate peroxidase to discover CRY interactions under dark and light conditions.

In this study, we first attached either TurboID or APEX2 to DmCry and expressed those fusion proteins in S2 cells and identified the proteins in proximity of DmCry. Since the C-terminal of DmCry plays an important role in the reaction mechanism by light-induced conformational change, we fused biotin labeling enzymes into the C-terminus of DmCry. We collected high-throughput MS data under dark vs light conditions and validated several novel cryptochrome interactions using classical co-IP technique. This part of study is completed and published under Photochemistry and Photobiology Journal with title: " Interactions of drosophila cryptochrome" [Ozcelik et al, 2024].

Having obtained a promising list of interaction candidates, we then analyzed DmCry N-terminal interaction partners by using another biotin ligase, miniTurbo which has less background compared to TurboID. For this purpose, we attached miniTurbo to N terminal of DmCry expressed in S2 cells and identified the proteins in proximity of respective proteins under darkness and light.

5.1. Generating Stable Lines to Express and Characterize DmCry

The cell lines were established in *Drosophila* S2 cell line. The stable expression of S2 cells were made by the selection of single-cell clones with limited dilution from hygromycin-selected cells as explained in the Methods section.

First, we generated the S2- 3xFLAG-DmCry cell line to determine optimal conditions such as light fluency to activate DmCry and analyze the DmCry behaviors and interactions. We determined appropriate doses of white light in comparison with black

light. We showed the degradation of tagged DmCry by exposing the cells to black light (366-nm, at a fluence rate of 0.125 $mW.cm^{-2}$) or white LED light (6000 lux) as functionality of overexpressed protein. Although a 366-nm black light source has been widely employed for inducing the degradation of DmCry in previous studies, we used white light in our experiments because of the anticipated subsequent experiments for interaction studies which needs overnight light exposure. Considering the potential cell toxicity associated with extended overnight exposure to 366-nm black light, which can vary depending on the treatment dose (fluency), we conducted initial tests to figure out white light treatment could effectively induce DmCry degradation at levels comparable to those achieved with black light treatment. Remarkably, we observed efficient light-dependent proteolysis of DmCry with both white and black light sources as shown in Figure 5.1. Light-dependent degradation (proteolysis) of 3xFLAG-DmCry was followed with immunoblotting using anti-FLAG antibody. The actin level was used to normalize DmCry levels in the samples (Figure 5.1A). The quantification of protein degradation over time is shown in Figure 5.1B. Although we observed an efficient light-dependent proteolysis of DmCry by both white and black light, we preferred to use white light in our experiments.

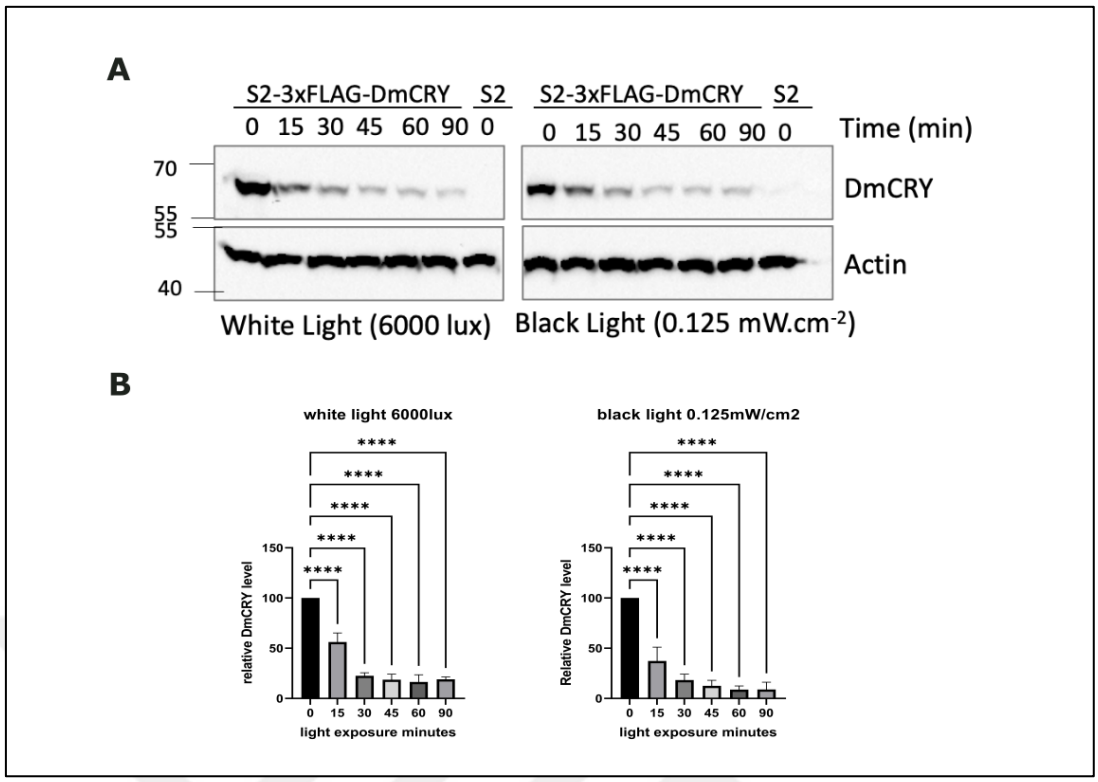

Figure 5.1: The light-dependent degradation of 3xFLAG-DmCry under white or 366-nm black light at the specified doses. (A) Representative western blot image of three independent experiment. Detection of 3xFLAG-tagged DmCry was performed via immunoblotting with an anti-FLAG antibody, and actin served as an internal control. The presented blot is a representative image from three independent replicates. (B) The accompanying graphs show the quantification of protein levels at various light-exposed time points, which was normalized to the dark control. The significance levels were determined as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) using One-way Annova.

5.2. Proximity Labeling of DmCry

The aim of this thesis is to identify the interactome of DmCry in the dark and under light. Considering the nature of DmCry and its known interactors, it is critical to choose fast-acting method to capture weak and transient interactions. Two recently developed biotin-labeling enzymes which act the scale of minutes to one hour, called APEX2 and TurboID (TID), met these criteria.

First, we investigated the C-terminal interactions of DmCry in *Drosophila* S2 cells. For this purpose, we generated stable S2 cell lines expressing APEX2, DmCry-APEX2, or TID, DmCry-TID. The plasmids that were used to generate stable cell lines are schemed in Figure 5.2A. In Figure 5.2B and C panels show the expression of fusion proteins and biotinylating activity of indicated stable cells. The V5 tag allowed us to

detect all fusion proteins in the same membrane. Streptavidin-HRP blot confirmed the activity of specific biotinylation when cells were supplied with biotin substrate. Since two enzymes use different biotin reagents to label preys, it is indicated as substrates in the results. This data indicates that all generated stable cells have comparable levels of recombinant proteins and fusions proteins are active regarding to biotin labeling.

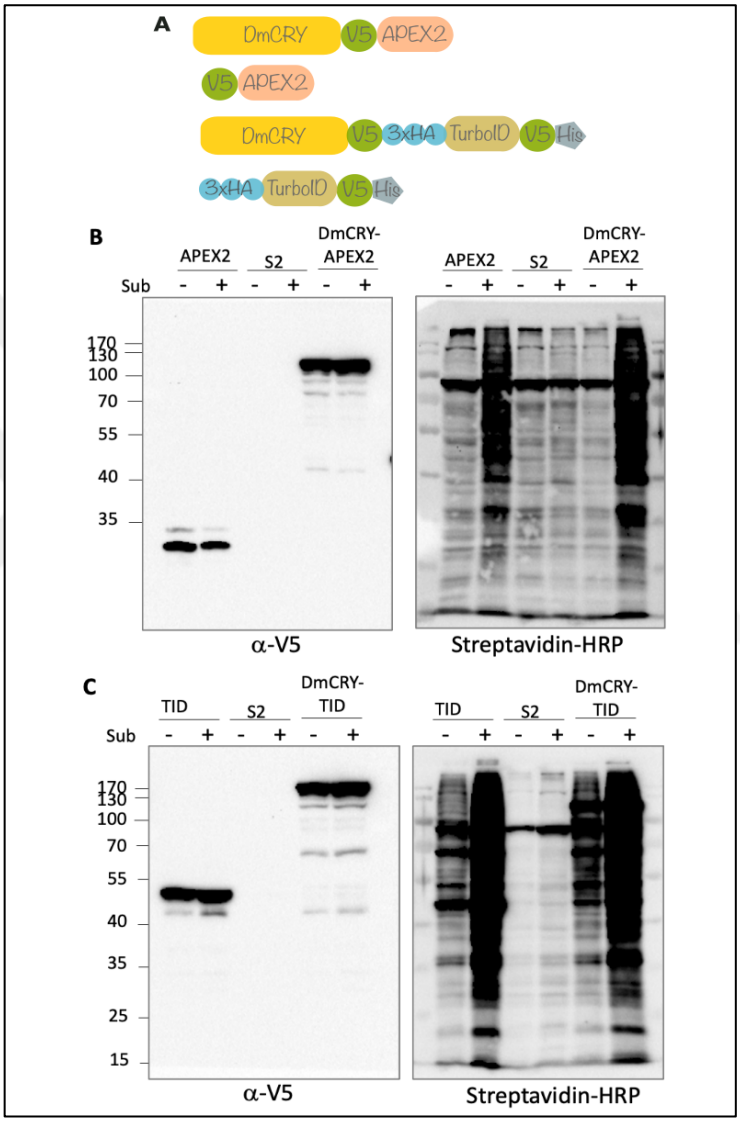

Figure 5.2: The expression pattern of DmCry-APEX2, APEX2, DmCry-TID and TID proteins in the generated stable cell lines. (A) The scheme of constructs used to express recombinant proteins in S2 cell lines. (B) The detection of DmCry-APEX2 and APEX2 was performed via immunoblotting with an anti-V5 antibody. APEX2 and DmCry-APEX2 run around 30kDa and 100kDa, respectively. Right Panel: The Streptavidin blot was performed to follow biotin labeling. (C) The detection of DmCry-TID and TID was performed via immunoblotting with an anti-V5 antibody. TID and DmCry-TID run around 50kDa and 120kDa, respectively.

After generating stable cells expressing recombinant proteins of interests, we want to confirm whether the creation of DmCry-enzyme fusions may impact the kinetics of protein degradation or not. This consideration arises due to differences in the labeling protocols: the APEX2 labeling protocol necessitates a 30-minute substrate incubation, while a 10-minute substrate incubation is sufficient for TID labeling. The time constraint of 30 minutes was critical as we aimed to use two distinct enzymes (APEX2 and TID) for labeling, ensuring that DmCry remains active during the labeling process. This approach allows capturing transient interactions from the folding to degradation stages.

As shown in Figure 5.3, light exposure of S2-APEX2, S2-DmCry-APEX2, S2-TID, and S2-DmCry-TID cell lines activated DmCry fusion proteins. This activation was evident through the partial degradation of recombinant DmCry fusions, while light treatment had no discernible effect on APEX2 or TID levels. The quantification of independent western blot experiments showed that relative DmCry fusion proteins level reduced approximately 60% upon light degradation in 30 min. Also, treating cells with substrates during light exposure did not influence the degradation of DmCry fusion proteins (Figure 5.3A and B).

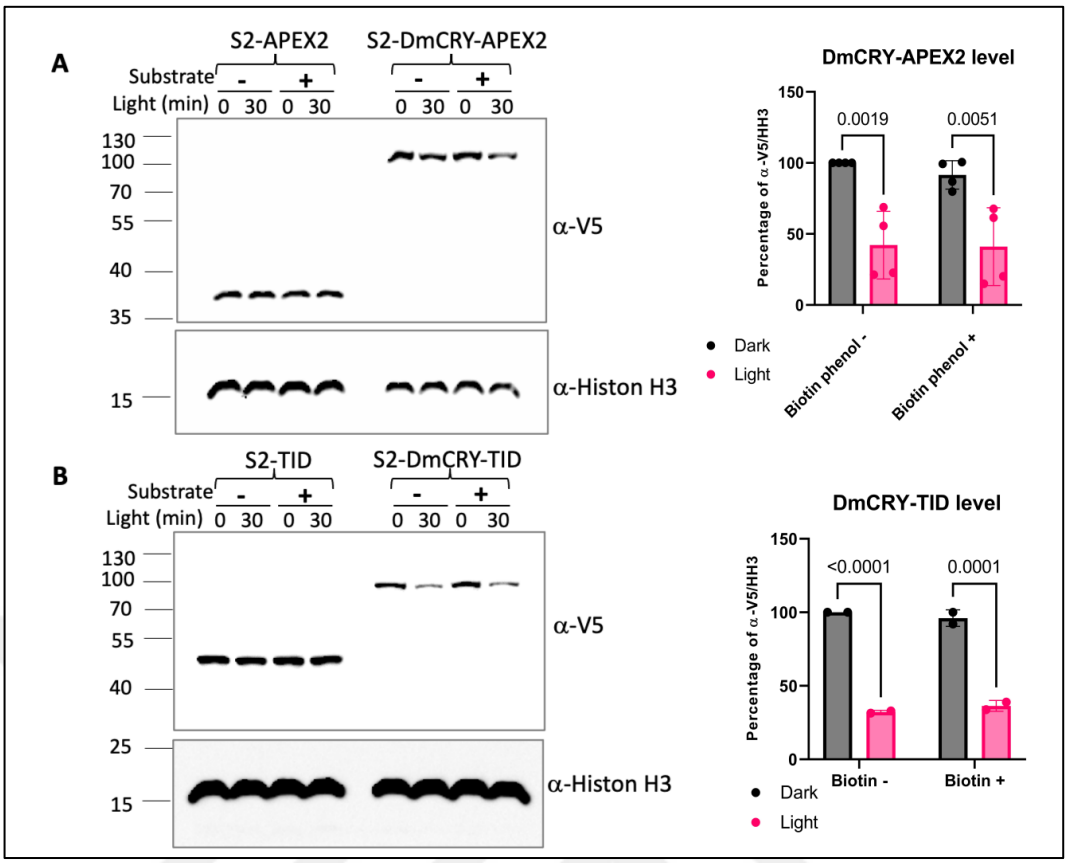

Figure 5.3: The degradation of DmCry-APEX2 and DmCry-TID proteins. (A) Representative western blot image of three independent experiment. Upon 30 min white light treatment, the DmCry-APEX2 and APEX2 levels were detected via anti-V5 antibody in either presence or absence of biotin substrate. The right panel shows the quantification of DmCry-APEX2 level under dark and light conditions with presence or absence of substrate. The individual dots represent different experiments. P-values are shown on the plot. (B) Representative western blot image of three independent experiment. Upon a 30-min white light treatment, the DmCry-TID and TID levels were detected via anti-V5 antibody in either presence or absence of biotin. The right panel shows the quantification of DmCry-TID level under dark and light conditions with presence or absence of substrate. The individual dots represent different experiments. P-values are shown on the plot.

Furthermore, we also showed that light treatment did not interfere with the biotinylation activities of the enzymes (Figure 5.4).

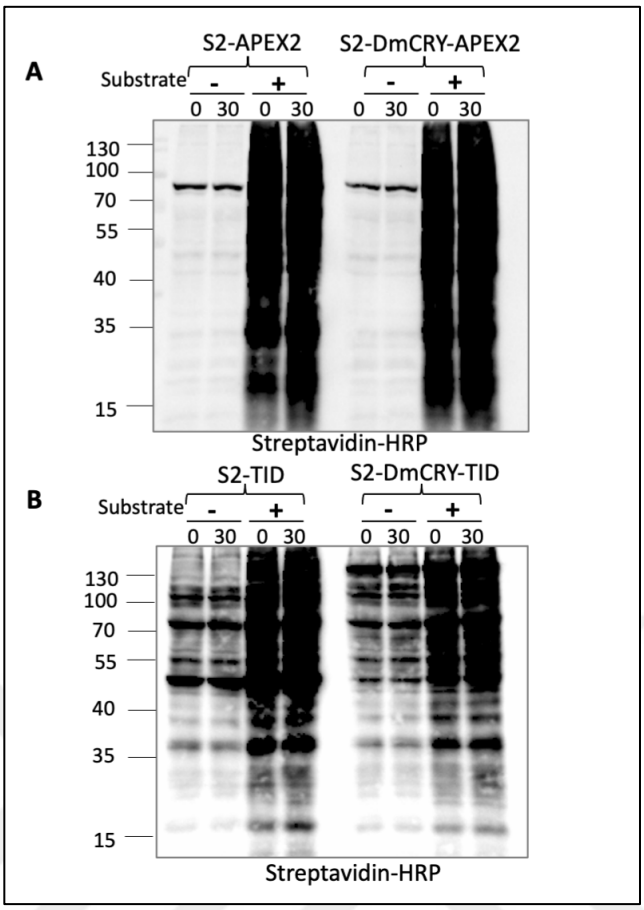

Figure 5.4: Biotinylation pattern of stable S2 cells under the light treatment. (A) Streptavidin-HRP immunoblot was used to detect biotinylated proteins in either APEX2 or DmCry-APEX2 expressing S2 cells. (B) Streptavidin-HRP immunoblot was used to detect biotinylated proteins in either TID or DmCry-TID expressing cells.

5.3. Identification of Proteins that Interact with DmCry

To analyze the protein interactions, we incubated the cells under light with or without aluminum covers as light and dark samples. To prepare samples for mass spectrometry analysis, cells were seeded in 15-cm dishes and biotinylated as explained in the Methods section and treated under conditions described in Figure 5.3. Biotinylated proteins were enriched on the streptavidin-coated beads and processed for mass spectrometry as described in the Methods section. The proteins were identified with label-free quantitation method after on-bead digestion of peptides. Each sample was run twice in the instrument and data quantified as technical replicates. Since more than two hundred proteins were identified in each condition, we first did principal component analysis (PCA) to investigate large dataset (Figure 5.5).

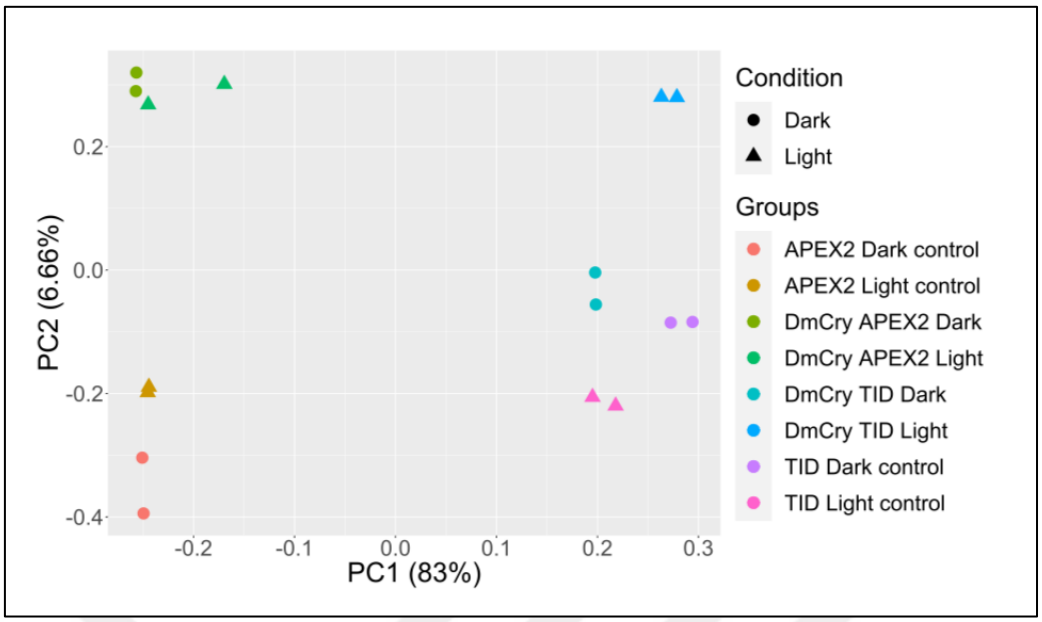

Figure 5.5: PCA plot shows the dimensions of groups DmCry-APEX2, APEX2, DmCry-TID, and TID and conditions: light and dark. Different colors show the sample groups as different cell types run in mass spectrometry, and different shapes indicate the same samples' light or dark conditions.

PCA analysis shows that proteins cluster separately in each group and nicely correlated with replicates. This analysis was done to control high throughput dataset, however, to qualify the data, statistical analysis performed with SAINT express tool as explained in the Methods section.

The SAINT express algorithm, which determines the likelihood of each protein interacting with the bait protein. In our experimental design, APEX2 and TID served as negative control to eliminate non-specific labeling through activity of enzymes in fusion proteins. A Saint score close to 1 suggests high confidence in the interaction, although it is often set a threshold for the Saint score to define a list of high-confidence interactions. We first chose the threshold based on the low stringency in catching possible interactions. To identify shared proteins across various groups, we applied a SAINT Express score threshold of ≥0.45. For DmCry-APEX2, we uncovered 196 proteins in the dark and 177 under light conditions meeting this score criterion. Similarly, for DmCry-TID, we identified 96 proteins in the dark and 158 under light conditions with a SAINT express score ≥0.45. The proteins are listed for each condition separately in Appendix table A.

To visualize the common and exclusive protein sets across different conditions, we plotted Venn diagrams representing the DmCry interaction candidates under light and

dark circumstances. The overlapping regions and distinctions between the two enzymes, considering both light and dark conditions, yielded robust candidates warranting investigation for light-dependent interactions of DmCry. Specifically, employing a Saint Score cutoff of ≥0.45, we observed 110 proteins common to both dark and light conditions for DmCry-APEX2, while 49 proteins exhibited overlap in the dark and light conditions for DmCry-TID (Figure 5.6A).

To investigate the strongest candidates, we did heatmap clustering analysis with a threshold SAINT express score ≥0.95. We identified 49 and 50 proteins in the dark and under light for DmCry-APEX2; while 21 and 41 proteins were identified in the dark and under light for DmCry-TID samples, respectively (Figure 5.6B). This cutoff allowed to illustrate strong candidates and obtained a less crowded clustering results (Figure 5.6C).

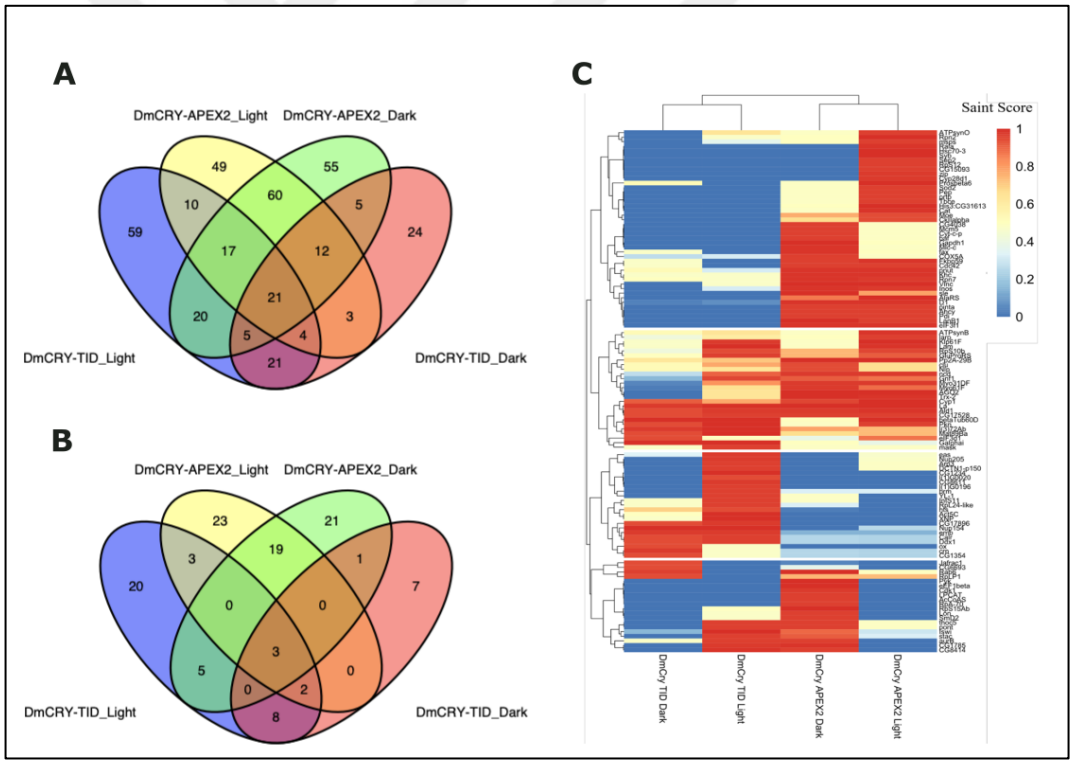

Figure 5.6: Saint express Analysis of DmCry-APEX2 and DmCry-TID groups in light and dark conditions. Venn diagram summarizing the total protein hits for each of the samples and the number of sample-exclusive or common hits for (A) saint score equal/greater than 0.45. (B) saint score equal/greater than 0.95. (C) Heatmap illustrating difference of protein clusters based on Saint Score scale from proteins listed in Figure 5.6B.

5.4. Network Analysis of Identified Protein Candidates

Employing two distinct biotinylation enzymes offers a comprehensive approach to detecting interaction partners, by eliminating limitations associated with various proximity labeling methods. To specifically explore light-dependent interactions involving DmCry, we combined the lists of interactors from DmCry-TID and DmCry-APEX2, categorized by dark and light conditions. Expanding on the DmCry interaction map, we conducted network analysis to unveil proteins that significantly interact with DmCry (Figure 5.7). Notably, the integration of certain proteins not initially detectable in our mass spectrometry data was facilitated through the KeyPathwayMiner (KPM) tool. This network analysis involves the computation of protein-protein interaction (PPI) properties and the identification of proteins with high betweenness centrality, referred to as bottleneck proteins. These bottleneck proteins exhibit elevated connectivity, indicative of interactions with numerous other proteins in the network.

In Figure 5.7, proteins identified in our proteomics data were shown in green nodes while pink nodes were additional proteins added by KPM. Our analysis highlights the top ten bottleneck proteins in the PPI network of DmCry under both dark and light conditions, providing insights into the functional integrity of the network.

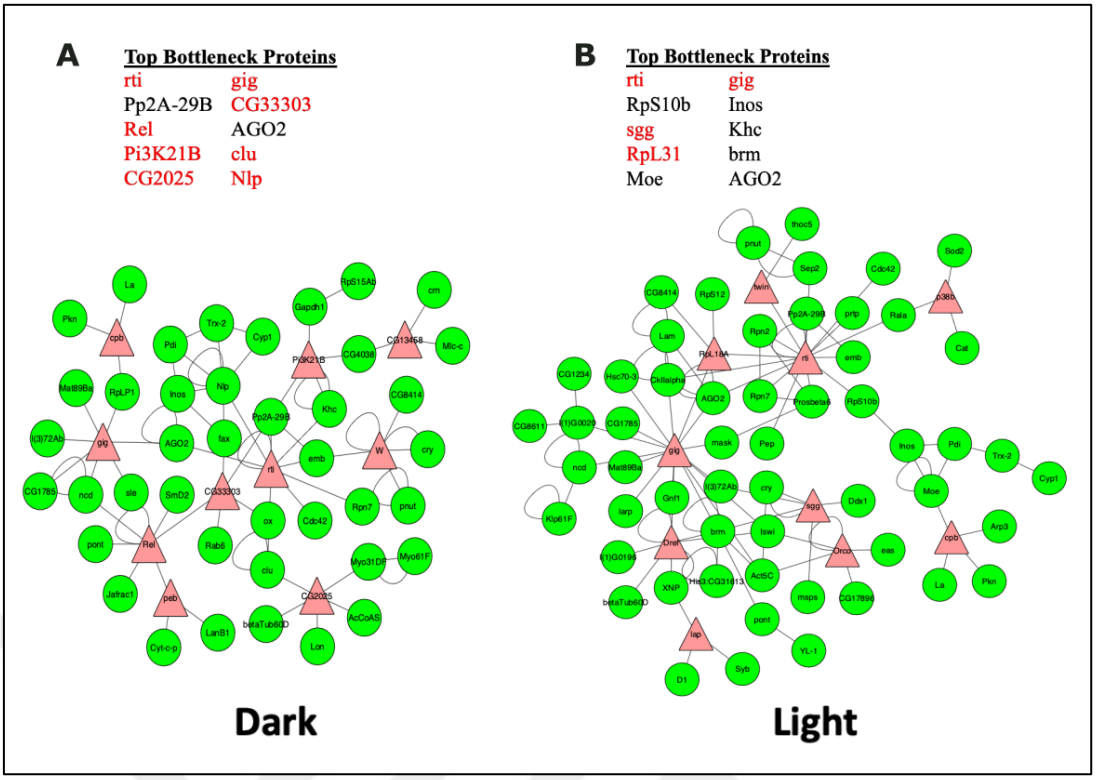

Figure 5.7: Network analysis of the interactome of DmCry. BIOGRID *Drosophila melanogaster* network analysis of significantly interacting proteins with DmCry under (A) the dark and (B) light. Two subnetworks were created using high-confidence proteins as dark (66 proteins) and light (83 proteins).

5.5. Validation of Selected Interactor Candidates

It is essential to experimentally validate protein-protein interactions identified via high-throughput analysis. Co-immunoprecipitation is one of the commonly used techniques for testing PPIs, although it is difficult to show interaction occurs weakly or transiently. Because proximity labeling method does not detect only physical protein interactions but also label the non-interactors in proximity, we wanted to validate interactors whose saint score higher than 0.95 which presumably have strong interaction via co-IP.

First, we recapitulated light-dependent known interactions of DmCry with Jet and Tim. In S2 cells, Tim is not expressed, and Jet is expressed weakly, while the endogenous DmCRY protein is unstable in this cell line. Hence, we preferred to overexpress V5-Jet and Tim-V5His in the S2-3xFLAG-DmCry cell line which already has been generated for characterization of light dependent activity of DmCry. Considering that not all S2-3xFLAG-DmCry cells might be expressing Jet or Tim, we precipitated Jet

or Tim from S2-3xFLAG-DmCry/Jet or Tim cells to increase the possibility of confirming the interactions because all Jet or Tim expressing cells were expected to express DmCry.

For proof-of-concept experiment, the cell lysate was split into two aliquots. These samples were subjected to light exposure to activate light-dependent interactions, relying on the conformational changes induced by light. Following the collection of samples for input, each aliquot was evenly distributed onto magnetic beads coated with anti-V5 antibody or control IgG and rotated under light exposure for 16 hours with or without aluminum foil covering as dark control (D) or light-treated (L) samples. After removing unbound proteins through washing, the protein complex on the beads were analyzed by immunoblotting. Notably, when V5-Jet was immunoprecipitated, the presence of DmCry was evident in the light sample, contrasting with its undetectability in the dark sample. Similarly, the Co-IP technique revealed the recapitulation of the light-dependent interaction between Tim and DmCry when Tim was precipitated (Figure 5.8).

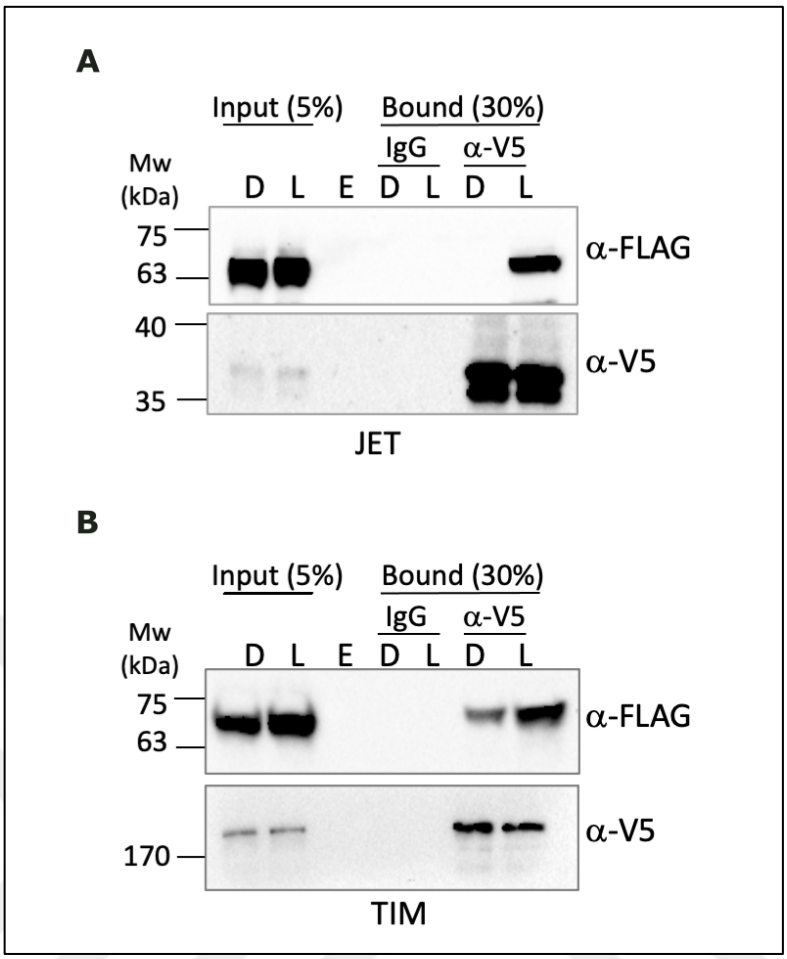

Figure 5.8: The proof of concept of light-dependent interactions between DmCry and its known interactors, Jet and Tim. Input samples indicate whole lysate and bound samples indicate immunoprecipitated fraction. Flag blot shows DmCry expression in the indicated conditions. (A) V5-Jet was expressed in 3xFLAG- DmCry expressing S2 cells, and the complexes were immunoprecipitated via anti-V5 antibody-magnetic beads under dark (D) or light (L) conditions. (B) Tim-V5 was expressed in 3xFLAG-DmCry expressing S2 cells. Co-IP with anti-V5 antibody showed that Tim interacts weakly in the dark, but this interaction is greatly enhanced by light.

This experiment stands out as an important part of our study, indicating the feasibility of reproducing light-dependent interactions involving DmCry and established interactors like Jet and Tim through the co-IP assay. While the interaction between Jet and DmCry was previously confirmed using purified proteins [Ozturk et al, 2011], our study extends this validation to cell lysates. This step is crucial, considering the difficulties associated with purifying all potential interactors for in vitro pull-down assays.

After establishing that we can show the light-dependent interaction between DmCry and Jet or Tim using the co-IP technique, we decided to test the selected novel

interactors using the same settings. The interactors, tagged with V5 and 3xHA, were subjected to co-IP, with the 3xHA tag being preferable due to its location in the most C-terminal part. Immunoprecipitation was conducted using anti-HA antibody-magnetic beads. To validate the interactions, candidates were chosen from the novel DmCry interactors with a SAINT express score ≥ 0.95 based on the analysis. Despite attempts to express and IP over eight interactors, only those feasible for co-IP due to adequate expression levels were tested. The co-IP results are categorized into four groups.

Light-induced interactions:

This group of interactions means that it is observed only under light conditions. In the first group, the interaction of DmCry with Nucleolar protein 53 NOP53 and Polynucleotide 5'-hydroxyl-kinase NOL9 could be observed only after treatment with light. This type of interaction could be observed between DmCry and Jet in our conditions (Figure 5.8A). This data shows DmCry might interact with the ribosomal regulation system.

Light-enhanced interactions:

In the second group, the interactions of DmCry with FK506-binding protein 59 and Acetyl-coenzyme A synthetase were evident in the dark but clearly enhanced by light. The interaction could be observed between DmCry and Tim (Figure 5.8B).

Light-independent interactions:

Interactions between DmCry and Failed axon connections protein as well as Casein kinase II subunit alpha protein remained unaffected by light and were observable in the dark. It can be hypothesized that the interaction in this group was already robust, precluding further enhancement by light in a non-specific manner.

Unproven interactions:

The interactions detected in proximity may not be validated via co-IP which validates mostly physical protein-protein interactions. Even though interactors having highest saint score presumably interact strongly with bait protein, it is possible that those interactions are false-positive. In this group, chosen candidates interacting DmCry, Endoplasmic reticulum chaperone BiP and Adenosylhomocysteinase cannot be

validated via co-IP. On the other hand, the absence of any interaction in this group confirmed that light did not cause nonspecific interactions.

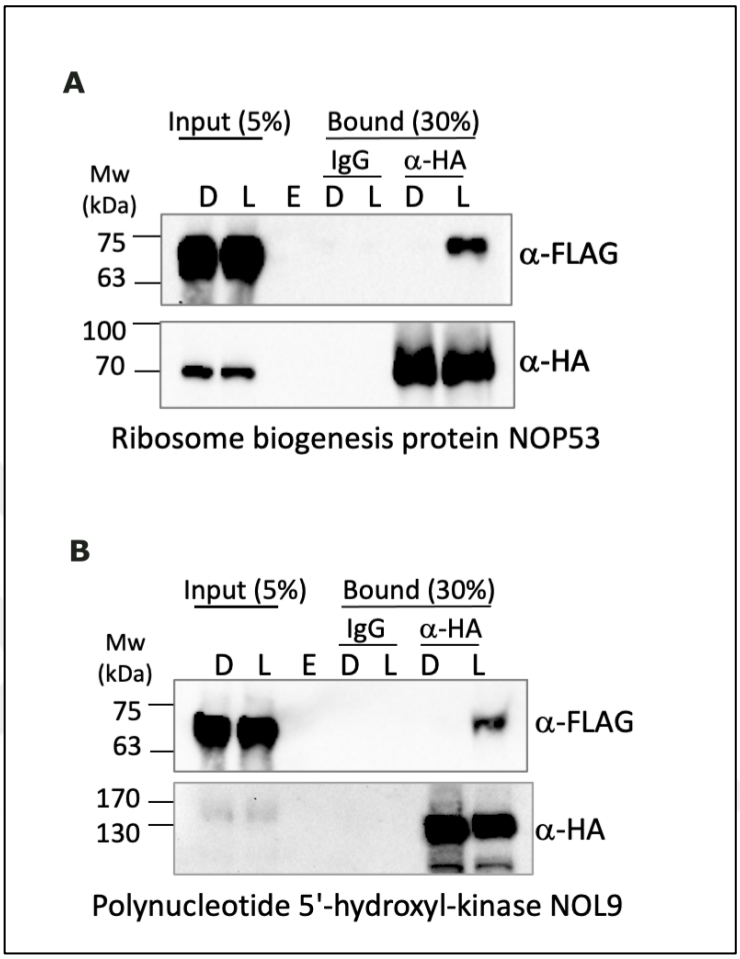

Figure 5.9: Testing the recapitulation of light-dependent interactions between DmCry and its novel interactors using co-IP technique. Input samples indicate whole lysate and bound samples indicate immunoprecipitated fraction. Flag blot shows DmCry expression, while HA blot shows interactors in the indicated conditions. The complexes were immunoprecipitated with anti-HA antibody-magnetic beads under dark (D) or light (L) conditions. (A) NOP53 (UniProt ID: Q9W3C2 Gene Name: *cg1785*) and (B) NOL9 (UniProt ID: A1ZA92, Gene Name: *cg8414*) proteins interaction could be recapitulated only in the presence of light in a similar way to V5-Jet.

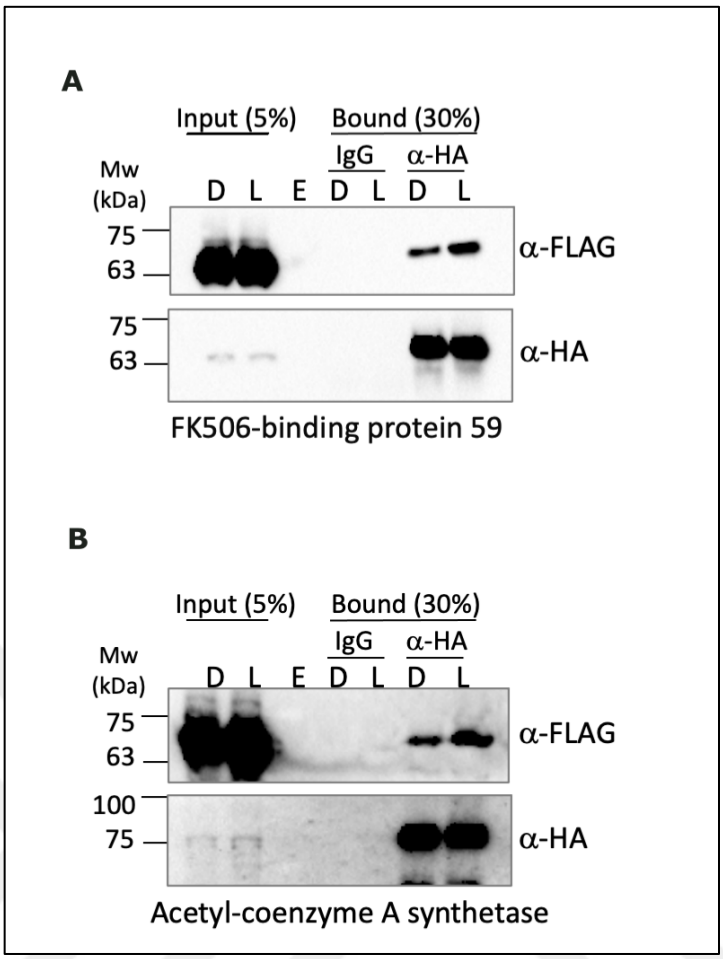

Figure 5.10: Testing the recapitulation of light-dependent (-enhanced) interactions between DmCry and its novel interactors using co-IP technique. Input samples indicate whole lysate and bound samples indicate immunoprecipitated fraction. Flag blot shows DmCry expression, while HA blot shows interactors in the indicated conditions. The complexes were immunoprecipitated with anti-HA antibody-magnetic beads under dark (D) or light (L) conditions. (A) FK506-binding protein 59 (UniProt ID: Q9VL78, Gene Name: *Fkbp59*) and Acetyl-coenzyme A synthetase (UniProt ID: Q9VP61, Gene Name: *AcCoAS*) interaction could be observed weakly under dark but greatly enhanced by light.

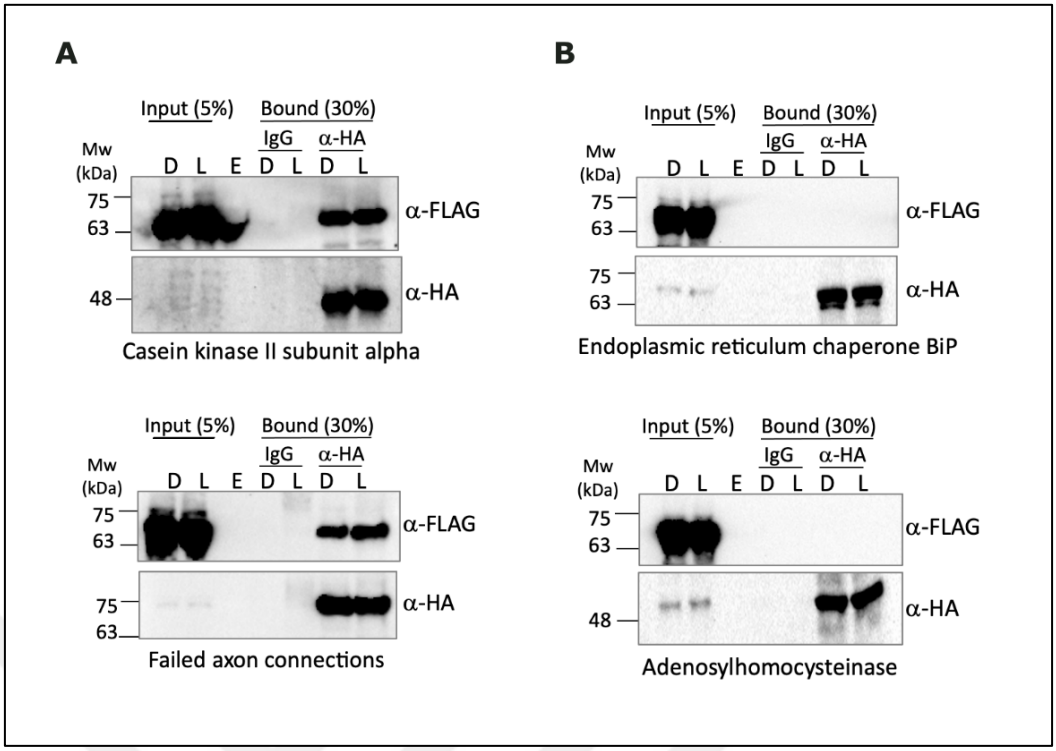

Figure 5.11: Testing the recapitulation of light-independent/unproved interactions between DmCry and its novel interactors using co-IP technique. Input samples indicate whole lysate and bound samples indicate immunoprecipitated fraction. Flag blot shows DmCry expression, while HA blot shows interactors in the indicated conditions. The complexes were immunoprecipitated with anti-HA antibody-magnetic beads under dark (D) or light (L) conditions. (A) Light-independent interactions of DmCry with Failed axon connections (UniProt ID: Q95RI5, Gene Name: *fax*) and Casein kinase II subunit alpha (UniProt ID: P08181, Gene Name: *CkIIalpha*). (B) A possible interaction between DmCry and interactors Endoplasmic reticulum chaperone BiP (UniProt ID: P29844, Gene Name: *Hsc70-3*) and Adenosylhomocysteinase (UniProt ID: Q27580, Gene Name: *ahcy*) couldn't be recapitulated.

The interactions of those selected candidates will be explained in detail in the discussion part. Overall, this part of thesis proves that proximity labeling methods could give broad and insightful results when they are used as complementary with conventional protein-protein interactions assays.

Second part of this study, we studied N-terminal of interactions of DmCry via different biotin ligase, miniTurbo.

5.6. Proximity Labeling of DmCry via N-terminal

Upon light activation, DmCry promptly transmits a light-induced resetting signal to Tim protein via Jet E3 ligase within minutes. Although the light-dependent degradations of DmCry and Tim are accelerated by increasing the light fluency, the order of first Tim degradation and then DmCry degradation suggests that a longer biotinylation reaction may help to probe all PPIs.

The miniTurbo (MT), another mutant biotin ligase enzyme, has slower kinetics than TurboID [Branon et al, 2018]. We kinetically studied the possible interaction candidates with longer biotin labeling up to 3hr. We inserted miniTurbo into N-terminus of DmCry, therefore with this strategy we increased the biotinylation time up to more than one hour. Alternatively, we also used 13x GGGGS linker to increase the labeling diameter as much as we can. This would increase the labeling zone but allows us to catch as many interactions as possible from N-terminus of protein. Using longer linkers is supposed to helpful for big size proteins as provides freedom of biotin ligase by preventing stuck in closed confirmation.

To prove light dependent reactions of fusion proteins, we conducted test experiments as in Figure 5.2 whether the creation of DmCry-enzyme fusion may impact the kinetics of protein degradation or not. Light-dependent degradation (proteolysis) of proteins of interest was followed with immunoblotting using anti-V5 antibody and quantified as in Figure 5.3 (Figure 5.12B). We observed efficient light-dependent proteolysis of fusion DmCry gradually, indeed, the quantification of independent western blot experiments showed that relative DmCry fusion protein level reduced approximately 80% upon light degradation in 120min.

Next, we biotinylated cells for up to 3 hrs and did streptavidin pulldown. After confirming of biotinylation and streptavidin pulldown allows detection of fusion protein under the labeling and light treatment conditions, cells were prepared for mass spectrometry analysis as described before in the Methods section for APEX and TID samples (Figure 5.12C).

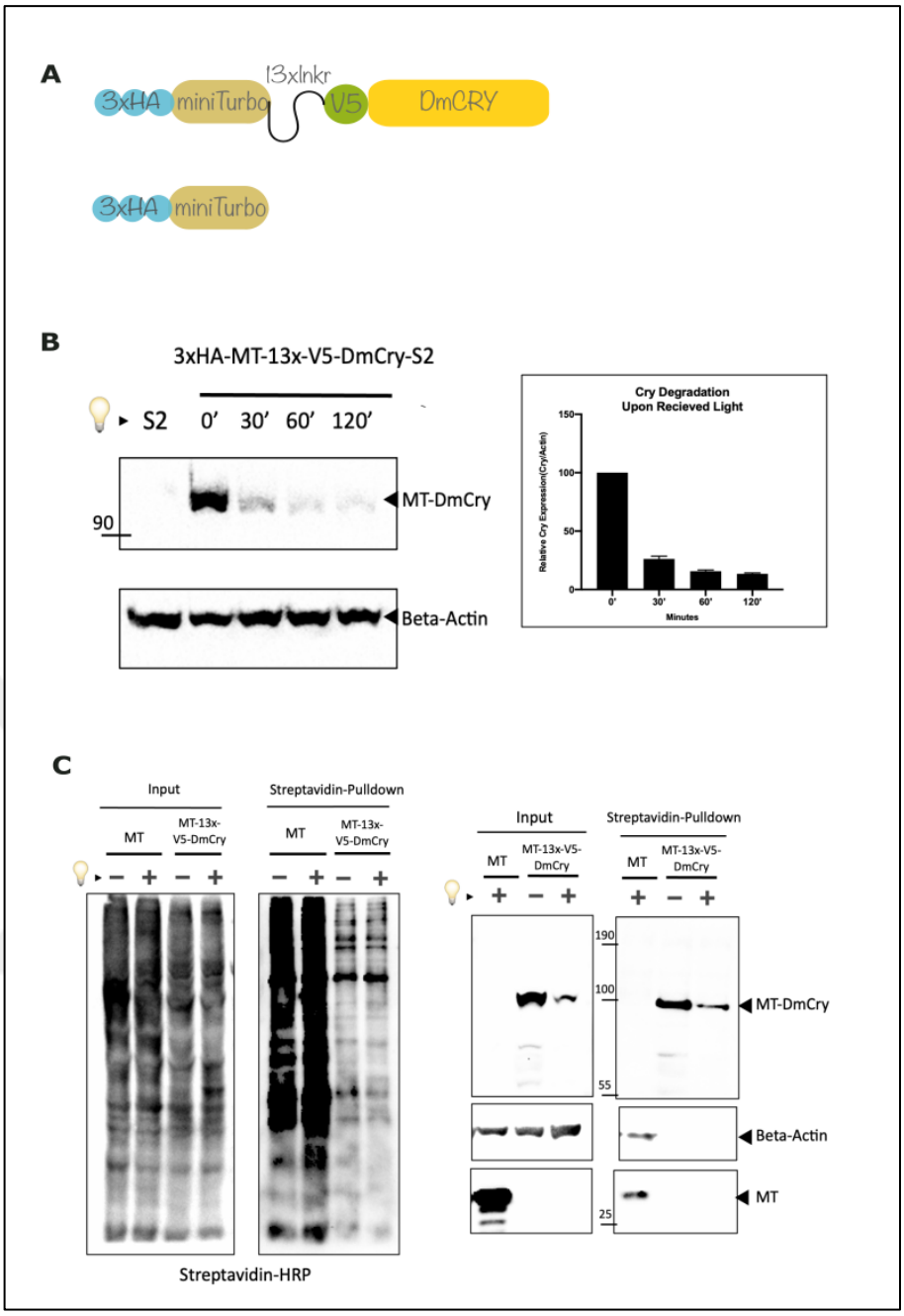

Figure 5.12: miniTurbo fused to N-terminus of DmCry. (A) The scheme of constructs used to integrate recombinant proteins in S2 cell lines. (B) Light degradation of MiniTurbo-DmCry. The detection of MT-DmCry was performed via immunoblotting with an anti-V5 antibody. MT size is around 27kDa and MT-DmCry runs around 100kDa. (C) Sample preparation for Mass Spec analysis. Streptavidin-HRP confirms the biotinylation of cells. Cells were biotinylated under dark or light conditions, while MT-S2 cells were treated with biotin only under light conditions since those cells are control group for unspecific biotinylation. Input samples indicate whole lysate and Streptavidin-pulldown samples indicate biotinylated fraction enriched with streptavidin beads. Anti-V5 blot (upper and bottom blot) shows miniTurbo-DmCry and miniTurbo expression, while beta actin (middle blot) was used internal control for loading.

5.7. Identification of Proteins Interact with DmCry via N-Terminal

To prepare samples for mass spectrometry analysis, cells were seeded in 15cm dishes and biotinylated as explained in the Methods section and treated under conditions one hour and three hours, separately. Biotinylated proteins were enriched on the streptavidin-coated beads and processed for mass spectrometry as described in the Materials and Methods section. The proteins were identified with label-free quantitation method after on-bead digestion of peptides. Each sample was run twice in the instrument and data quantified as technical replicates. To qualify the data, statistical analysis was performed with SAINT express tool as explained in the Methods section. In this experimental setup, miniTurbo served as negative control to eliminate non-specific labeling through activity of enzymes in fusion proteins.

To identify shared proteins across various groups, we first applied a SAINT Express score threshold of ≥0.45. First, we analyzed three hours labeling dataset. For MT-DmCry, we uncovered 204 proteins in the dark and 162 under light conditions. Next, we analyzed one hour labeling dataset, and we uncovered 6 proteins in the dark and 6 under light conditions. To visualize the common and exclusive protein sets across different time-points, we plotted Venn diagrams representing the DmCry interaction candidate numbers under light and dark circumstances, separately (Figure 5.13A). We detected very few numbers of proteins with one hour labeling of MT-DmCry cells. This would be because of combinatory effect of low labeling kinetics of miniTurbo and fast degradation of DmCry within one hour. With Saint Score cutoff of ≥0.45, we observed 61 proteins common to both dark and light conditions for MT-DmCry in 3hr labeling conditions, while none of proteins exhibited overlap in the dark and light conditions for MT-DmCry in one-hour labeling (Figure 5.13A).

To investigate the strongest candidates, we applied a threshold SAINT express score ≥0.95. Those proteins can be listed as highly significant for each condition. For one-hour labeling of MT-DmCry cells, none of proteins identified as strong candidate under light condition, and only one protein had saint score 1 under dark. Thus, we only analyzed three hours labeling of MT-DmCry candidates and seventeen proteins were identified common to both dark and light conditions (Figure 5.13B).

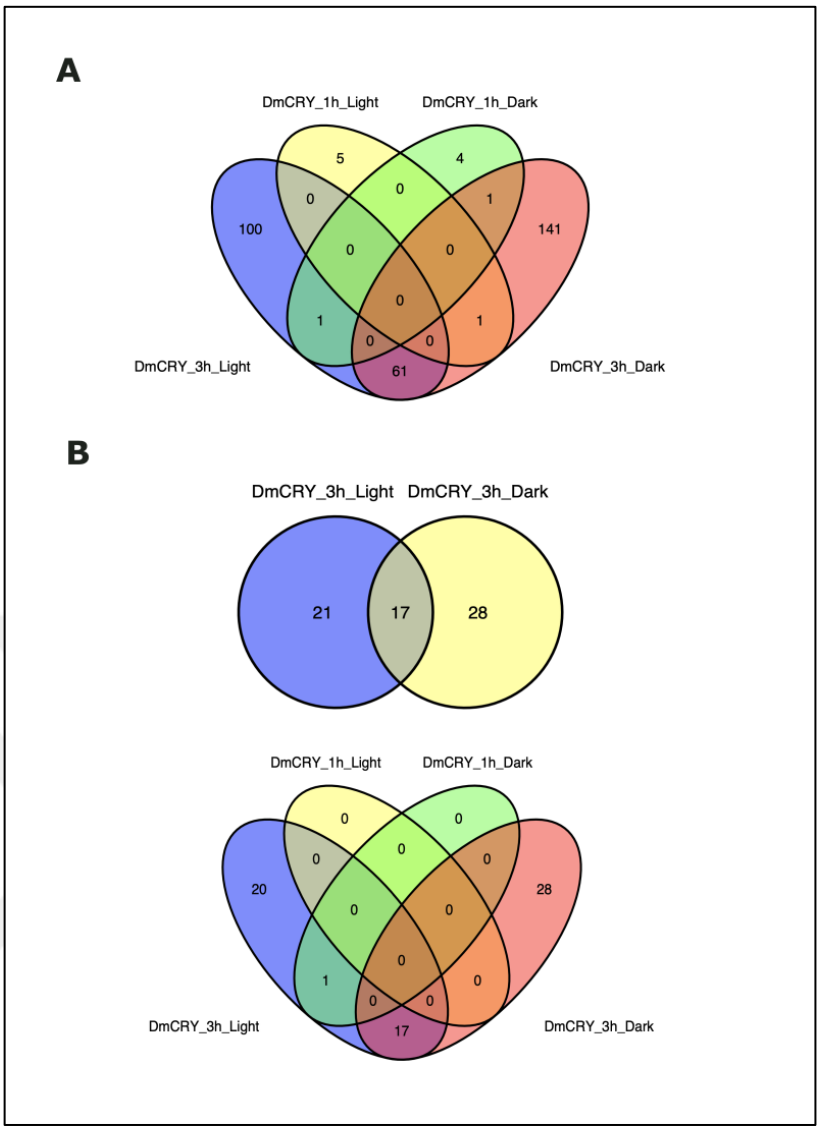

Figure 5.13: SAINT express Analysis of DmCry interactors in light and dark conditions for one-hour or three-hour labeling. Venn diagram summarizing the total protein hits for each of the samples and the number of sample-exclusive or common hits for (A) saint score equal/greater than 0.45 for MT-DmCry, (B) saint score equal/greater than 0.95 for MT-DmCry.

To investigate the strongest candidates, we did heatmap clustering analysis with a threshold SAINT express score ≥0.95 for three hours labeling of MT-CRY expressing cells. This cutoff allowed to illustrate the abundance of strong interaction candidates under dark or light conditions of DmCry (Figure 5.14A). to visualize one hour labeling candidates, we used threshold SAINT express score ≥0.45, since none of the proteins strongly detected for this condition. There we also have been able to detect DmCry itself (Figure 5.14B). This heatmap results showed us strong hits differently under light

or dark conditions to follow up to confirm interactions happen through N-terminus of DmCry.

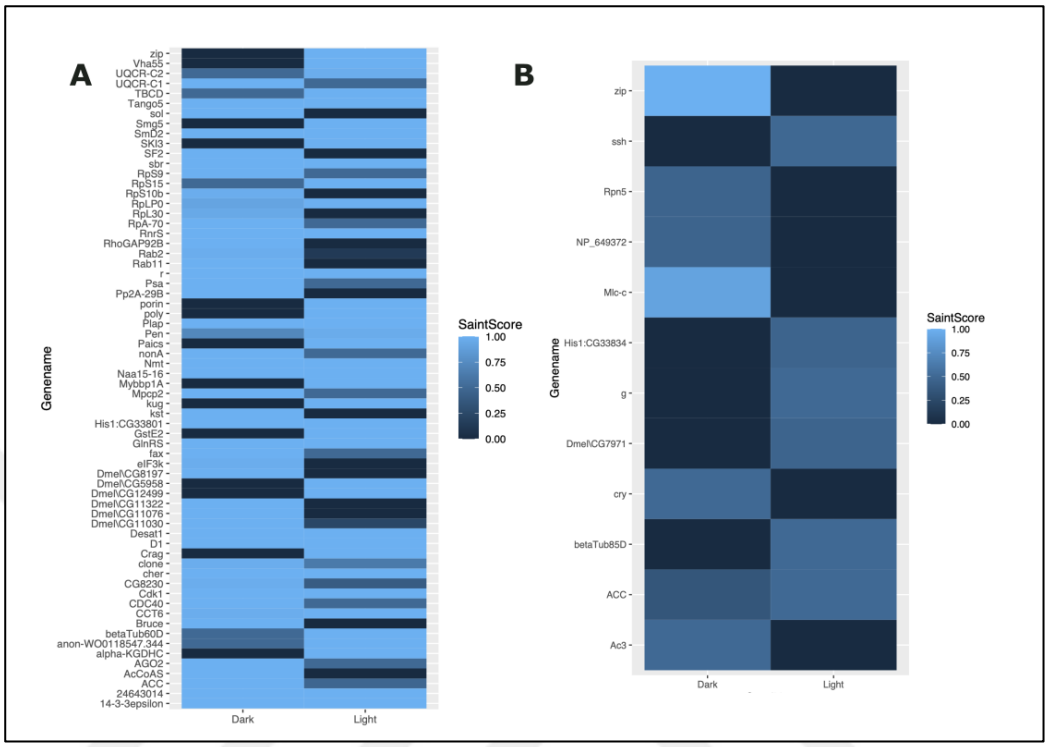

Figure 5.14: Heatmap illustrating difference of protein clusters based on saint score scale. (A) the candidates with a threshold SAINT express score ≥0.95 for three hours labeling, (B) the candidates with a threshold SAINT express score ≥0.45 for one-hour labeling.

6. DISCUSSION

The objective of this investigation was to uncover new interactions of DmCry. Despite the identification of certain interactions through methods like yeast-2-hybrid and genetic screens, we predicted that transient or weak interactions caused by light pose a persistent challenge with classical methods. Upon exposure to light, DmCry undergoes a conformational change [Ozturk et al, 2011]. This light-induced conformational alteration is crucial for subsequent biochemical events leading to the resetting of the circadian clock [Koh et al, 2006]. Initially, the light-induced conformational change exposes interaction sites on DmCry, allowing interactors such as Jet, Tim, and BRWD3 to bind to DmCry. It was previously demonstrated that the half-life of this conformational change is 15 minutes, as determined by partial trypsinization or in vitro pulldown assays. Conversely, the half-life of the light-activated (Lit state of) DmCry in S2 cells was approximately 27 minutes [Ozturk et al, 2011, Ozturk et al, 2013, Ozturk et al, 2014]. Notably, the half-life of the lit state refers to the duration during which the active form can persist when not coupled to subsequent biochemical events. For instance, DmCry can be activated by a one-millisecond flash of light at 0°C, and 50% of DmCry proteins will be in the lit state, with the remaining 50% returning to the dark state after 27 minutes following the light treatment [Ozturk et al, 2011]. Consequently, when the cells are warmed up to physiological temperature (25°C for S2 cells) after 27 minutes, the 50% of DmCry in the lit state begins to be degraded by the proteasome system, while the other 50% is not degraded, as they revert to the basal (non-active) state. Assuming that DmCry degradation is completed in 1 hour and Tim is degraded in 10 minutes after light exposure in S2 cells, we hypothesized that light-induced interactions, especially those related to light-dependent changes, should be captured by a method capable of recording them within a time scale of 30 minutes. Moreover, the FAD cofactor of purified DmCry is reduced within minutes by light and oxidized back again in 30 minutes in darkness [Ozturk et al, 2014]. Although the in vitro light-induced photoreduction of FAD was suggested not to be a prerequisite for conformational change, as blocking the photoreduction did not affect the light-induced conformational changes as detected by partial trypsinization or pull-down of DmCry by JET-GST [Ozturk et al, 2014].

According to the proposed model, interactions start and terminate within a timeframe of less than 1 hour. Recent developments in proximity labeling techniques, specifically the introduction of TurboID and APEX2 encouraged us to discover novel interactions of the DmCry. The success of TurboID and APEX2 in mammalian cells, particularly with labeling incubations for 10 and 30 minutes, respectively, indicated their suitability for our investigation [Brannon et al, 2018, Lam et al, 2015]. This time scale aligned with our objective, allowing us to label interactors before DmCry undergoes complete degradation due to light treatment.

While black light (366-nm) has traditionally been employed to activate DmCry in assays due to its alignment with the maximum absorption of the FAD co-factor, we opted for white light in our experiments. This choice was motivated by concerns that black light might contribute to the generation of reactive oxygen species, potentially interfering with our labeling protocol, especially concerning the APEX2 enzyme. After establishing stable lines of S2 cells to express DmCry and biotinylating enzymes alone or fused to DmCry, we initially determined suitable doses of white light in comparison to black light (Figure 5.1A). Using DmCry with a 3xFLAG tag at the N-terminus, we observed highly efficient light-dependent degradation with low light intensity (0.125 mW/cm²). Previous studies utilizing DmCry tagged at the C-terminus required higher light intensity (>0.5 mW/cm²) to achieve a similar degradation profile [Ozturk et al, 2011, Ozturk et al, 2014, Ozturk et al, 2008]. This observation was anticipated as the C-terminal tail of DmCry plays a crucial role in the reaction mechanism, undergoing a light-induced conformational change. A fluorescent white light source, considered more practical and biologically relevant than the 366 nm black light source, indeed, resulted in similar degradation profiles for both 6000 lux white and 366 nm black light treatments.

Assessment of enzyme biotinylation activities in S2 cells demonstrated their efficacy, with consistent expression levels observed for both the enzyme alone and DmCry-enzyme fusions (Figure 5.2B and 5.2C). TurboID (TID) exhibited higher biotinylation, yielding background labeling even in the absence of substrate due to the presence of biotin in FBS and culture media (Figure 5.2C). While biotin depletion in the culture medium is possible, we decided not to perform this procedure, assuming that background labeling would be excluded from the results. Evaluation of light-dependent degradation profiles for fusion proteins (DmCry-APEX2 or DmCry-TID)

revealed that the presence or addition of substrates for these enzymes did not influence their light-dependent degradation characteristics (Figure 5.3) This step was crucial to confirm that light-activated degradation of the DmCry fused to biotinylating enzymes before submitting for MS analysis. This result ensured DmCry fusions are functional, although addition of the enzymes to the C-terminus slows down DmCry degradation.

Main proposal and novelty of this thesis is to provide high throughput data regarding to identification of DmCry interactors in resting and active states using proximity-dependent labeling via mass spectrometry analysis. The effective controls, TurboID or APEX2 alone, assist in excluding proteins identified through TurboID-DmCry or APEX2-DmCry. Before the determination of interactors, we tried to understand data behavior of mass spectrometry results with principal component analysis (PCA). The principal component analysis (PCA) plot effectively distinguished between the dark and light sample groups (Figure 5.5), and the Venn diagram illustrated a significant overlap in interactors identified by both methods (Figure 5.6A and 5.6C). For DmCry-APEX2, we identified 195 and 176 proteins in the dark and under light, respectively, while for DmCry-TID, 95 and 157 proteins were identified in the dark and under light, respectively, all with a SAINT express score ≥0.45 (Figure 5.6A). Given our primary interest in identifying light-dependent partners of DmCry, the S2 cell line emerges as the most suitable choice currently. As of our current knowledge, a *Drosophila* cell line containing the complete core circadian clock components is not accessible. However, the establishment of such a cell line would enhance the physiological relevance of our findings, as the inclusion of core clock components, Tim and Jet could increase the identification of interactors. Despite S2 being an embryonic cell line, the insights gained from in vitro interactions of DmCry are notably informative. Although proximity labeling tools have been predominantly employed in cultured cells, their potential application in intact flies presents an innovative in vivo approach.

In the APEX2 labeling group, there is a distinct clustering of dark and light conditions, contrasting the APEX2 control group. A notable difference in biotinylation behavior is observed with the APEX2 enzyme compared to the TID enzyme. Using a 0.45 cutoff for Saint Score, 110 proteins are common in both dark and light conditions for DmCry-APEX2, while 51 proteins are shared in the dark and light conditions of DmCry-TID (Figure 5.6A), possibly indicating variations in biotinylation patterns between APEX2 and TID systems. Evaluation of Saint Scores across all groups with the same cutoff

reveals that the APEX2 system identifies proteins with higher Saint Scores, effectively distinguishing differences between dark and light conditions (Figure 5.6A and 5.6C). This distinction may be attributed to the inherent characteristics of the labeling methods, where TID continuously labels proximate proteins, while the APEX2 enzyme predominantly labels proteins near DmCry at the moment of labeling during the 30-minute incubation. Despite a higher number of interactors in APEX2, this could be influenced by increased background labeling with TID, as TID-labeled proteins are excluded from the list of DmCry-TID-labeled proteins.

Networks for light and dark samples were built where green ones were from our mass spectrometry results and pink nodes were added by KPM tool (Figure 5.7). Among the key bottleneck proteins identified in our mass spectrometry data, Pp2A-29B is the regulatory subunit of Serine/threonine-protein phosphatase PP2A with a molecular weight of 65 kDa. In *Drosophila*, Pp2A counters the activity of doubletime (Dbt), a homolog of casein kinase Iϵ, to stabilize Per, suggesting that the rhythmic expression of Pp2A plays a role in Per cycling [Sathyanarayanan et al, 2004]. *Drosophila* Ago1 is involved in miRNA-guided RNA cleavage, while Ago2 primarily facilitates cleavage guided by siRNAs [Höck et al, 2008]. As an effector in the siRNA and microRNA pathways, *Drosophila* Ago2 regulates the transcription of developmentally regulated genes [Cernilogar et al, 2011]. Notably, Gw182, which interacts with Ago1 and is crucial for miRNA-mediated translation, was shown to influence circadian behavior and PDF neuropeptide receptor (PDFR) signaling, impacting the circadian neural network's response to light input [Zhang et al, 2013]. Therefore, exploring the impact of light and DmCry on the RNA silencing mechanism would be valuable. Although the RpS10b gene encodes a structural component of ribosomes, the interaction between Ribosome biogenesis protein NOP53 and DmCry should be further investigated especially considering the confirmed interaction between Nucleolar protein 58 (NOP58) and BMAL1 in mammals [Cervantes et al, 2020].

The Moesin (Moe) protein, a member of the Ezrin, Radixin, Moesin (ERM) family, is implicated in cortical cytoskeleton stability, and its interaction with DmCry might be essential for DmCry docking or may contribute to light-induced cytoskeletal rearrangements. Myo-inositol-phosphate synthase (Inos) mediates the conversion of glucose 6-phosphate to 1-myo-inositol 1-phosphate in an NAD-dependent manner [Park et al, 2000]. Notably, in plants, FHY3 (FAR-RED ELONGATED

HYPOCOTYL3) and FAR1 (FARRED IMPAIRED RESPONSE1) directly bind to the promoter of myo-inositol 1-phosphate synthase 1, activating its expression to prevent light-induced oxidative stress and salicylic acid-dependent cell death [Ma L et al. 2016]. A direct interaction between DmCry and Inos suggests potential roles for their FAD and NAD cofactors, respectively, in modulating this interaction. Khc (kinesin heavy chain) functions as a microtubule-associated force-producing protein involved in organelle transport [Yang et al, 1989]. While establishing a specific functional connection between DmCry and Khc is challenging, it is conceivable that a subset of DmCry may utilize Khc for self-docking under certain conditions. Another prominent bottleneck protein, Brahma (Brm), serves as the catalytic subunit of Brm chromatin-remodeling complexes and has been demonstrated to interact with Clock protein, fine-tuning Clock-dependent transcription levels and maintaining circadian clock robustness [Kwok et al, 2015]. Given the absence of endogenous Clock, Tim, and Per in S2 cells, the observed interaction between DmCry and Brm appears to be direct. Considering DmCry's proposed role as a repressor of Clock–Cycle-mediated transcription in peripheral tissues [Collins et al, 2006], the interaction with Brm suggests a potential mechanism whereby DmCry inhibits Clock–Cycle-mediated transcription through Brm interaction. The increased direct interactions observed in the light-treated samples align with the assumption that light-induced conformational changes in DmCry expose interaction surfaces, facilitating binding with its interactors.

The most critical experiment in this study was that we could recapitulate the light-dependent interactions between DmCry and its known interactors such as Jet and Tim (Figure 5.8). The interaction between Jet and DmCry was previously shown using the purified proteins [Ozturk et al, 2011]. However, we here confirmed these interactions using cell lysates and this was critical because it is difficult to purify all the candidate interactors for *in vitro* pull-down assays. Two interactors of DmCry, Jet and Tim are well known to have light-dependent interactions. Keeping in mind that some of the transient or weak interactions can be difficult or impossible to detect using classical methods such as the co-IP technique, we still used the co-IP assay as the confirmatory experiment. Indeed, we were able to confirm interactions of some candidates that can be easily cloned and expressed in S2 cells. For this purpose, initially, we managed to show the effect of light on the interaction between Cry and Jet/Tim. Theoretically, FLAG-tagged DmCry and V5-tagged Jet or Tim can interact only after light treatment.

Light treatment causes a conformational change in DmCry to expose the interaction sites for Jet or Tim, and it was calculated that the half-life of the active confirmation is 15 minutes *in vitro*. Therefore, the washing steps in the co-IP assay had to be finished quickly assuming that the active conformations are lost with a half-life of 15 minutes, which results in the loss of 50% of the interactions every 15 minutes. Even though DmCry would be degraded *in vivo*, we could not see the degradation in cell-free extracts (*in vitro* conditions) as the lysate was kept at 4 ^{o}C during the co-IP, which supports the active conformation but slows down or totally blocks biochemical steps of DmCry degradation in a similar logic to the experiment done to calculate the half-life of the *in vivo* lit state [Ozturk et al, 2011]. Therefore, we could expose the cell free extracts with the antibody and magnetic beads to white light overnight so continuous induction of the active conformation of DmCry will allow Jet or Tim bind to DmCry while Jet or Tim binds to magnetic beads by Protein G-anti-V5 antibody interaction. The critical point was to remove the unbound fraction before the active conformation is lost during the washing steps. Therefore, we briefly spun down the beads and placed them onto a magnetic rack and removed the supernatant. Still to help with preserving the active conformation, we continued the washing steps under ambient white light (~500 lux) after first washing. The weak interaction of Tim with DmCry under dark conditions as seen in Figure 10 may make the initial substrate (Tim) available for ubiquitylation by the JET-containing E3 ligase complex as soon as Jet interacts with DmCry.

Next, we started to test whether we can obtain similar results with the newly identified interactors. Even though all interactors were expressed in the same vector, the expression levels were quite different. Therefore, we co-expressed the plasmids for the interactors with blasticidin resistance providing pCoBlast plasmid and selected the cells with blasticidin for one week to enrich the interactor expression. We have not tested interactions between DmCry and bottleneck proteins using co-IP, however, we selected 8 interactors based on their interaction with the DmCry as representatives of the interactome, potentially to draw conclusions about their roles in DmCry-related pathways. The result of interactors is further divided into four groups.

When referring to light-induced interaction, we specifically mean interactions observed exclusively under light conditions. Even though this is still a subjective classification, it helps with differentiating the ones which are functional under light

conditions. The observation of DmCry interacting with the ribosome biogenesis protein NOP53 may seem unexpected. However, considering that the human homologue of the *Drosophila* Cycle protein, BMAL1, has a non-canonical role in regulating ribosomal RNA, controlling NOP58-associated Snord118 nucleolar levels and cleavage of unique pre-rRNA intermediates [Cervantes et al, 2020], the interaction between DmCry and the ribosomal regulation system is not unprecedented. Furthermore, the identification of Polynucleotide 5'-hydroxyl-kinase NOL9 as a light-induced interactor lends support to the hypothesis of DmCry's involvement in RNA processing.

In the second group, we could see a weak interaction under dark conditions, but it could be enhanced greatly by the presence of light. In the case of the light-enhanced interaction, dFKBP59 has been previously documented to interact with Inactivation No Afterpotential D (Inad) in S2 cells or *Drosophila* head extracts [Goel et al, 2001]. InaD plays a crucial role in organizing the core components of the phototransduction pathway into a supramolecular complex [Huber 2001, Montell 2012]. The suggested functional importance of the interaction between DmCry and InaD in fly vision further supports its significance [Mazzotta et al, 2013]. Additionally, it has been proposed that calmodulin, DmCry, and InaD form a complex in the fly retina, with their interaction being strengthened by light exposure [Mazzotta et al, 2018]. On the other hand, AceCS plays a role in activating acetate for utilization in lipid synthesis or energy generation [Miao et al, 2022]. The regulatory role of miR-210 in lipid metabolism and neurodegeneration prevention in the *Drosophila* retina has been established, with Acyl-CoA synthetase identified as a target of miR-210 [Lyu et al, 2021]. Considering that genetic manipulation to reduce AceCS levels in miR210 mutants partially rescues photoreceptor degeneration defects, the interaction between DmCry and AceCS may play a role in lipid metabolism.

The observation of the third group is light-independent interactions which means Two interactors could bind DmCry under dark conditions and their affinity to DmCry could not be enhanced by light. Between DmCry with Failed axon connections protein and CKII-alpha showed that the interactions in light-induced or -enhanced groups are real light-induced ones because, in the same conditions, the interactions in the light-independent group were not enhanced by light. One can speculate that the interaction was so strong in this group that it could not be further enhanced by light non-

specifically. The protein associated with failed axon connections plays a role in embryonic axonal development [Hill et al, 1995]. Anticipated functions of CKII-alpha in the DmCry-containing photoreceptor complex stem from the recognized pivotal role of protein kinase CK2 (CKII) in the evolutionarily conserved clockwork mechanism across diverse taxonomic groups. CK2 governs the stability and nuclear localization of crucial clock proteins in mammals, fungi, and insects [Mizoguchi et al, 2006]. The CK2 holoenzyme comprises a tetrameric complex with two catalytic (α) and two interacting regulatory (β) subunits [St-Denis et al, 2009]. The β subunits stabilize the α subunits, which exhibit constitutive kinase activity. In *Drosophila*, CK2α and CK2β influence the abundance and subcellular localization of Per and Tim, aligning with the documented direct phosphorylation of both proteins by the CK2 holoenzyme in vitro [Akten et al, 2009, Smith et al, 2008, Lin et al, 2005, Akten et al, 2003, Lin et al, 2002].

The observation of no interaction in the fourth group (endoplasmic reticulum chaperone GRP78 and Ahcy) confirmed that light did not induce interactions non-specifically. In BioID results, we could see GRP78 and Ahcy proteins as possible DmCry interactors. Because we could not see these interactions at the condition that we could detect other interactions, it can be speculated that either they do not interact physically strongly, or the type of the interactions could not be detected by the co-IP protocol that we used. If there is an interaction which is dependent on the redox status of FAD, then it is possible to lose the interactions easily because the redox status of FAD can change on the minute scale. However, this minute-scale will not affect *in vivo* biotinylation because of continuous light exposure. Endoplasmic reticulum chaperone GRP78 plays a key role in protein folding and quality control in the endoplasmic reticulum lumen. It is involved in the correct folding of proteins and degradation of misfolded and acts as a key repressor of the unfolded protein response (UPR). Ahcy is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions; therefore, Ahcy may play a key role in the control of methylation via regulation of the intracellular concentration of adenosylhomocysteine. In mammals, AHCY (adenosylhomocysteinase) cyclically associates with CLOCK-BMAL1 at chromatin sites and promotes circadian transcriptional activity.

Some alternative methods such as the yeast-2-hybrid assay would be worth testing the last group (GRP78 and Ahcy) for light-dependent interactions with DmCry. However, the absence of any interaction between DmCry and Endoplasmic reticulum chaperone BiP or Adenosylhomocysteinase in the co-IP can be explained most likely due to positives of proximity-dependent labeling.

7. CONCLUSION

Our findings suggest that employing proximity-dependent labeling coupled with mass spectrometry (MS) offers a viable approach for uncovering light-dependent protein-protein interactions (PPIs). While our study utilized a 30-minute labeling protocol, the exploration of shorter labeling durations followed by MS-based identification may unveil finer mechanistic details underlying the light-dependent interactions of DmCry. Consideration of fusing the labeling enzymes to proteins such as Tim or Jet, as opposed to DmCry, holds potential for expanding the scope of light-dependent reactions, overcoming technical limitations associated with DmCry. As DmCry has been proposed as a potential magnetoreceptor, our results provide impetus for investigating potential magnetic field-induced interactions involving DmCry [Bradlaugh A et al, 2021, Gegear et al, 2010, Yoshii et al, 2009]. Regardless of the magnetoreceptor mechanism, if DmCry is implicated, it is likely to involve protein-protein interactions during the relay of the initial signal to downstream effector pathways. Given that our data represent the in vitro interactome of DmCry in S2 cells lacking most core clock components, extending this methodology to in vivo applications in *Drosophila* could yield more physiologically relevant insights. The identification of novel interactors, including those contributing to ribosomal regulation, lipid metabolism, and cytoskeletal stability, enriches our comprehension of DmCry functions. Furthermore, network analysis has pinpointed key bottleneck proteins with potential roles in circadian regulation, offering avenues for in-depth investigation. The demonstrated efficacy of proximity-dependent labeling coupled with MS in uncovering light-dependent PPIs underscores its utility, and its application in *Drosophila* in vivo holds promise for garnering additional physiological insights. Our findings also prompt the exploration of potential magnetic field-induced interactions involving DmCry, aligning with its proposed role as a magnetoreceptor.

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BIOGRAPHY

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Ozcelik G., Koca MS., Sunbul B., Yilmaz-Atay F., Demirhan F., Tiryaki B., Cilenk K., Selvi S., Ozturk N., (2024), “Interactions of drosophila cryptochrome”, Photochem Photobiol., 2024;00:1-20.

APPENDICES

APPENDIX-A: SAINT express Analysis Results

Table A1: SAINT express analysis of DmCry-APEX2_Dark data.

Bait	Prey	PreyGene	Spec	Spec Sum	AvgSpec	SaintScore	FoldChange	BFDR	Sig	Highly sig.
DmCry	O62619-2	Pyk	20|22	42	21.00	1.00	210.00	0.00	+	+
DmCry	Q8INM3	sle	16|10	26	13.00	1.00	6.50	0.00	+	+
DmCry	P36179	Pp2A-29B	8|7	15	7.50	1.00	75.00	0.00	+	+
DmCry	Q7KR04	RpS15Ab	16|12	28	14.00	1.00	140.00	0.00	+	+
DmCry	P07486	Gapdh1	6|5	11	5.50	1.00	55.00	0.00	+	+
DmCry	Q9VUQ5	AGO2	10|8	18	9.00	1.00	90.00	0.00	+	+
DmCry	O96827	eEF1beta	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	Q9V429	Trx-2	6|4	10	5.00	1.00	50.00	0.00	+	+
DmCry	P40796	La	4|6	10	5.00	1.00	50.00	0.00	+	+
DmCry	O46037	Vinc	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	O18334	Rab6	5|3	8	4.00	1.00	40.00	0.00	+	+
DmCry	P11046	LanB1	3|3	6	3.00	1.00	30.00	0.00	+	+
DmCry	O97477	Inos	10|10	20	10.00	0.99	8.00	0.00	+	+
DmCry	P40797	pnut	4|2	6	3.00	0.99	30.00	0.00	+	+
DmCry	P22058	D1	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	P17210	Khc	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q9VN50	eIF3f1	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q95RI5	fax	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q9V3G7	Rpn7	2|5	7	3.50	0.99	35.00	0.00	+	+
DmCry	Q9W3C2	CG1785	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	P54357	Mlc-c	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q23978	Myo31DF	10|6	16	8.00	0.98	8.00	0.01	+	+
DmCry	P20480	ncd	6|8	14	7.00	0.98	7.00	0.01	+	+
DmCry	Q23979	Myo61F	8|8	16	8.00	0.98	4.57	0.01	+	+
DmCry	P07764	Ald1	2|3	5	2.50	0.98	25.00	0.01	+	+
DmCry	P23572	Cdk1	2|3	5	2.50	0.98	25.00	0.01	+	+
DmCry	Q27580	Ahcy	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q94514	COX5A	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P84029	Cyt-c-p	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q7KUT2	Lon	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VP61	AcCoAS	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q24492	RpA-70	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q7PLI7	CG17528	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	A1ZA92	CG8414	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P54399	Pdi	2|2	4	2.00	0.97	20.00	0.01	+	+

Table A.1: Continued.

DmCry	Q9W1F4	thoc5	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VKN7	aurB	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VL78	Fkbp59	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q7KVQ0	CG4038	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q0KHU5	LPCAT	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VH07	pont	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VI10	SmD2	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VLU0	baf	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VD09	pinta	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P40793	Cdc42	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VGW6	Mcm5	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P25007	Cyp1	4|10	14	7.00	0.96	14.00	0.02	+	+
DmCry	A1ZAB5	clu	6|4	10	5.00	0.95	10.00	0.02	+	+
DmCry	Q27415	Nlp	4|6	10	5.00	0.95	10.00	0.02	+	+
DmCry	P35600	Gnf1	8|6	14	7.00	0.92	7.00	0.02	+	
DmCry	Q24186	RpS5a	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	Q9VBY8	stac	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	Q9VTU4	eIF3l	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	Q24368	Iswi	8|8	16	8.00	0.90	4.00	0.02	+	
DmCry	P08879	awd	8|4	12	6.00	0.89	6.00	0.02	+	
DmCry	P07909	Hrb98DE	6|4	10	5.00	0.88	5.00	0.03	+	
DmCry	Q9VLM8	AlaRS	10|6	16	8.00	0.87	4.57	0.03	+	
DmCry	Q9VHS8	CG7483	6|8	14	7.00	0.85	4.67	0.03	+	
DmCry	Q9I7S8	Paics	10|10	20	10.00	0.84	4.00	0.03	+	
DmCry	O62621	beta'COP	6|8	14	7.00	0.84	4.00	0.03	+	
DmCry	Q8IRH5	CG2199	8|6	14	7.00	0.80	3.50	0.04	+	
DmCry	Q7KLV9	Rpn6	4|4	8	4.00	0.79	4.00	0.05	+	
DmCry	Q24151	Stat92E	4|4	8	4.00	0.79	4.00	0.05	+	
DmCry	Q9VUV9	l(3)72Ab	4|2	6	3.00	0.79	6.00	0.04	+	
DmCry	Q7KNA0	CG8230	4|4	8	4.00	0.79	4.00	0.05	+	
DmCry	Q9VNE2	kra	4|4	8	4.00	0.79	4.00	0.05	+	
DmCry	P40320	Sam-S	4|2	6	3.00	0.79	6.00	0.04	+	
DmCry	Q9VFE6	CG3817	2|4	6	3.00	0.79	6.00	0.04	+	
DmCry	Q9VUR3	AIMP2	2|4	6	3.00	0.79	6.00	0.04	+	
DmCry	P46150	Moe	44|38	82	41.00	0.77	3.73	0.06	+	
DmCry	Q9VWG3	RpS10b	7|6	13	6.50	0.75	3.25	0.06	+	
DmCry	P28668	GluProRS	3|2	5	2.50	0.75	5.00	0.06	+	
DmCry	P08570	RpLP1	3|2	5	2.50	0.75	5.00	0.06	+	
DmCry	P18173	Gld	2|3	5	2.50	0.75	5.00	0.06	+	
DmCry	P92177	14-3-3epsilon	12|12	24	12.00	0.72	3.43	0.07	+	
DmCry	P36241	RpL19	6|8	14	7.00	0.71	3.50	0.07	+	

Table A.1: Continued.

DmCry	P12982	Pp1-87B	4|6	10	5.00	0.69	3.33	0.08	+	
DmCry	Q9NG98	Top3alpha	6|4	10	5.00	0.68	3.33	0.08	+	
DmCry	Q8SXY6	bai	6|6	12	6.00	0.68	3.00	0.08	+	
DmCry	Q9V595	Urod	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	Q8IH00	Mat89Ba	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	Q9VHG4	ATP6AP2	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	P08181	CkIIalpha	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	Q9VSF3	UbcE2M	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	P54351	Nsf2	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	P25160	Arl1	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	Q9VAQ5	CG11837	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	P18935	CYTB	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	P48554	Rac2	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	O76902	rush	2|2	4	2.00	0.67	4.00	0.09	+	
DmCry	Q9VIF0	CG9246	4|3	7	3.50	0.66	3.50	0.12	+	
DmCry	Q05344	Ssrp	6|6	12	6.00	0.65	3.00	0.12	+	
DmCry	Q9W5E4	Mybbp1A	6|4	10	5.00	0.63	3.33	0.12	+	
DmCry	Q9VL96	CG4364	4|6	10	5.00	0.63	3.33	0.12	+	
DmCry	P13607	Atpalpha	6|2	8	4.00	0.61	4.00	0.13	+	
DmCry	Q8T079	Ndf	19|14	33	16.50	0.57	2.87	0.13	+	
DmCry	Q9VIU7	CG10166	4|2	6	3.00	0.52	3.00	0.13	+	
DmCry	Q9VKY2	Cand1	2|4	6	3.00	0.52	3.00	0.13	+	
DmCry	Q9VAM6	Cisd2	2|4	6	3.00	0.52	3.00	0.13	+	
DmCry	Q9VMW7	CG8891	2|4	6	3.00	0.52	3.00	0.13	+	
DmCry	Q27331	Vha68-2	8|20	28	14.00	0.50	3.11	0.15	+	
DmCry	P02299	His3:CG31613	19|0	19	9.50	0.50	95.00	0.15	+	
DmCry	P48601	Rpt2	6|10	16	8.00	0.50	2.67	0.18	+	
DmCry	P20353	Galphai	7|4	11	5.50	0.50	2.75	0.20	+	
DmCry	Q9VAW5	larp	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9NFP5	Sh3beta	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9V5C6	Prosalpha7	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	O02195	eIF3i	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9VL18	eEF1delta	3|0	3	1.50	0.50	15.00	0.19	+	
DmCry	Q9VEZ3	msps	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9VP47	Tsr1	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q24179	Slh	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	P54352-2	NA	3|0	3	1.50	0.50	15.00	0.19	+	
DmCry	P48810	Hrb87F	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9VKJ3	Nup160	3|0	3	1.50	0.50	15.00	0.19	+	
DmCry	P08841	betaTub60D	0|14	14	7.00	0.50	70.00	0.15	+	

Table A.1: Continued.

DmCry	Q9V3P6	Rpn2	0|4	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9VUC6	Frl	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9V468	CG9776	4|12	16	8.00	0.49	2.91	0.20	+	
DmCry	P41073	Pep	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VLV5	SmE	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P48610	Argk	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VF87	Sra-1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P08928	Lam	2|1	3	1.50	0.49	15.00	0.20	+	
DmCry	O18404	scu	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VYV3	prtp	2|0	2	1. 00	0.49	10.00	0.20	+	
DmCry	Q24439	ATPsynO	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q94516	ATPsynB	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q8SX37	Pis	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P61851	Sod1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9W1H4	DNAlig1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P08645	Rap1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P26802	Dbp73D	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VVX5	nes	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q86BY9	rig	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9V8R9	cora	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q94517	HDAC1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9V7N5	Vha44	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VWE6	Elys	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q7KMJ6	nito	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P20477	Gs1	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VXK0	Nipsnap	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VMQ7	CG6907	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VLC0	scat	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	P22468	gdl	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9W5N2	RpL38	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VJJ7	Trpgamma	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9W058	SCOT	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	O97394	sdk	2|0	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VMD9	Tig	1|2	3	1.50	0.49	15.00	0.20	+	
DmCry	A1Z6J5	Tbce	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VAH9	IntS11	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P34739	lds	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P40417	rl	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P22465	AnxB10	0|2	2	1.00	0.49	10.00	0.20	+	

Table A.1: Continued.

DmCry	Q9VCA8	mask	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9GQQ0	spin	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	O77059	cry	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VQ62	Npc2a	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P17719	Dhfr	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VSS2	Srp68	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q8IQ56	Pmi	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VKK1	Ge-1	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VCF0	CG5902	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VH95	p23	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	A1Z7T0	Pkn	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VMX1	CG14043	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q00637	Sod2	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P40304	Prosbeta6	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P91679	yin	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P17336	Cat	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VKM6	YL-1	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	A1Z9L3	pea	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VWE9	Sec61gamma	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9V4Q8	U2A	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	A1Z7A8	coil	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q9VRJ2	Fitm	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	O76324	dco	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q8T3X9	MagR	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	Q24292	ds	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	P42207	Sep1	0|2	2	1.00	0.49	10.00	0.20	+	
DmCry	O18373	Sps1	3|1	4	2.00	0.48	8.00	0.31	+	
DmCry	Q94518	Nacalpha	0|3	3	1.50	0.48	6.00	0.31	+	
DmCry	Q7K7A9	Fen1	0|6	6	3.00	0.48	3.00	0.31	+	
DmCry	O77460	Nurf-38	4|4	8	4.00	0.47	2.67	0.32	+	
DmCry	P04359	RpL32	4|4	8	4.00	0.47	2.67	0.32	+	
DmCry	P39018	RpS19a	4|10	14	7.00	0.46	2.33	0.32	+	
DmCry	Q9VEX6	bor	4|4	8	4.00	0.46	2.67	0.32	+	
DmCry	P25161	Rpn3	4|0	4	2.00	0.46	4.00	0.32	+	
DmCry	Q9VTP4	RpL10Ab	8|10	18	9.00	0.45	2.57	0.32	+	
DmCry	Q9VBX1	Clbn	0|6	6	3.00	0.45	2.00	0.32	+	
DmCry	Q99323	zip	0|5	5	2.50	0.45	3.33	0.32	+	

Table A2: SAINT express analysis of DmCry-APEX2_Light data.

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	SaintScore	FoldChange	BFDR	sig.	highly sig.
DmCry	P29844	Hsc70-3	30|31	61	30.50	1.00	2.98	0.00	+	+
DmCry	Q9VLM8	AlaRS	12|12	24	12.00	1.00	6.86	0.00	+	+
DmCry	P08841	betaTub60D	10|12	22	11.00	1.00	110.00	0.00	+	+
DmCry	P25007	Cyp1	8|10	18	9.00	1.00	18.00	0.00	+	+
DmCry	P36179	Pp2A-29B	5|5	10	5.00	1.00	50.00	0.00	+	+
DmCry	O46037	Vinc	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P40304	Prosbeta6	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P02299	His3:CG31613	12|18	30	15.00	1.00	150.00	0.00	+	+
DmCry	Q9VUQ5	AGO2	5|6	11	5.50	1.00	55.00	0.00	+	+
DmCry	P40796	La	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	Q9V3P6	Rpn2	3|3	6	3.00	1.00	30.00	0.00	+	+
DmCry	P46863	Klp61F	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P18489	Syb	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P17336	Cat	6|2	8	4.00	0.99	40.00	0.00	+	+
DmCry	Q9VL78	Fkbp59	6|2	8	4.00	0.99	40.00	0.00	+	+
DmCry	P07764	Ald1	6|2	8	4.00	0.99	40.00	0.00	+	+
DmCry	P17210	Khc	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q9V429	Trx-2	4|2	6	3.00	0.99	30.00	0.00	+	+
DmCry	P40797	pnut	2|5	7	3.50	0.99	35.00	0.00	+	+
DmCry	Q94516	ATPsynB	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q9VN50	eIF3f1	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	P08928	Lam	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	P22058	D1	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	P48555	Rala	2|4	6	3.00	0.99	30.00	0.00	+	+
DmCry	Q99323-4	NA	29|28	57	28.50	0.98	6.71	0.01	+	+
DmCry	Q23978	Myo31DF	8|6	14	7.00	0.98	7.00	0.01	+	+
DmCry	P20480	ncd	6|8	14	7.00	0.98	7.00	0.01	+	+
DmCry	O97477	Inos	8|10	18	9.00	0.98	7.20	0.01	+	+
DmCry	Q9V3G7	Rpn7	2|3	5	2.50	0.98	25.00	0.01	+	+
DmCry	Q9VMT5	Cyp28d1	3|2	5	2.50	0.98	25.00	0.01	+	+
DmCry	Q27580	Ahcy	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P80455	RpS12	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q7PLI7	CG17528	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VYV3	prtp	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	A1Z6J5	Tbce	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9V8M5	CG15093	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q24439	ATPsynO	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P41073	Pep	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VAW5	larp	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VD09	pinta	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VEZ3	msps	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P40793	Cdc42	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	A1Z7T0	Pkn	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P54399	Pdi	2|2	4	2.00	0.97	20.00	0.01	+	+

Table A.2: Continued.

DmCry	P54359	Sep2	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q00637	Sod2	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P11046	LanB1	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VWG3	RpS10b	9|8	17	8.50	0.96	4.25	0.02	+	+
DmCry	P46150	Moe	46|45	91	45.50	0.95	4.14	0.02	+	+
DmCry	P08181	CkIIalpha	6|4	10	5.00	0.95	10.00	0.02	+	+
DmCry	Q24186	RpS5a	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	P28668	GluProRS	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	Q9VUR3	AIMP2	4|4	8	4.00	0.92	8.00	0.02	+	
DmCry	Q9W5E4	Mybbp1A	12|6	18	9.00	0.89	6.00	0.02	+	
DmCry	Q23979	Myo61F	8|6	14	7.00	0.89	4.00	0.02	+	
DmCry	P35600	Gnf1	6|6	12	6.00	0.88	6.00	0.02	+	
DmCry	Q9VCK0	eIF3d1	5|8	13	6.50	0.88	4.33	0.03	+	
DmCry	Q9VMW7	CG8891	4|6	10	5.00	0.87	5.00	0.03	+	
DmCry	P08879	awd	6|4	10	5.00	0.87	5.00	0.03	+	
DmCry	P07909	Hrb98DE	4|6	10	5.00	0.87	5.00	0.03	+	
DmCry	P20432	GstD1	4|3	7	3.50	0.87	7.00	0.03	+	
DmCry	Q9VEN1	cher	87|90	177	88.50	0.86	2.62	0.03	+	
DmCry	Q94518	Nacalpha	2|2	4	2.00	0.85	8.00	0.04	+	
DmCry	Q9VIF0	CG9246	5|4	9	4.50	0.84	4.50	0.04	+	
DmCry	P92177	14-3-3epsilon	16|12	28	14.00	0.82	4.00	0.04	+	
DmCry	Q9XZJ4	Prosalpha1	8|8	16	8.00	0.81	4.00	0.04	+	
DmCry	O77460	Nurf-38	6|6	12	6.00	0.80	4.00	0.04	+	
DmCry	Q8INM3	sle	6|8	14	7.00	0.79	3.50	0.05	+	
DmCry	Q9V9X4	CG11334	4|2	6	3.00	0.79	6.00	0.05	+	
DmCry	P25161	Rpn3	4|2	6	3.00	0.79	6.00	0.05	+	
DmCry	P40320	Sam-S	4|2	6	3.00	0.79	6.00	0.05	+	
DmCry	Q24133	DnaJ-1	2|4	6	3.00	0.79	6.00	0.05	+	
DmCry	Q9VN25	eIF3a	14|14	28	14.00	0.76	2.80	0.06	+	
DmCry	P15007	Eno	10|10	20	10.00	0.75	3.33	0.06	+	
DmCry	Q9I7S8	Paics	10|8	18	9.00	0.74	3.60	0.07	+	
DmCry	Q7JVI3	eIF3m	2|3	5	2.50	0.74	5.00	0.06	+	
DmCry	Q8IH00	Mat89Ba	2|3	5	2.50	0.74	5.00	0.06	+	
DmCry	Q9VUV9	l(3)72Ab	2|3	5	2.50	0.74	5.00	0.06	+	
DmCry	P08570	RpLP1	2|3	5	2.50	0.74	5.00	0.06	+	
DmCry	Q94522	Scsalpha1	10|8	18	9.00	0.71	3.00	0.08	+	
DmCry	P39018	RpS19a	8|10	18	9.00	0.71	3.00	0.08	+	
DmCry	P36241	RpL19	8|6	14	7.00	0.70	3.50	0.08	+	
DmCry	O62621	beta'COP	7|5	12	6.00	0.70	3.43	0.09	+	
DmCry	P12881	Prosalpha6	8|6	14	7.00	0.70	3.50	0.08	+	
DmCry	P54622	mtSSB	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q3LHL9	wge	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q9VIW7	RtcB	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q9VKY2	Cand1	3|4	7	3.50	0.66	3.50	0.12	+	

Table A.2: Continued.

DmCry	P54351	Nsf2	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q9VHG4	ATP6AP2	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	A1ZAB5	clu	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q27415	Nlp	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	P48592	RnrS	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q9VTU4	eIF3l	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	P48554	Rac2	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q6J5K9	armi	2|2	4	2.00	0.66	4.00	0.09	+	
DmCry	Q8IRH5	CG2199	5|8	13	6.50	0.65	3.25	0.12	+	
DmCry	Q7K7A9	Fen1	6|2	8	4.00	0.60	4.00	0.12	+	
DmCry	Q8MQJ9	brat	2|2	4	2.00	0.54	2.67	0.13	+	
DmCry	P25171	Rcc1	4|2	6	3.00	0.51	3.00	0.13	+	
DmCry	Q9VNE2	kra	4|2	6	3.00	0.51	3.00	0.13	+	
DmCry	Q9VAM6	Cisd2	2|4	6	3.00	0.51	3.00	0.13	+	
DmCry	P82712	Cyp12d1-p	6|0	6	3.00	0.50	30.00	0.14	+	
DmCry	Q95RI5	fax	6|0	6	3.00	0.50	30.00	0.14	+	
DmCry	O18334	Rab6	4|0	4	2.00	0.50	20.00	0.16	+	
DmCry	Q9VGK7	Elp1	3|0	3	1.50	0.50	15.00	0.16	+	
DmCry	Q9VL18	eEF1delta	1|3	4	2.00	0.50	20.00	0.16	+	
DmCry	Q99323	zip	0|55	55	27.50	0.50	36.67	0.14	+	
DmCry	P07486	Gapdh1	0|6	6	3.00	0.50	30.00	0.14	+	
DmCry	Q9VKK1	Ge-1	0|5	5	2.50	0.50	25.00	0.15	+	
DmCry	Q9GSR1	DNApol-zeta	0|3	3	1.50	0.50	15.00	0.16	+	
DmCry	P45888-2	NA	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P84029	Cyt-c-p	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q94514	COX5A	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VCA8	mask	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P48610	Argk	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VLU0	baf	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	A1Z713	Vps13	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VMQ7	CG6907	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VSS2	Srp68	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VP47	Tsr1	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P32392	Arp3	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P54357	Mlc-c	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9U1H9	sbr	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VH07	pont	2|1	3	1.50	0.49	15.00	0.17	+	
DmCry	P48598	eIF4E1	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9W1H4	DNAlig1	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P26802	Dbp73D	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P42207	Sep1	2|0	2	1.00	0.49	10.00	0.17	+	

Table A.2: Continued.

DmCry	O96860	Cdlc2	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9XZU1	Cse1	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VCN1	DNApol-epsilon255	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VXK0	Nipsnap	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q7KVQ0	CG4038	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VCF0	CG5902	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P38040	Ggamma1	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VM75	l(2)k09022	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	P13496	DCTN1-p150	1|2	3	1.50	0.49	15.00	0.17	+	
DmCry	Q8T3X9	MagR	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VYD1	Set2	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VKX7	gny	2|0	2	1.00	0.49	10.00	0.17	+	
DmCry	O76902	rush	0|6	6	3.00	0.49	6.00	0.17	+	
DmCry	Q86BY9	rig	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9W4X9	Zw10	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9W252	rad50	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9V492	CG11077	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	P91938	Trxr-1	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9W1F4	thoc5	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9U915	Adk2	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	P34739	lds	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q04047	nonA	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q24179	Slh	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VGW6	Mcm5	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9V5C6	Prosalpha7	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VRJ2	Fitm	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	P22465	AnxB10	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	A1YK02	Nup75	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	A0A0B4K7J2	Nup358	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VQ62	Npc2a	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VFM9	twf	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9V9K7	Ars2	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	P08182	CkIIbeta	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VA73	aralar1	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9VPH7	eRF1	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q8IN94	osa	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	A1Z6M6	Dpit47	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q9W1C9	EbpIII	0|2	2	1.00	0.49	10.00	0.17	+	

Table A.2: Continued.

DmCry	P18091	Actn	0|2	2	1.00	0.49	10.00	0.17	+	
DmCry	Q8IQV9	Nup205	0|5	5	2.50	0.48	5.00	0.29	+	
DmCry	P54352	eas	8|8	16	8.00	0.47	2.67	0.29	+	
DmCry	P37276	Dhc64C	3|5	8	4.00	0.47	2.67	0.29	+	
DmCry	O02373	sgl	4|4	8	4.00	0.46	2.67	0.29	+	
DmCry	O18388	Fs(2)Ket	4|4	8	4.00	0.46	2.67	0.29	+	
DmCry	Q0E940	eIF3b	0|4	4	2.00	0.46	4.00	0.29	+	
DmCry	Q9VIM5	Arpc2	4|4	8	4.00	0.45	2.67	0.29	+	
DmCry	Q9VBX1	Clbn	4|4	8	4.00	0.45	2.67	0.29	+	

Table A3: SAINT express analysis of DmCry-TID_Dark data.

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	SaintScore	FoldChange	BFDR	Sig.	highly sig.
DmCry	P08841	betaTub60D	8|10	18	9.00	1.00	90.00	0.00	+	+
DmCry	Q9V463	Nup154	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P20353	Galphai	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	Q9VUV9	l(3)72Ab	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	P40796	La	2|4	6	3.00	0.98	30.00	0.00	+	+
DmCry	Q9TVM2	emb	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	P17886	crn	2|4	6	3.00	0.98	30.00	0.00	+	+
DmCry	O18334	Rab6	2|3	5	2.50	0.97	25.00	0.01	+	+
DmCry	P29413	Calr	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q9V3P0	Jafrac1	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q8IH00	Mat89Ba	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q9XY35	ox	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	P07764	Ald1	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q9VGR7	CG6693	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q9VCK0	eIF3d1	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q7PLI7	CG17528	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q9VNV3	Ddx1	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q7KW39	CG17896	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	Q8SWU7	CG1354	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	A1Z7T0	Pkn	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	P08570	RpLP1	2|2	4	2.00	0.95	20.00	0.02	+	+
DmCry	P25007	Cyp1	4|6	10	5.00	0.92	10.00	0.03	+	
DmCry	P91926	AP-2alpha	4|4	8	4.00	0.87	8.00	0.04	+	
DmCry	Q24186	RpS5a	4|4	8	4.00	0.87	8.00	0.04	+	
DmCry	Q02645	hts	4|2	6	3.00	0.70	6.00	0.04	+	
DmCry	Q9NG98	Top3alpha	4|2	6	3.00	0.70	6.00	0.04	+	
DmCry	Q9VBX1	Clbn	2|4	6	3.00	0.70	6.00	0.04	+	
DmCry	Q9VUR3	AIMP2	4|2	6	3.00	0.70	6.00	0.04	+	
DmCry	Q95029	Cp1	2|4	6	3.00	0.70	6.00	0.04	+	
DmCry	Q9VMW8	CG3792	2|4	6	3.00	0.70	6.00	0.04	+	
DmCry	P54367	CkIalpha	4|4	8	4.00	0.68	4.00	0.10	+	
DmCry	Q9VPQ2	shv	8|8	16	8.00	0.66	3.20	0.10	+	
DmCry	P36179	Pp2A-29B	6|4	10	5.00	0.63	4.00	0.11	+	
DmCry	P48591	RnrL	2|6	8	4.00	0.55	4.00	0.12	+	
DmCry	Q86BY9	rig	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	P49858	cni	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	P40797	pnut	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q94516	ATPsynB	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q24151	Stat92E	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	P40304	Prosbeta6	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	A1ZAB5	clu	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q9VSF3	UbcE2M	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q9VP47	Tsr1	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q27415	Nlp	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q24133	DnaJ-1	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q24325	Taf2	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q9NBK5	trc	2|2	4	2.00	0.53	4.00	0.13	+	
DmCry	Q9VLT5	poe	3|4	7	3.50	0.53	3.50	0.22	+	

Table A.3: Continued.

DmCry	P61857	betaTub85D	16|0	16	8.00	0.50	80.00	0.24	+	
DmCry	P28668	GluProRS	4|0	4	2.00	0.50	20.00	0.24	+	
DmCry	Q9V3G7	Rpn7	3|0	3	1.50	0.50	15.00	0.25	+	
DmCry	Q6J5K9	armi	3|0	3	1.50	0.50	15.00	0.25	+	
DmCry	P10987	Act5C	0|26	26	13.00	0.50	130.00	0.23	+	
DmCry	P05303	eEF1alpha2	0|24	24	12.00	0.50	120.00	0.23	+	
DmCry	O18405	Surf4	0|4	4	2.00	0.50	20.00	0.24	+	
DmCry	Q8SX83	spen	0|3	3	1.50	0.50	15.00	0.25	+	
DmCry	Q9VKD3	Nfs1	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	O02373	sgl	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9GQN5	XNP	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VKN7	aurB	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	P17210	Khc	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VAH9	IntS11	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	O16810	Orc1	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VKK1	Ge-1	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VCA8	mask	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9GU68	eEF5	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q09332	Uggt	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VWE6	Elys	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	P40320	Sam-S	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	Q7JV70	Arc2	2|0	2	1.00	0.48	10.00	0.27	+	
DmCry	O02193	mof	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q24562	U2af50	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9W1H4	DNAlig1	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VKV8	Bug22	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q24592	hop	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VL78	Fkbp59	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VIW7	RtcB	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VX91	Ubr1	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VDG2	Rlip	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P08928	Lam	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P48592	RnrS	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P40793	Cdc42	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P46863	Klp61F	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VGN9	RpL24-like	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P51140	dsh	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	P05389	RpLP2	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q95RI5	fax	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VAQ5	CG11837	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VFE6	CG3817	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VW09	Ltn1	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9VH89	dmt	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q95RJ9	ebi	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q8IRB5	Claspin	0|2	2	1.00	0.48	10.00	0.27	+	
DmCry	Q9NFU0-2	NA	4|3	7	3.50	0.46	2.80	0.37	+	
DmCry	Q9XZJ4	Prosalpha1	4|2	6	3.00	0.46	3.00	0.37	+	

Table A4: SAINT express analysis of DmCry-TID_Light data.

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	SaintScore	FoldChange	BFDR	sig	highly sig
DmCry	P10987	Act5C	38|36	74	37.00	1.00	370.00	0.00	+	+
DmCry	Q9VI82	CG1234	28|26	54	27.00	1.00	4.15	0.00	+	+
DmCry	Q9VUV9	l(3)72Ab	16|8	24	12.00	1.00	120.00	0.00	+	+
DmCry	P08841	betaTub60D	14|18	32	16.00	1.00	160.00	0.00	+	+
DmCry	Q24368	Iswi	11|11	22	11.00	1.00	11.00	0.00	+	+
DmCry	Q9V463	Nup154	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	Q9GQN5	XNP	6|6	12	6.00	1.00	60.00	0.00	+	+
DmCry	P20353	Galphai	6|4	10	5.00	1.00	50.00	0.00	+	+
DmCry	Q8IH00	Mat89Ba	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	A1Z7T0	Pkn	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P08928	Lam	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	P40796	La	4|4	8	4.00	1.00	40.00	0.00	+	+
DmCry	A1ZA92	CG8414	12|7	19	9.50	0.99	9.50	0.00	+	+

Table A.4: Continued.

DmCry	P54352	eas	6|8	14	7.00	0.98	7.00	0.01	+	+
DmCry	Q86B47	CG8611	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	Q9TVM2	emb	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	Q9W3C2	CG1785	6|2	8	4.00	0.98	40.00	0.00	+	+
DmCry	P07764	Ald1	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	Q7KW39	CG17896	4|2	6	3.00	0.98	30.00	0.00	+	+
DmCry	Q8IQV9	Nup205	2|5	7	3.50	0.98	35.00	0.00	+	+
DmCry	P46863	Klp61F	2|3	5	2.50	0.98	25.00	0.01	+	+
DmCry	P29413	Calr	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P32392	Arp3	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VNV3	Ddx1	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q7PLI7	CG17528	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P25439	brm	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VAH9	IntS11	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VGN9	RpL24-like	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VR59	l(1)G0196	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9W1F4	thoc5	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P28668	GluProRS	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VKN7	aurB	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VKM6	YL-1	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VBY8	stac	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VH07	pont	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P13496	DCTN1-p150	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	Q9VCA8	mask	2|2	4	2.00	0.97	20.00	0.01	+	+
DmCry	P35600	Gnf1	6|6	12	6.00	0.96	6.00	0.02	+	+
DmCry	Q9W3C1	l(1)G0020	26|35	61	30.50	0.95	4.07	0.02	+	+
DmCry	Q02645	hts	6|4	10	5.00	0.95	10.00	0.02	+	+
DmCry	Q9VWG3	RpS10b	5|9	14	7.00	0.95	7.00	0.02	+	+
DmCry	Q9VUM0	Msh6	12|15	27	13.50	0.92	3.86	0.02	+	
DmCry	Q9VM75	l(2)k09022	4|2	6	3.00	0.92	12.00	0.02	+	
DmCry	Q24186	RpS5a	4|4	8	4.00	0.91	8.00	0.02	+	
DmCry	P20480	ncd	4|4	8	4.00	0.91	8.00	0.02	+	
DmCry	P32234	128up	4|4	8	4.00	0.91	8.00	0.02	+	
DmCry	Q9W0T1	E(bx)	2|3	5	2.50	0.90	10.00	0.03	+	
DmCry	Q9V411	Non1	17|18	35	17.50	0.89	2.92	0.03	+	
DmCry	Q9VPC0	Pitslre	6|6	12	6.00	0.89	4.00	0.03	+	
DmCry	P55162	Hem	4|8	12	6.00	0.88	6.00	0.03	+	
DmCry	Q7K2B0	CG7137	4|6	10	5.00	0.87	5.00	0.03	+	
DmCry	Q9V460	Spt5	9|10	19	9.50	0.84	3.17	0.04	+	
DmCry	Q9NFU0-2	NA	5|8	13	6.50	0.84	5.20	0.04	+	
DmCry	Q8SXY6	bai	5|4	9	4.50	0.83	4.50	0.04	+	
DmCry	Q23978	Myo31DF	10|8	18	9.00	0.82	3.27	0.04	+	
DmCry	P91926	AP-2alpha	2|6	8	4.00	0.81	8.00	0.05	+	
DmCry	Q9VEB3	Non3	22|14	36	18.00	0.79	3.43	0.05	+	
DmCry	P25007	Cyp1	4|2	6	3.00	0.78	6.00	0.05	+	
DmCry	A1ZAB5	clu	2|4	6	3.00	0.78	6.00	0.05	+	
DmCry	Q7KU24	Chd1	2|4	6	3.00	0.78	6.00	0.05	+	
DmCry	P25843	chic	4|4	8	4.00	0.77	4.00	0.06	+	
DmCry	Q9VLT5	poe	4|4	8	4.00	0.73	4.00	0.07	+	
DmCry	Q7K4H4	NO66	2|3	5	2.50	0.73	5.00	0.06	+	
DmCry	P36179	Pp2A-29B	6|4	10	5.00	0.72	4.00	0.07	+	
DmCry	Q9VEX9	Bin1	3|3	6	3.00	0.70	4.00	0.07	+	

Table A.4: Continued.

DmCry	Q9VN25	eIF3a	11|13	24	12.00	0.68	3.20	0.08	+	
DmCry	Q9V468	CG9776	2|4	6	3.00	0.67	4.00	0.08	+	
DmCry	Q23979	Myo61F	6|4	10	5.00	0.66	3.33	0.08	+	
DmCry	A8JUV0	sno	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q9VMW8	CG3792	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q27333	Alg3	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q24325	Taf2	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q27415	Nlp	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q94516	ATPsynB	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q9VUQ5	AGO2	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	P25161	Rpn3	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q9VEJ2	l(3)07882	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q9VUR3	AIMP2	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q24439	ATPsynO	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	Q9V429	Trx-2	2|2	4	2.00	0.64	4.00	0.09	+	
DmCry	P22769	Prosalpha4	4|6	10	5.00	0.61	3.33	0.13	+	
DmCry	Q9VAW5	larp	2|6	8	4.00	0.60	4.00	0.13	+	
DmCry	P12982	Pp1-87B	5|7	12	6.00	0.56	3.00	0.13	+	
DmCry	P27619	shi	10|9	19	9.50	0.55	2.71	0.14	+	
DmCry	Q7KR04	RpS15Ab	14|0	14	7.00	0.50	70.00	0.14	+	
DmCry	O18405	Surf4	4|0	4	2.00	0.50	20.00	0.15	+	
DmCry	Q07327	Rop	4|2	6	3.00	0.50	3.00	0.16	+	
DmCry	Q9XZ06	Nup93-1	4|2	6	3.00	0.50	3.00	0.16	+	
DmCry	P22813	Hsf	3|0	3	1.50	0.50	15.00	0.17	+	
DmCry	Q9VL72	Etl1	2|4	6	3.00	0.50	3.00	0.16	+	
DmCry	Q9VMN5	Hsp60C	0|4	4	2.00	0.50	20.00	0.15	+	
DmCry	Q7JVI3	eIF3m	0|6	6	3.00	0.50	30.00	0.15	+	
DmCry	Q9VCK0	eIF3d1	0|4	4	2.00	0.50	20.00	0.15	+	
DmCry	Q9V3H2	Rpn11	0|3	3	1.50	0.50	15.00	0.17	+	
DmCry	Q9VXK6	eIF5	10|14	24	12.00	0.48	2.67	0.30	+	
DmCry	Q9VPH7	eRF1	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9NHD5	san	2|1	3	1.50	0.48	15.00	0.18	+	
DmCry	Q7K2G1	Rpn13	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q7KUT2	Lon	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q960W6	CG8306	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9NEF6	Vha36-3	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q24154	RpL29	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	P48592	RnrS	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VGC7	eIF3d2	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VR89	l(1)G0004	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VIU7	CG10166	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	O77277	Torsin	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	P18935	CYTB	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9V3P6	Rpn2	2|1	3	1.50	0.48	15.00	0.18	+	
DmCry	Q9VMX1	CG14043	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	P49071	MAPk-Ak2	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	P40795	fog	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q8T8R1	CNBP	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9W5N2	RpL38	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	P36188	wupA	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q7PLS8	IntS3	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VFE6	CG3817	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q8MT80	PIG-Z	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	Q24592	hop	2|0	2	1.00	0.48	10.00	0.18	+	
DmCry	O02373	sgl	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q7KNA0	CG8230	0|5	5	2.50	0.48	5.00	0.30	+	

Table A.4: Continued.

DmCry	Q9VKV8	Bug22	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q24319	Ost48	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9XY35	ox	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9W1H4	DNAlig1	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9W4X9	Zw10	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q7KLV9	Rpn6	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q8SWU7	CG1354	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	P08645	Rap1	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	O46037	Vinc	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q24168	Orc2	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	P17210	Khc	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9W2F2	mahj	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q7K1V5	jagn	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	P17886	crn	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9Y0Y2	AdSS	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VH95	p23	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VI10	SmD2	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q24595	Xpc	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q26454	dpa	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q95RJ9	ebi	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VCU9	Dcr-1	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9V3G7	Rpn7	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	P38040	Ggamma1	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VS05	bin	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	O16102	Chd3	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9XTM1	Sec10	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q6J5K9	armi	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	O16810	Orc1	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	Q9VKJ3	Nup160	0|2	2	1.00	0.48	10.00	0.18	+	
DmCry	P08879	awd	6|2	8	4.00	0.47	2.67	0.30	+	
DmCry	Q9U5L1	SrpRalpha	4|0	4	2.00	0.46	4.00	0.31	+	
DmCry	O62621	beta'COP	5|3	8	4.00	0.46	2.67	0.31	+	
DmCry	Q9V5P6	CG7637	1|4	5	2.50	0.46	5.00	0.31	+	
DmCry	Q9VL18	eEF1delta	4|0	4	2.00	0.46	4.00	0.31	+	
DmCry	P13709	fs(1)h	0|4	4	2.00	0.46	4.00	0.31	+	
DmCry	Q9VKB4	CG6712	0|5	5	2.50	0.45	2.50	0.31	+	