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CICLO XXV

Fly Cryptochrome and the Visual System

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ABSTRACT

Cryptochromes are flavoproteins, structurally and evolutionarily related to photolyases, involved in development, magnetoreception and temporal organization of a variety of organisms. Drosophila cryptochrome (dCRY) mediates light synchronization of the master circadian clock, and is an integral component of circadian clocks in fly's peripheral tissues, where it works as a transcriptional repressor. The C-terminus of dCRY plays an important role in modulating light sensitivity and activity of the protein. The activation of dCRY by light requires a conformational change, but it has been suggested that it could be mediated also by specific “regulators” that bind the C-terminus of the protein. This region harbors several protein-protein interaction motifs, likely relevant for the regulation of signal transduction.

Linear motifs (LMs) are short sequences (4-5 amino acidic residues long) evolved to mediate molecular (protein-protein) interactions. Often, LMs are present in disordered non-globular protein regions with a particular preference for protein C-terminus localization. LMs have the tendency to be unstable over long evolutionary distances and, within distinct non-globular regions, it can be hypothesized that they might evolve by “jumping” between different sequence positions. Some functional linear motifs are evolutionarily conserved in the C-terminus of cryptochromes and, specifically, class III PDZ binding sites are selectively maintained in animals. A co-immunoprecipitation assay followed by mass spectrometry analysis has revealed that dCRY interacts with Retinal DeGeneration A (RDGA) and Neither Inactivation Nor Afterpotential C (NINAC). Both proteins belong to a multi-protein complex (the Signalplex) that includes visual signaling molecules. In this work, using bioinformatic and molecular approaches, it has been found that dCRY interacts with proteins of the visual cascade through INAD (Inactivation No Afterpotential D) and that the CRY-INAD interaction, mediated by specific domains of the two proteins, is light-dependent. Moreover, an impairment of the visual behavior in flies mutants for dCRY was detected, indicating a role, direct or indirect, for this photoreceptor in fly vision.

RIASSUNTO

I criptocromi sono flavoproteine, strutturalmente ed evolutivamente legate alle fotoliasi, coinvolte nello sviluppo, magnetorecezione e organizzazione temporale di una varietà di organismi. Il criptocromo di Drosofila (dCRY) media la sincronizzazione luminosa dell'orologio circadiano principale ed inoltre è un componente fondamentale degli orologi circadiani nei tessuti periferici dell'insetto dove agisce come repressore trascrizionale.

Il C-terminale di dCRY ha un ruolo importante nel modulare la sensibilità alla luce e l'attività della proteina. L'attivazione di dCRY attraverso la luce richiede un cambiamento conformazionale, ma è stato ipotizzato che può essere mediata anche da specifici "regolatori" che si legano al suo C-terminale. Questa regione contiene diversi domini di interazione proteina-proteina probabilmente rilevanti per la regolazione della trasduzione del segnale. Alcuni motivi lineari sono evolutivamente conservati nel C-terminale dei criptocromi e siti di legame per i motivi PDZ di classe III sono particolarmente conservati negli animali. Esperimenti di co-immunoprecipitazione seguiti da analisi di spettrometria di massa hanno mostrato che dCRY interagisce con due proteine: Retinal DeGeneration A (RDGA) e Neither Inactivation Nor Afterpotential C (NINAC). Entrambe queste proteine appartengono ad un complesso multipeptidico (il Signalplex) che include anche molecole della cascata visiva. In questo lavoro, utilizzando un approccio sia bioinformatico che molecolare si è scoperto che dCRY interagisce con le proteine della cascata visiva attraverso Inactivation No Afterpotential D (INAD) e che l'interazione dCRY-INAD, mediata da specifici domini nelle due proteine, è luce dipendente. Si è osservato che esiste un impedimento visivo nelle mosche mutanti per dCRY, il che indica un ruolo, diretto od indiretto, di questo fotorecettore nella visione delle mosche.

INTRODUCTION

1.0 CIRCADIAN CLOCKS

1.1 Introduction to Circadian Clocks

Organisms from cyanobacteria to mammals and throughout the whole nature kingdom have the capacity to anticipate daytime and consequently organize their physiology and metabolism to get a competitive advantage. This capacity derives from particular molecular oscillators called: “circadian clocks” (“*circa diem*” or “about a day”). Circadian clocks exhibit an oscillatory period of about 24 hours and they are constantly synchronized to the geophysical time by environmental factors. In almost all the investigated organisms, light is the dominant synchronizing agent or “time giver” (*Zeitgeber*), entraining the phase of circadian oscillators.
We, as human beings, have a strong perception of these internal clocks when we travel across time zones or twice a year, when we re-synchronize for daylight savings. For a short period we recognize that common habits, such as lunch or sleep times are slightly shifted. This condition persists for a brief period until we re-adapt to the new routine.
The intuition of the presence of circadian clocks is ancient (Androsthenes, 400 BC), but the first scientific study ever reported was performed only in the XVIII century when a French geophysicist, Jean-Jacques de Mairan, in his “*Observation Botanique*” dated 1729 [de Mairan, 1729], studied the daily cycle of leaf opening and closing. He observed that by placing the plants in his dark wine cellar, imposing then constant darkness (DD) phase, the leaves kept the opening/closing rhythm. He intuited the presence of an internal clock driving this mechanism, which works even by removing the environmental stimuli (*free running*).

In the model organism *Drosophila melanogaster* for example, adults eclose from pupals in the early morning, taking advantage of the higher moisture and lower temperature and thus minimizing the risk of desiccation. Pioneering experiments on this topic were performed by Colin Pittendrigh in the mid 1950’s [Pittendrigh, 1954] showing that *Drosophila*’s eclosing clock has a 24 hours period. He found out that this clock can be reset by a light input during the darkness phase and the rhythm is temperature independent (temperature compensated) within a physiologic range.

Seymour Benzer and his student Ron Konopka at the beginning of 1970s gave the first molecular look inside the circadian clock, via *Drosophila* chemical mutagenesis

[Konopka and Benzer, 1971]. In their screening they found insects with a longer eclosion period (29 hours), flies displaying a shorter period (19 hours), and flies not showing any rhythm at all. The gene locus in which the mutation occurred was the same for all the mutants and located in the X-chromosome. They designated this locus as "*Period"* and consequently they got mutants respectively: "*period long*", "*period short*" and "*period-01*". Since this first study, the controlling mechanism beyond circadian clocks has been deeply investigated and expanded in several model organisms such as: fungi, cyanobacteria, zebra fish and mice.

Drosophila melanogaster is currently particularly well suited for circadian system investigation [Peschel and Helfrich-Förster, 2011], for the following reasons:

- *Drosophila's* genetics is so far one of the most studied and many molecular and biochemical techniques are available to understand complex molecular networks and oscillation mechanisms.
- The fruit fly exhibits many different and easily scalable circadian patterns of behavior to challenge: Locomotor activity above all, but also eclosion rhythm, olfactory sensitivity, egg laying, gustatory sensitivity, learning and memory capacity.
- The simplicity of *Drosophila's* neuronal network organization, with just 150 cells expressing clock genes per brain hemisphere, is easy to ménage and allows an easy tissue dissection or single cell isolation.

1.2 Molecular Insights into Circadian Clock Functioning

1.2.1 First Molecular System: Transcription-Translation Feedback Loop (TTFL)

At the current knowledge status, the principle of transcription-translation feedback loop (TTFL) is considered as a universal building block among circadian oscillators. In its general features, it has been identified in all the model organisms studied so far [Brown *et al.*, 2012] (Figure 1).
In *Drosophila*, the TTFL has been extensively studied and it is now modeled around two feedback loops [Peschel and Helfrich-Förster, 2011]:

1) *Period* (PER) and *Timeless* (TIM) feedback loop:

The loop starts with *per* and *tim* expression. Their expression is regulated primarily by a heterodimer of two trans-activator partners: *Clock* (CLK) and *Cycle* (CYC). CLK and CYC display a basic-helix-loop-helix/PAS domain that binds to E-box regulatory elements in a time frame ranging from mid-day to early night. The CLK-CYC heterodimer activates *per* and *tim* transcription: mRNAs for both genes peak early in the evening, while protein levels reach their maximum at late night. Two protein kinases called, *Double Time* (DBT) and casein Kinase 2 (CK2) act on PER and modulate the delay between mRNA and protein levels. Phosphorylated forms of PER are in fact unstable, and only the binding of TIM preserve PER from degradation. Together with DBT the two proteins form a heterotrimeric complex that is driven into the nucleus. Here PER acts as transcriptional repressor by binding to CLK and inhibiting the trans-activation activity of CLK-CYC heterodimer (Figure 1C).

2) The *Clock* (CLK) feedback loop:

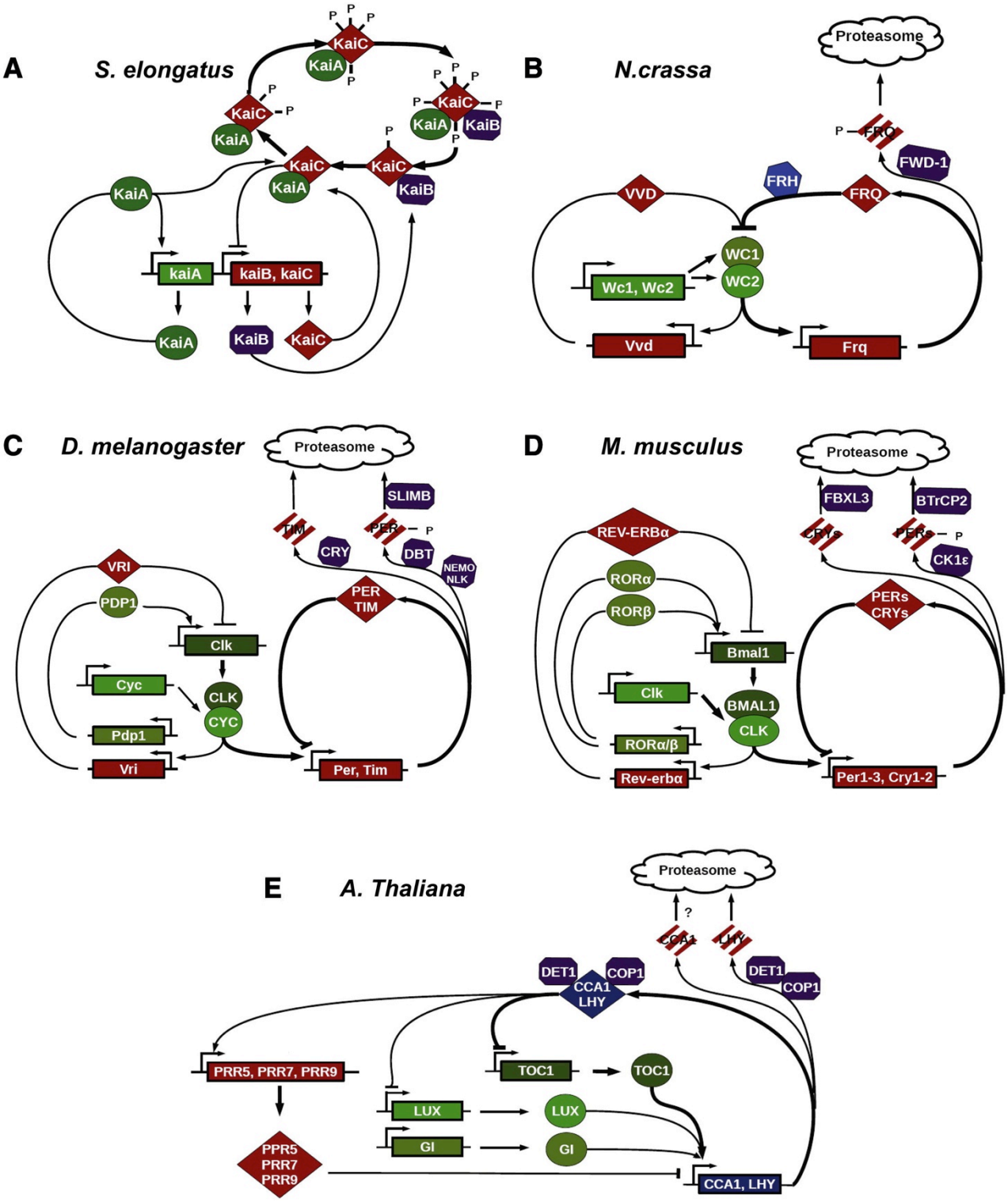
This loop involve the heterodimer CLK-CYC, which by binding the E-box elements, promotes expression of a series of proteins including *Vrille* (VRI), a basic-leucine zipper transcriptional repressor, and a basic leucine zipper transcription factor named *Par Domain Protein 1ε* (PDP1ε), during late day, early night. VRI accumulates and binds to the Clock's regulatory element inhibiting CLK expression. On the other hand, PDP1ε accumulates at high levels at mid to late evening promoting CLK expression (Figure 1C).

In mammals, circadian clock functions in a similar way, with *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*) that repress their own expression. Transcription of *Per1-3* and *Cry1-2* is due to trans-activators CLOCK and BMAL1/NPAS2 (a heterodimer). Once again, PER and CRY repress their own transcription [Ripperger and Brown, 2010]. In *Neurospora crassa* [Backer *et al*., 2011], plants [McWatters and Devlin, 2011] and cyanobacteria [Ishiura *et al*., 1998], a similar feedback loop system was found. In figure 1 the functioning models of the circadian oscillator systems *Neurospora crassa*, *Drosophila melanogaster*, *Mus musculus* and *Arabidopsis thaliana* are schematized. A principal loop (bold lines) shares common components with its assisting loops (light lines). In eukaryotes the main circadian loop relies on transcription-translation feedback systems.

1.2.2 Second Molecular System: Posttranslational Feedback Loop (PTFL)

Circadian clock speed in both prokaryotic and eukaryotic organisms is related to posttranslational modifications. These modifications are so many that can be defined as a second functional loop of regulation. The concentration of transcripts and proteins is not the only feature that is clock controlled, but also phosphorylation state of some partners as well as acetylation, methylation, sumoylation, ubiquitination and superoxidation, enter in the system as part of the clock machinery [Zhang *et al*., 2012].
Rhythmic transcriptional events have even shown to not be necessary for a functional molecular pacemaker in particular organisms. Cyanobacteria are an important example, with a circadian system relying on a PTFL based on kai genes. The isolated partners of the cyanobacteria clock system, proteins KaiA-B-C, have shown to undergo rhythmic phosphorylation events even if purified and mixed in a test tube [Nakajima et al., 2005]. KaiC protein, which presents both kinase and phosphatase activities, arranges in hexamers and generates rhythmic, temperature compensated, phosphorylation/dephosphorylation events that are modulated by KaiB and C. Figure 1 reports a schematic representation of the cyanobacterium Synechoccus elongatus circadian clock loop essentially relying on KaiC posttranscriptional modification events.

Figure 1



[Reprinted from Developmental Cell, 22, March 13, 2012, Brown *et al*., (Re)inventing the Circadian Feedback Loop, 477-487., Copyright 2012, with permission from Elsevier]

Figure 1: Schemes of circadian clock regulation in different model organisms. A principal loop (bold lines) is assisted by several parallel loops (lighter lines) sharing common activator (green) or repressor (red) proteins. In cyanobacteria *Synechococcus elongatus* (A), the oscillatory system relies on a posttranslational feedback loop (PTFL), based KaiC cyclic phosphorylation-dephosphorylation events. In the four eukaryotes: *Neurospora crassa* (B), *Drosophila melanogaster* (C), *Mus musculus* (D) and *Arabidopsis thaliana* (E), the primary mechanism is constituted of a transcription-translation feedback loop (TTFL).

1.2.3 Light Entrainment of the Clock.

The circadian clock system needs to be constantly adjusted by environmental stimuli to maintain a correct oscillatory period of 24 hours per cycle.

Light among others (temperature, food or social interactions) is considered the strongest and most pervasive factor for clock entrainment. In *Drosophila*, the blue light photoreceptor cryptochrome (dCRY) triggers TIM phosphorylation and subsequent degradation into the proteasomes in response to light. The light dependent degradation of TIM produces circadian rhythm advance or delay as a function of *tim* mRNA. In the early evening (early dark phase), TIM levels can be restored after a light pulse because of the high levels of mRNA to be translated producing a circadian rhythm delay. During the night (late dark phase) the TIM levels cannot rebound after a light pulse because of the low levels of mRNA present. In this way the rhythm can be pushed forward to the next phase [Hardin, 2005].

2.0 CRYPTOCHROMES

2.1 Introduction to Cryptochromes

Cryptochromes derive form photolyases, which are a class of enzymes that utilize light energy to repair DNA damages [Chaves *et al*., 2011]. Cryptochromes are far less widespread than photolyases and along the evolution course, they lost the DNA repairing activity and gained new roles as signaling molecules. Two families of cryptochromes have been discovered so far coming from two different photolyase ancestors: plant cryptochromes and animal cryptochromes.

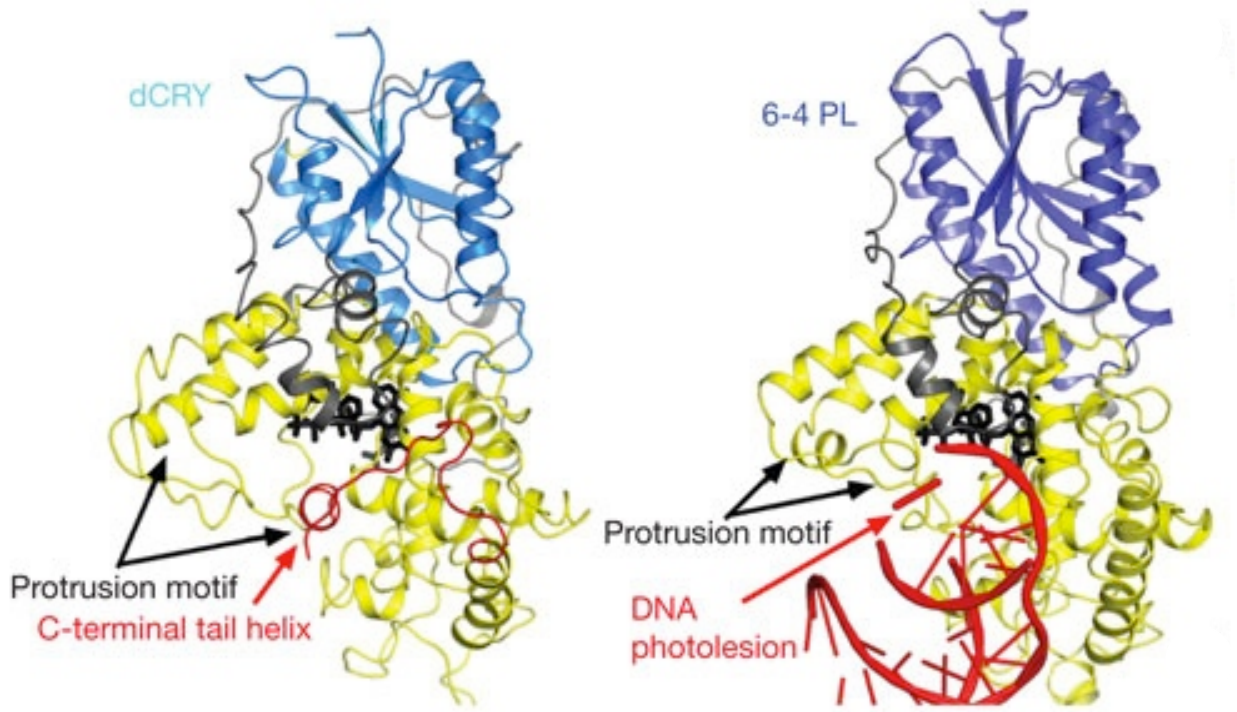
Cry-DASH cryptochromes are a third class of proteins that show single-strand DNA repair activity [Selby and Sancar, 2006]. They might be classified as the link between canonical cryptochromes and photolyases and their role in signaling has still to be unveiled.

2.2 Cryptochromes Structure

Cryptochrome structural backbone consists substantially of two regions: an N-terminal photoreactive domain homologous to photolyases (Photolyase Related, PHR), in which Flavin Adenine Dinucleotide (FAD) is bound as cofactor, and a very evolutionary divergent short C-terminal tail [Cashmore *et al.,* 1999]. Structural studies on photolyases revealed that the N-terminal domain has a mixed α/β topology, in which five β-sheet strands are surrounded by a crown of α-helices. This region hosts the nucleotide-like cofactor, which acts as antenna chromophore. The C-terminal tail is highly variable and a hotspot for possible molecular interactions: in fact, by *in silico* analysis and experimental validation it was possible to identify several protein-protein interaction motifs in this region [Hemsley *et al*., 2007].

The structure of *Drosophila* cryptochrome (dCRY) was recently obtained at 2.3 Å resolution by X-ray chrystallography [Zoltowski *et al*., 2011]. The backbone is overall similar to *Drosophila* 6-4 photolyase (6-4 PL) with major discrepancies in the cofactor binding site and in the variable C-terminal tail (figure 2). In dCRY, the flexible C-terminal tail replaces the DNA substrate and the FAD cofactor is positioned such that changes in its electronic state likely influence the interaction of the binding pocket and the tail.

Figure 2



[Reprinted by permission from Macmillan Publishers Ltd: Nature, 480, 2011, Zoltowski *et al*., Structure of full-length *Drosophila* cryptochrome, 396-399. Copyright 2012]

Figure 2: Comparison between dCRY and 6-4 photolyase structures. *Drosophila* cryptochrome resembles 6-4 photolyase with the C-terminal tail replacing the DNA substrate. The N-terminal α/β domain (blue) is coupled to the C-terminal helical domain (yellow) through a long linker (grey). In dCRY a C-terminal helix (red) docks to the photolyase DNA binding cleft beside the flavin cofactor (black).

2.3 Light Activation of Cryptochromes

Purified dCRY exhibits two main absorption peaks in the violet-blue portion of the light spectrum, a main one centered at about 365 nm and a secondary one at about 450 nm Although the mechanism is not fully understood yet, the activation of dCRY by light implies a conformational rearrangement.

Considering purified dCRY, two mechanisms are possible to explain its photo-induced activation:

1) Upon exposure to light, oxidized FAD is converted to reduced FAD with a conformational change in the protein structure that generates the “signaling form” of dCRY. Re-oxidation of the cofactor in the dark allows dCRY to return back to the ground state.
2) dCRY already contains a reduced FAD in the dark and it is the excitation of the cofactor by light that causes the conformational change responsible for signaling.

The second mechanism is supported by *in vitro* experimental data [Ozturk *et al*., 2011] and is currently considered as the most plausible.

It has been hypothesized that *in vivo* activation of dCRY by light is mediated also by specific “regulators” that bind its C-terminus, which is known to regulate the light-dependence of dCRY activity [Rosato *et al*., 2001].

2.4 Cryptochromes within the Circadian Clock Mechanism

Cryptochromes’ role in the circadian clock differs among the different species. In fact, they have a merely blue light photoreceptor activity in plants, while in mammals they are part of the central clock mechanism, and this function is not light dependent [Lin and Todo, 2005].

2.4.1 Plant Cryptochromes

In *Arabidopsis thaliana,* two cryptochromes were identified (CRY1 and CRY2) that are predominantly nuclear proteins and act as transcriptional regulators of gene expression and entrain the circadian clock in response to light. In response to light, they drive gene

expression in particular on the plant photomorphogenesis, such as stem elongation, leaf expansion and floral initiation by daylight. Gene transcription can be affected in two ways:

a) Directly: CRY molecule interacts directly with the transcriptional machinery to affect transcription [Cutler *et al*., 2000].
b) Indirectly: CRY molecules interacts with proteins exerting different cellular functions to regulate the stability, modification, trafficking of transcriptional regulators [Wang *et al*., 2001]

2.4.2 Mammalian Cryptochromes

Mammalian cryptochromes (two cryptochromes were identified in mammals, named CRY1 and CRY2) can be found in the nucleus as well as in the cytosol and perform both light-dependent and light independent regulatory functions within the circadian clock.

Mice mutated in *Cry* genes showed reduced or even completely abolished *Period* expression and the pupils of mice lacking the cryptochromes have reduced response to light stimuli [Selby *et al*., 2000]. Looking at the circadian rhythm, mice knocked out for both *Cry1* and *Cry2* showed usual circadian oscillation in normal light-dark cycles, but they lost completely the rhythm and went to *free-running* once placed in constant dark conditions [van der Horst *et al*., 1999]. Mammalian CRYs are classified as integral components of the clock negative feedback loop by physically interacting, in a light independent fashion, with the other components of the oscillator mechanism [Griffin *et al*., 1999].

2.4.3 *Drosophila* Cryptochrome

On the basis of the tissue in which it is expressed dCRY can act as a circadian photoreceptor in the master clock [Lin *et al*., 2001; Busza *et al*, 2004], or be a component of the circadian pacemaker in the peripheral clocks [Ivanchenko *et al*., 2001], functioning as a transcriptional repressor [Collins *et al,* 2006]. dCRY plays a fundamental role also in the fly's perception of Earth's magnetic field that they use for orientation and navigation (fly's magnetosensitivity) [Yoshii *et al*., 2009].

dCRY shares with the other clock components an oscillatory expression level [Emery *et al*., 1998] during the 24 hours. Flies entrained in normal light/dark cycling conditions exhibit oscillating dCRY levels, The gene is rhythmically expressed, with a maximum at

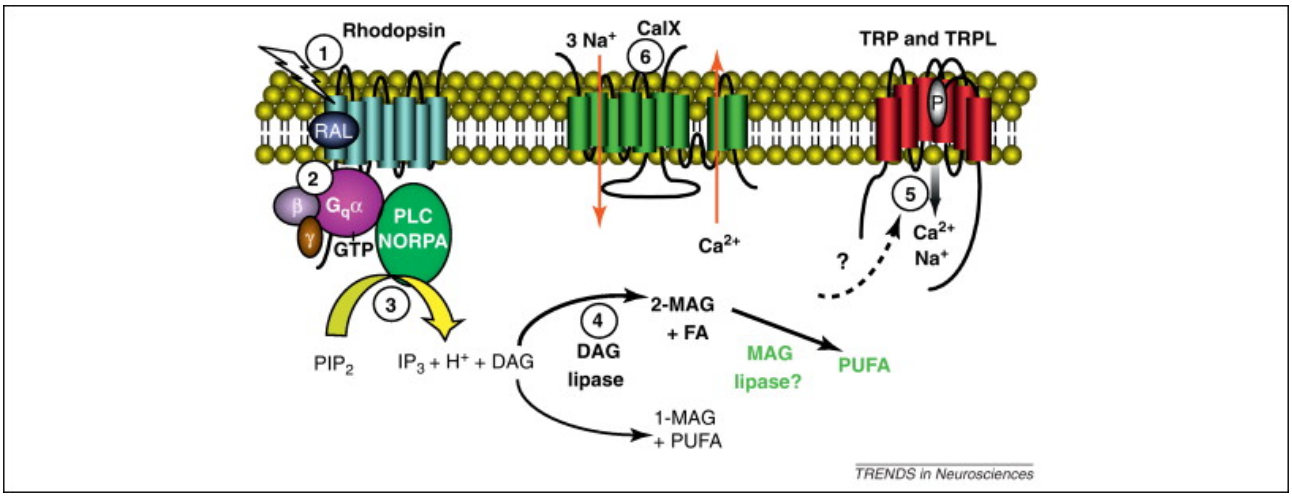
the beginning of the day (ZT1-6). This oscillation is completely abolished in clock mutants. dCRY protein levels oscillate only in LD cycles while in constant light the protein is rapidly degraded and in constant dark it accumulates continuously. These data suggest a double regulation for *cryptochrome*: a clock controlled gene expression and a post-transcriptional and post-translational control on the protein exerted by light.
Within the *Drosophila* circadian mechanism, as depicted in figure 1, cryptochrome function is to reset the clock by interacting with TIM in the presence of light: following to this interaction, TIM is phosphorylated and targeted for degradation through a ubiquitin-proteasome mechanism. Upon light activation, dCRY interacts also with JETLAG (JET, a component of E3 ubiquitin ligase complex) and is degraded via proteasome [Peschel *et al*., 2009].
dCRY interacts also with the kinase SGG (shaggy/GSK3), and the cryptochrome's stability in the light is considerably increased by this interaction while the inactivation of the kinase leads to the degradation of dCRY in the darkness [Stoleru *et al*., 2007].

3.0 *Drosophila* VISUAL CASCADE

3.1 Introduction to *Drosophila* Visual Cascade

To amplify photon responses and allow cells to adapt to variations in light intensity, Nature has developed a phototrasduction cascade mechanism.
The visual cascade functioning mechanism is still controversial (Figure 3) [Montell, 2012]. In *Drosophila*, this signaling pathway occurs at the level of the rhabdomere membrane and depends initially on light sensors that are constituted by a transmembrane protein, rhodopsin, linked to a chromophore (3-hydroxy-11-*cis*-retinal). An all-*trans* configuration is induced on the chromophore upon energy transfer by photons, thereby promoting a conformational change in the rhodopsin subunit. Rhodopsin is coupled with a heterotrimeric G-protein complex that associates with GTP and stimulates no receptor potential A (NORPA), a phospholipase type C (PLC) bound to the G-protein complex [Waldo *et al*., 2010]. It effects the hydrolysis of phosphatidylinosiltol 4,5-bisphosphate (PIP_2) to inositol and diacylglycerol (DAG). A diacylglycerol lipase hydrolyzes DAG to produce monoacylglycerol (MAG). NORPA triggers TRP channel and, in photoreceptor cells, also the related TRP-like (TRPL) cation channel activation (figure 4) [Cook *et al*., 2000].

Figure 3



[Reprinted from Trends in Neuroscience, 35, June 2012, Craig Montell, *Drosophila* visual transduction, 356-363., Copyright 2012, with permission from Elsevier]

Figure 3: Model and sequence of the events in the *Drosophila* phototransduction cascade. (1) Rhodopsin is light-activated; (2) the light-activated rhodopsin is coupled with the heterotrimer Gq protein and GTP is bound to the activated Gqα subunit; (3) hydrolysis of PIP2 and production of IP3, DAG and H^+ occurs after stimulation of phospholipase type C (PLC); (4) gene inaE encodes for a DAG lipase, which hydrolyzes DAG to produce 2-MAG and FA and in lower amounts also 1-MAG and PUFA. The 2-MAG might be converted into PUFA by an unknown metabolic mechanism, likely due to a MAG lipase; (5) PLC stimulation is followed by TRP and TRPL activation, although the mechanism remains controversial; (6) following activation of the channels, a Na^+-Ca^{2+} cation exchanger pump (CalX) extrudes Ca^{2+} out of the photoreceptor cell membrane.

Abbreviations: DAG, diacylglycerol; FA, saturated fatty acid; IP3, inositol 1,4,5-trisphosphate; MAG, monoacylglycerol; PIP2, phosphatidylinositol 4,5-bisphosphate; P, pore loop indicated in TRP; PLC, phospholipase C; PUFA, polyunsaturated fatty acid; RAL, the chromophore (3-OH-11-cis-retinal); TRP, transient receptor potential (channel); TRPL, TRP-like.

3.2 The Signaplex and INAD

The designation “Signaplex” defines a large macromolecular assembly of proteins that generate the visual transduction cascade in the rhabdomeres. The complex is held by Inactivation No After-Potential D (INAD) a protein that acts as scaffold [Tsunoda *et al*., 1997].

In the sequence of INAD, five distinct structural domains of about 90 amino acids each, can be identified. These structural domains fall within the category of PDZ binding domains and have the characteristic to recognize and bind to specific amino acidic short

linear motifs within the sequence of other proteins, most often present at the C-termini. The main interacting partners of INAD were shown to be: TRP [Chevesich *et al*., 1997], PLC (NORPA), and a protein kinase C (PKC) [Xu *et al*., 1998]. Loss of INAD destabilizes these partners and disrupts their localization in the rhabdomeres [Tsunoda *et al*., 2001].

Several other proteins gave indication of possible INAD binding such as calmodulin, TRPL, and rhodopsin [Wes *et al*., 1999], but if this is the case, their interaction should be dynamic in contrast to the members of the core complex. The dynamic interaction of INAD with some molecular partners was demonstrated recently [Liu et al., 2011]. Specifically, PDZ5 (the closest to INAD C-terminus) was shown to undergo light dependent conformational changes, in this way shifting from an open (binding) form to a closed (unbinding) one. Since TRP is above all the main partner of INAD, the mechanism proposed is that the TRP-INAD interaction, which occurs via INAD's PDZ3 and PDZ5, is very strong in the darkness and so TRP is maximally sensitive to activation. Upon light irradiation, INAD's PDZ5 dissociates from TRP and the PDZ3 interaction alone is too weak to maintain a stable TRP-INAD association, thus TRP sensitivity to activation is reduced [Mishra et al., 2007].

3.3 PDZ Domains

PDZ domains are very abundant modular domains mediating protein-protein interactions that play a crucial role in the assembly of large protein complexes involved in signaling processes. Usually constituted of about 80-100 amino acidic residues, PDZ domains have a conserved fold consisting of 6 β-strands (βA – βF), a short α-helix (αA) and a long α-helix (αB). Although the PDZ class has a highly conserved fold (figures 5A and B) their secondary structure can be very variable in length. More than 200 PDZ structures have been defined so far mainly by NMR and X-ray crystallography techniques [Lee *et al*., 2010]. Detailed information is now available on their ligand recognition and selectivity. PDZ domains typically recognize characteristic short sequences (linear motifs, LMs) at the extreme C-terminus of target proteins, but cases of binding to internal sequence motifs were also reported [Hillier *et al*., 1999].

Some PDZ domains, instead of acting as independent structures, were found to form homodimers with each other. This dimerization occurs in different ways: distinct PDZ domains can simply interact via conserved structural portions [Im YJ *et* al., 2007] (figure 5D) or they can share a part of the sequence [Wu *et al*., 2007] (figure 5C).

Homodimerization of PDZs does not involve the binding groove, which stays open, but regards different parts of the sequence, and they remain fully active toward their target linear motifs. Recent studies have shown that some PDZs need another PDZ domain in tandem to fold properly and be able to recognize the target linear motif [Long J *et al.* 2008].

Structural analysis of known binding sites of PDZ domains and their ligands has provided insight into the specificity of PDZ protein-protein interactions [Songyang et al., 1997]. It is now known that a groove, with a highly conserved carboxylate binding loop, is formed between αB and βB. This is the “PDZs active site”, in which the specific linear motif is recognized and bound.

The preference of each residue of a binding peptide is related to the physical-chemical characteristics of different relevant residues on specific secondary structure elements forming the PDZ binding pocket [Chen *et al*. 2008]. The binding specificity of a PDZ domain is crucially determined by its αβ helix first residue and the side chain of the -2 residue in the linear motif recognized [Hung *et al*., 2002]. This constitutes the basis for PDZ classification. Three major classes of PDZ with different binding motif specificities have been established, but other classes are likely to be distinguished with further research [Tonikian *et al*., 2008]. According to the current classification, PDZ domains can then be subdivided in:

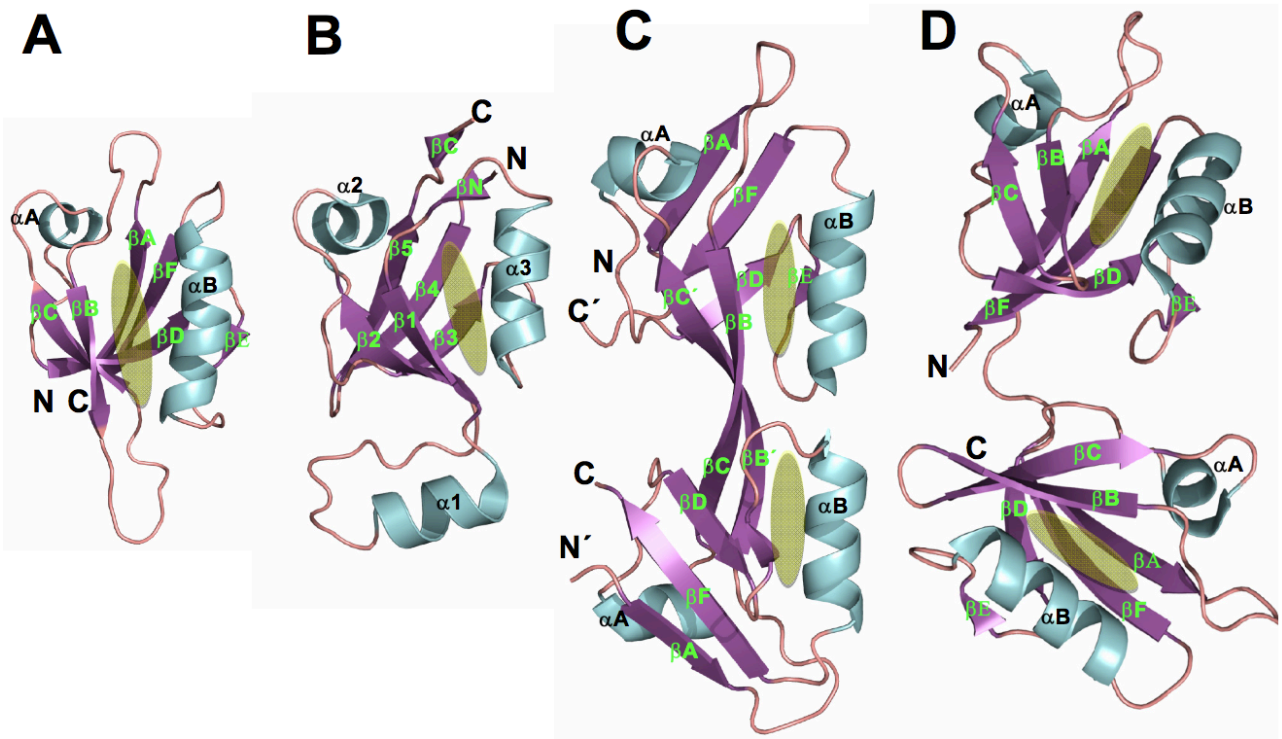
Class I: PDZs class I recognize linear motifs the sequence of which matches the following scheme for the last four residues: -X-Ser/Thr-X-Φ
(in which Φ is any hydrophobic amino acid)

Class II: For class II PDZs the sequence of the last four residues in the linear motif must be: -X-Φ-X-Φ

Class III: For class III PDZs the recognized sequence is: -X-Asp/Glu-X-Φ

For those PDZ domains that bind to internal sequences of target proteins, docking happens via a two-stranded β-hairpin, which enters into a structural groove of the binding partner [Brenman *et al*., 1996].

Figure 4



[Reprinted from Lee HJ *et al*., (2010) PDZ domains and their binding partners: structure, specificity, and modification. *Cell Communication and Signalling* 8:8]

Figure 4: A) PDZ domain classic structure: specifically the ribbon diagram of Dvl-1 PDZ (PDB code: 2KAW) is reported. B) PDZ-like domain structure: the diagram refers to HtrA2 PDZ (PDB code: 1LCY). C) PDZ-PDZ dimeric structure: specifically the ZO-1 PDZ2 (PDB code: 2RCZ) is depicted. D) Tandem PDZ domains. The GRIP-1 PDZ1+2 (PDB code: 2QT5) is shown. A yellow oval represents the linear motif binding site of each PDZ domain.

4.0 PRELIMINARY DATA

4.1 Evolutionary Conservation of Cryptochrome C-Terminal Tail across Species

An un-rooted neighbor-joining phylogenetic tree was constructed using amino acid sequences from various members of the cryptochrome family from plants to human. The phylogenetic analysis (figure 5) shows five distinct groups in the cryptochrome family. Four of them are clustered as animal cryptochromes (Vertebrate, Vertebrate-like, Cry4, Drosophila-like) while plant cryptochromes are present only in one group. In both Vertebrate-like and Plant clusters, two subclusters including CRY1 and CRY2 types are reported.

Focalizing on *Drosophila* cryptochrome, it bears a highly variable C-terminal tail that has undergone rapid evolution while maintaining overall similar roles in circadian rhythmicity.

Figure 5

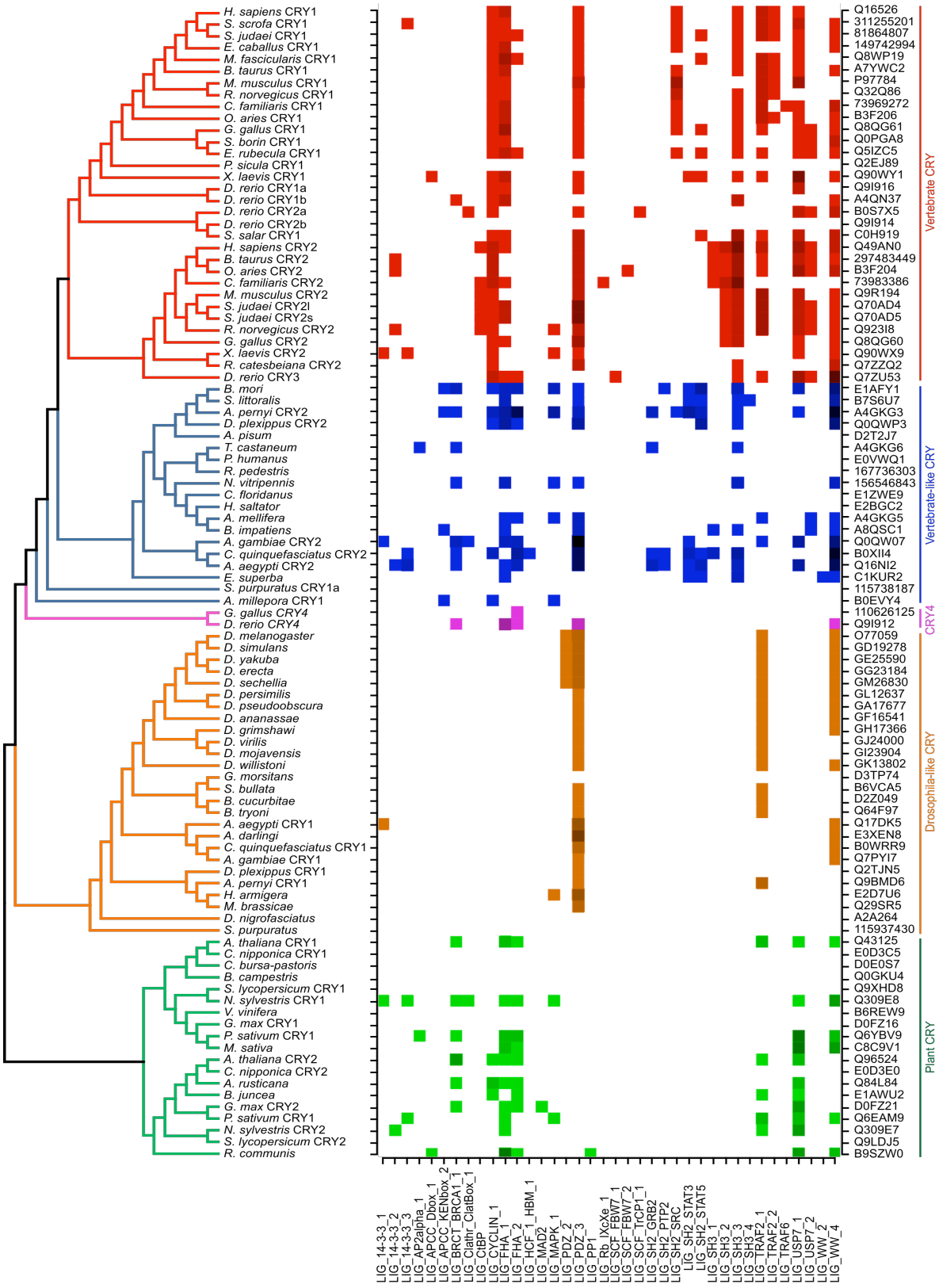


Figure 5: The phylogenetic tree of 98 known cryptochromes and cryptochrome-like proteins is reported on the left side. The tree refers to the N-terminal photolyase-like domain and is related, through colored squares, to the presence of functional linear motifs, identified in the highly variable C-terminus (center). Listed with their names on the bottom row, from the ELM database, only functional motifs with limited true

binding sequences are reported. UniProt sequence accession numbers are depicted on the right with the high level taxonomic grouping of the sequences. More motifs of the same type in the sequence correspond to darker box colors. In case a particular functional motif is evolutionarily conserved long vertical stripes are drawn. As can be deduced from the figure, class III PDZ binding motifs are very conserved among organisms and correspond to the longest of such stripes. This is of particular relevance, given the potentially high error rate of single motif instances.

Within the dCRY C-terminal residues, several sequences (Linear motifs, LMs) were detected that could be recognized by PDZ structural domains of other molecular partners. Linear motifs (LMs) are short sequences (4-5 amino acidic residues long) evolved to mediate molecular (protein-protein) interactions. Often, LMs are present in disordered non-globular protein regions with a particular preference for protein C-teminus localization. LMs have the tendency to be unstable over long evolutionary distances and, within distinct non-globular regions, it can be hypothesized that they might evolve by “jumping” between different sequence positions.

An important ingredient for the system-level understanding of a cellular process, is its protein-protein interaction network. It was hypothesized that a protein partner of *Drosophila* cryptochrome could be a PDZ domain containing protein and a search on STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database [Szklarczyk *et* al., 2011] for possible candidates was performed.

Numerous sources contribute to the STRING database, which includes experimental data, public text collections and prediction methods. Information on about 5.2 million proteins from 1133 species is stored in this database, which is regularly updated and maintained by a consortium of academic institutions. Analysis on STRING provides as output a network of interaction with the protein of interest that can be used for filtering and assessing functional genomics data or for providing a platform of structural, functional and evolutionary properties. Predicted interaction can improve the consistency of experimental results providing new directions for further research. The predictions can be formulated with the following guidelines:

Neighborhood: similar genomic context in different species might suggest a similar function of proteins.

Fusion-fission events: proteins that are fused in some genomes are very likely functionally linked (as in other genomes where the genes are

not fused).

Occurrence: proteins that have a similar function or an occurrence in the same metabolic pathway, must be expressed together and have a similar phylogenetic profile.

Co-expression: predicted association between genes based on observed patterns of simultaneous expression of genes.

Figure 6 shows the distribution of selected interactors for dCRY with high confidence levels using the neighborhood approach. The results showed a weak hypothetical connection to No Receptor Potential A (NORPA), a protein belonging to the phototransduction complex [Wang and Montell, (2007)].

Figure 6

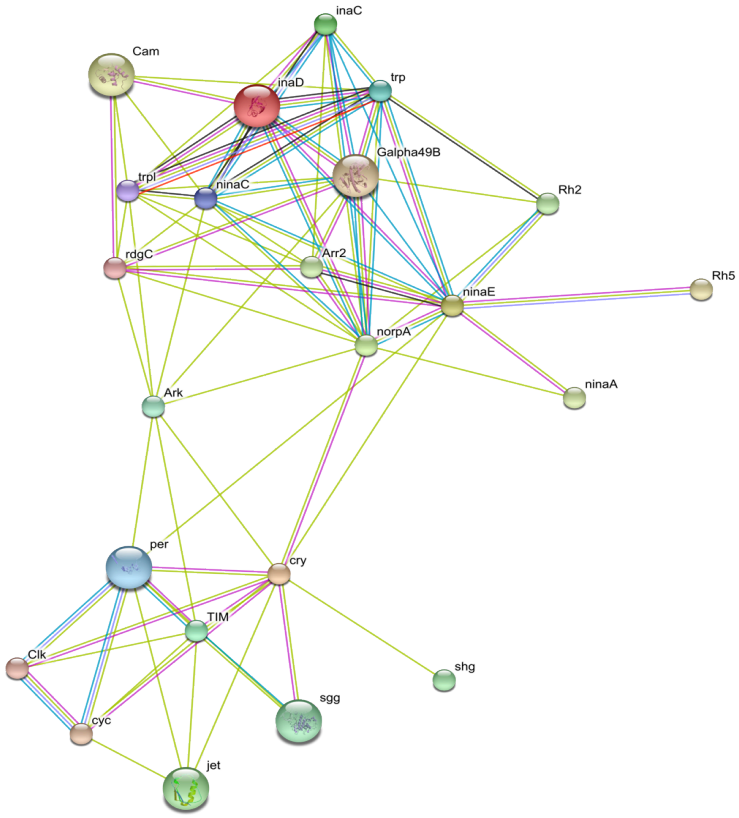


Figure 6: Protein interaction network surrounding dCRY and INAD. The STRING interaction network is shown for dCRY, INAD and their main interaction partners with edge colors representing different detection methods. Note that the edge between dCRY and NORPA is based on phenotypic enhancement assays (Stanewsky et al., 1998) and thus may not necessarily represent a true physical interaction.

4.2 *Drosophila* Cryptochrome Interacts with the Signaplex

In order to identify new partners of *Drosophila* cryptochrome, a co-immunoprecipitation assay, followed by mass spectrometry analysis, was performed on transgenic flies overexpressing a HA-tagged form of dCRY [Dissel *et al*., 2004]. The flies were raised in 12:12 light:dark cycles (LD 12:12) and collected at ZT24, before lights on, and after a 15 min light pulse. A ~115 kDa species was observed in the dark sample and a ~180 kDa species after the light pulse, that were not present in the respective negative controls (figure 7). These protein bands were in-gel digested and the peptide mixtures were analyzed by LC-MS/MS using an ESI-QTOF mass spectrometer [Wilm *et al*., 1996]. Analysis of the MS/MS data using the MASCOT software yielded the identification of Retinal DeGeneration A (RDGA) in the dark and Neither Inactivation Nor Afterpotential C (NINAC) in the light**.** These two proteins are both involved in the fly visual signaling pathway.

Figure 7

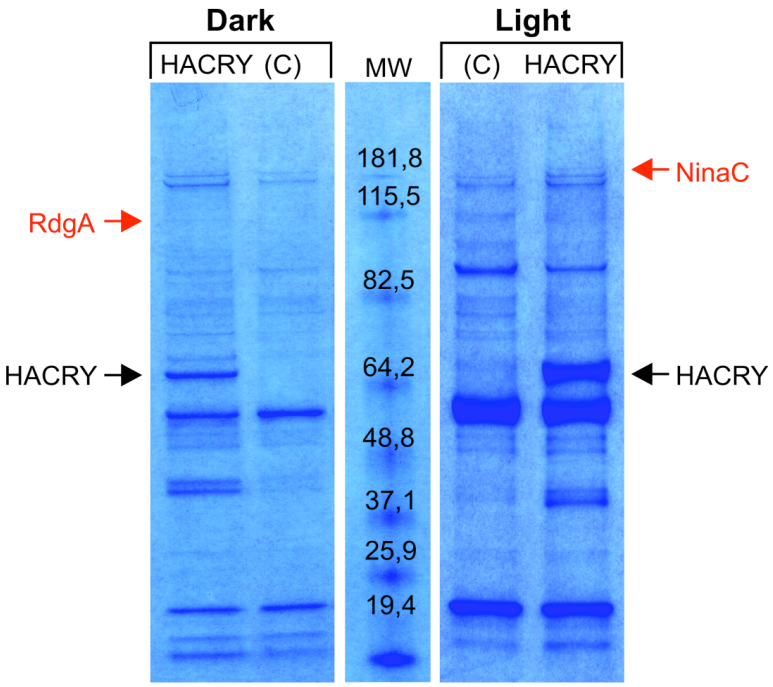


Figure 7: *Drosophila* head protein extracts, Coomassie blue-stained on gel were co-immunoprecipitated with an anti-HA antibody. HACRY overexpressing flies (HACRY, yw;tim-GAL4/+; UAS-HAcry/+) and relative controls (C, yw;tim-GAL4) were reared in 12:12 LD and collected in the dark (ZT24) and in the light (ZT24+15 min light pulse). Molecular masses of markers are indicated (MW: BenchMark Pre-Stained Protein Ladder, Invitrogen). Black arrows indicate bands corresponding to HACRY while red arrows indicate stained proteins excised and characterized by mass spectrometry. RdgA stands for Retinal Degeneration A, while NinaC for Neither Inactivation Nor Activation C.

To explore the structural organization of the proteins, experimentally or *in silico* identified as potential interactors of dCRY, the interactive view of the STRING network was used. In figure 8 is depicted a schematic draw of the domain organization of *Drosophila* cryptochrome and its potential partners of the phototrasduction complex. dCRY C-terminus was analyzed with CSpritz [Walsh *et al*., 2011], which predicts intrinsic disorder in the sequence as well as secondary structure preferences.

Figure 8

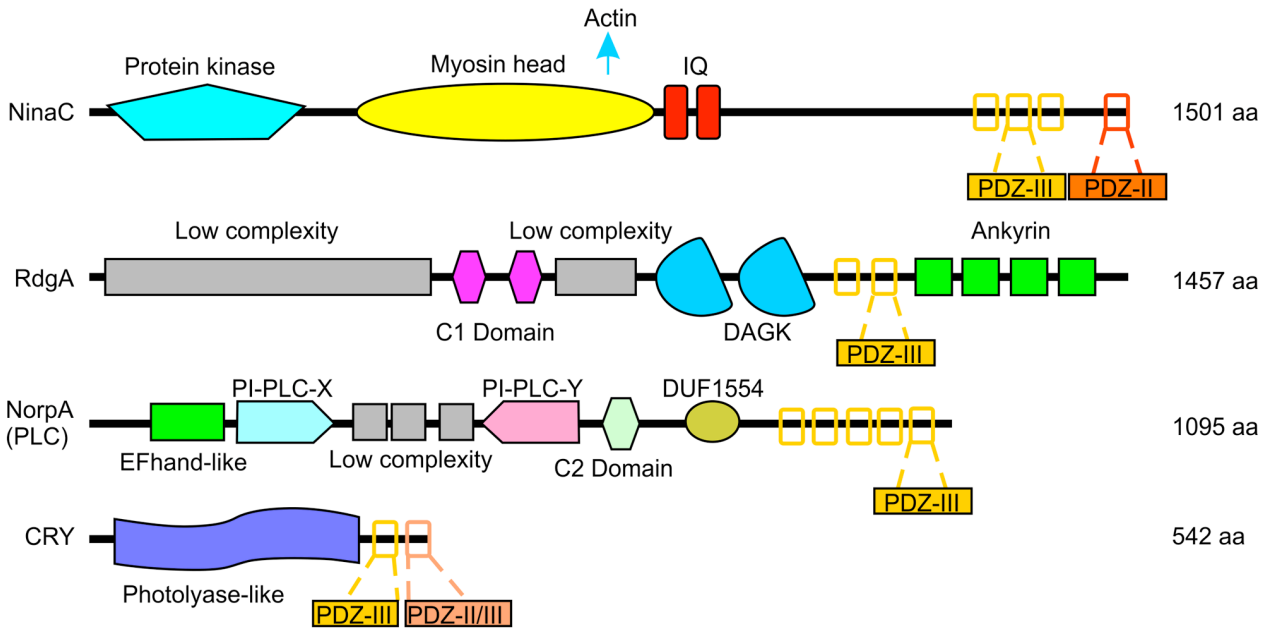


Figure 8: Schematic domain distribution for known and putative INAD interacting proteins. Each protein is drawn proportional to its size, with solid shapes representing different protein domains and their name from the Pfam database. Low complexity regions, shown in picture as light grey rectangles, are not proper domains. PDZ binding linear motifs are shown as empty rectangles with yellow (for binding with class III PDZ domains), orange (with type II PDZ) or peach (overlapping classes II/III PDZ) borders.

5.0 AIM OF THIS WORK

The preliminary data reported in the previous chapter were a solid starting base to hypothesize a possible interaction between INAD and dCRY and then a possible involvement of cryptochrome in the visual cascade.

The following considerations

a) many of the elements of *Drosophila* visual cascade are assembled in the Signalplex organized by INAD;

b) none of the proteins experimentally detected after co-immunoprecipitation and MS/MS assay with cryptochrome present PDZ domains in their sequence;
c) instead, they show linear motifs along their sequence that can match the characteristics to be recognized by specific classes of PDZs as well as dCRY C-terminus

led to the conclusion that a third partner could mediate the interaction between dCRY and the detected proteins of the visual cascade. This partner could be INAD, which presents several PDZ domains that potentially might recognize all the linear motifs of the supposed partners.

The aim of this work was to discover if the scaffolding protein INAD, beside its well know role in assembling the Signalplex, could also mediate the interaction between CRYPTOCHROME and the visual cascade in *Drosophila*.

RESULTS

1.0 CONFIRMATION OF dCRY INTERACTION WITH NINAC

In *Drosophila,* NINACp174 is normally localized in the rhabdomere membranes of photoreceptor cells of the fly eye [Porter *et al.,* 1992]. The presence of NINACp174 (longer isoform) in the complex with dCRY was confirmed by western-blot with an antibody specifically raised against this protein isoform. In figure 9, the results of co-immunoprecipitation and western-blot analysis are reported. A clear signal corresponding to NINACp174 is present in HACRY overexpressing flies. Flies for testing were collected in both dark and light conditions.

Figure 9

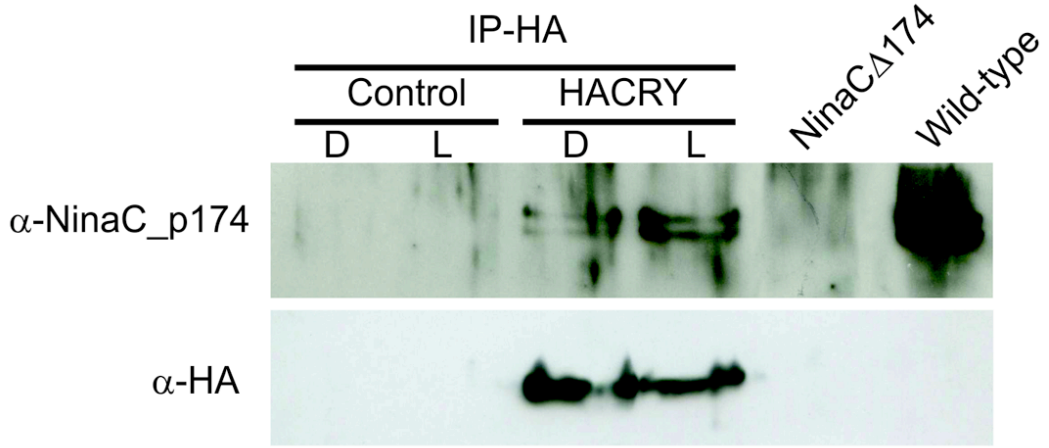


Figure 9: Co-immunoprecipitation and western-blot confirming the interaction between HACRY and NinaC in HACRY overexpressing flies (yw;tim-GAL4/+; UAS-HAcry/+). *Tim-GAL4* flies were used as control. Heads were collected in the dark (ZT24) and in the light (ZT24+15 min light pulse) as previously reported. Membranes were probed with anti-NINACp174 and anti-HA antibodies. *NinaC*$^{\Delta 174}$ and w^{118} flies were used as negative and positive control, respectively.

Antibodies specifically raised against *Drosophila* RdgA were not available at the time of this study. To confirm the presence of RdgA in HACRY co-immunoprecipitate commercial antibodies against the human RdgA were tested unsuccessfully.

2.0 INTERACTIVE PROPERTIES OF INAD PDZ DOMAINS

2.1 *Drosophila* Cryptochrome Interacts with INAD

A direct interaction between *Drosophila* cryptochrome and the phototransduction complex through INAD was hypothesized and to test this idea, a search for INAD in the immunocomplex formed by dCRY was performed. Western-blot analysis, with anti-INAD antibody [Wes *et al*., 1999], was performed on head protein extracts from HACRY over-expressing *Drosophila* flies immunoprecipitated with anti-HA antibody.

The presence of INAD *in vivo* associated with dCRY was detected by co-immunoprecipitation and western-blot assays. Heads extracts of HACRY overexpressing flies, collected in both light and dark conditions, were analyzed using anti-INAD and anti-HA antibodies (figure 10).

Figure 10

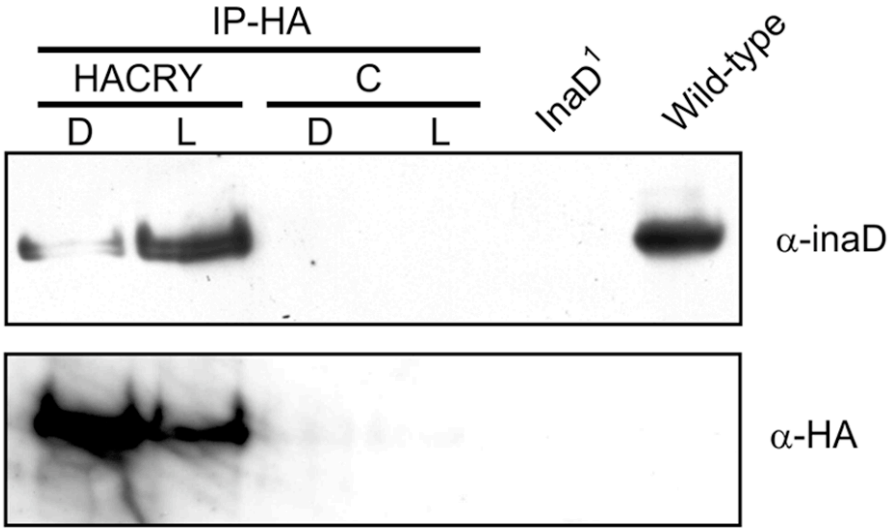


Figure 10: Co-immunoprecipitation and western-blot analysis highlighting the interaction between HACRY and INAD in flies overexpressing HACRY (*yw;tim*-GAL4/+; UAS-*HAcry/*+). In column C, *tim-GAL4* flies were used as control. For head protein extraction, *Drosophila* flies were collected in the dark (D; ZT24) and in the light (L; ZT24+15 min light pulse). Anti-INAD and anti-HA antibodies were used to probe the membranes. Flies $inaD^{1}$ and w^{1118} (wild-type) were used respectively, as negative and positive control of the antibodies.

A yeast two-hybrid system was designed to further analyze the physical interaction between dCRY and INAD: a full-length dCRY (bait) was initially challenged with the full-length INAD (prey) (figure 11) in both light and dark conditions. The expression of the

reporter gene (β-galactosidase) was detected in yeasts cultured in the light (500 Lux) and no reporter expression was observed in yeasts grew in darkness (0 Lux) confirming that an interaction occurs between the two proteins and, remarkably, this interaction occurs in a light-dependent fashion.

In a second two-hybrid set-up, the two portions of dCRY, i.e., the conserved photolyase domain and the short C-terminal tail (baits), were split and separately challenged with the full-length INAD (prey). In figure 11, the results of this analysis are reported and they highlight that the interaction is completely abolished when the dCRY C-terminus tail (amino acids from 521 to 540) is removed. On the other hand, the interaction between INAD and the extreme C-terminal tail of dCRY is light independent, as this region contains the binding motifs for PDZ domains.

Figure 11

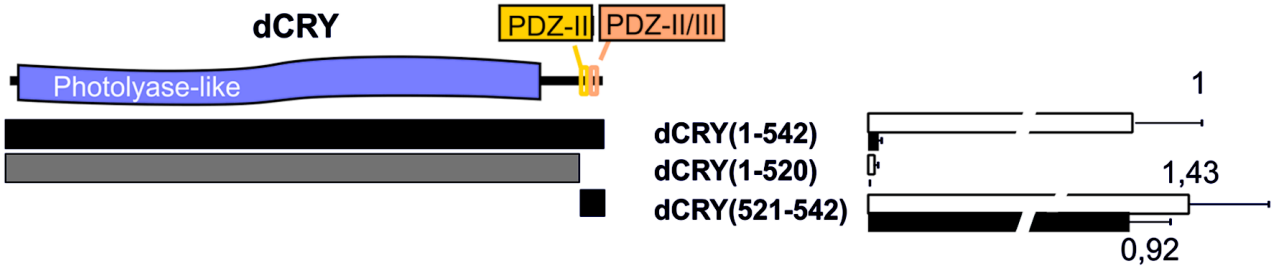


Figure 11: Different portions of the dCRY sequence were tested for interaction with the full-length INAD under both light and dark conditions. On the left, the interacting fusions are shown in black while on the right, relative β-galactosidase activities are reported for each fusion (in this case, white and black rectangles refer to light and dark conditions, respectively). The β-galactosidase activity obtained with dCRY full-length is set to 1 as reference point. The values reported are the mean ± SEM of at least 7 independent clones for each fusion, analyzed in triplicates. The results obtained report a light dependent interaction between dCRY and INAD, with the C-terminus of dCRY being crucial. On the other hand, these 22 C-terminal amino acids of cryptochrome showed a light-independent affinity for INAD, with a significantly stronger interaction in the light compared to the dark (t_{13}=2.6, P=0.02).

2.2 *Drosophila* INAD Interacts with Cryptochrome through a Specific Portion of its Sequence

To identify the regions of INAD specifically responsible for the interaction with dCRY, the binding specificity of each INAD PDZ domains was investigated. First, prey fusions expressing individual PDZs or different combinations of them were generated and were tested for the interaction with full-length dCRY as bait. In table 1, the expected molecular masses for the various constructs are reported.

Table 1

FUSION	SHORT NAME	MM (kDa)
A-inaD (17-106)	PDZ1	21.7
A-inaD (249-332)	PDZ2	20.8
A-inaD (364-448)	PDZ3	20.9
A-inaD (489-577)	PDZ4	21.5
A-inaD (584-664)	PDZ5	20.6
A-inaD (17-332)	PDZ1-2	46.9
A-inaD (249-448)	PDZ2-3	33.1
A-inaD (364-577)	PDZ3-4	35.6
A-inaD (489-664)	PDZ4-5	31.2
A-inaD (207-332)	PDZ2bis	28.2
A-inaD (207-448)	PDZ2-3bis	40.5
A-inaD (17-448)	PDZ1-2-3	59.2
A-inaD (249-577)	PDZ2-3-4	47.8
A-inaD (364-664)	PDZ3-4-5	45.1
A-inaD (1-674)	INAD	86.1
A-NinaC	NINAC	186.1
Nuclear FLAG-inaD (1-674)	NFLAG-INAD	82.1

Table 1: Expected molecular masses of the constructs generated in the yeast two hybrid assays. A-: Acid Blob (prey fusion)

Prior to the β-galactosidase assay, the correct expression of each fusion proteins in yeast cells was checked by western-blot analysis on lysates with anti-HA antibodies (Figure 12). For PDZ1, PDZ3 and PDZ4, besides the expected signal, a band of molecular mass compatible with a dimeric organization was observed.

Figure 12

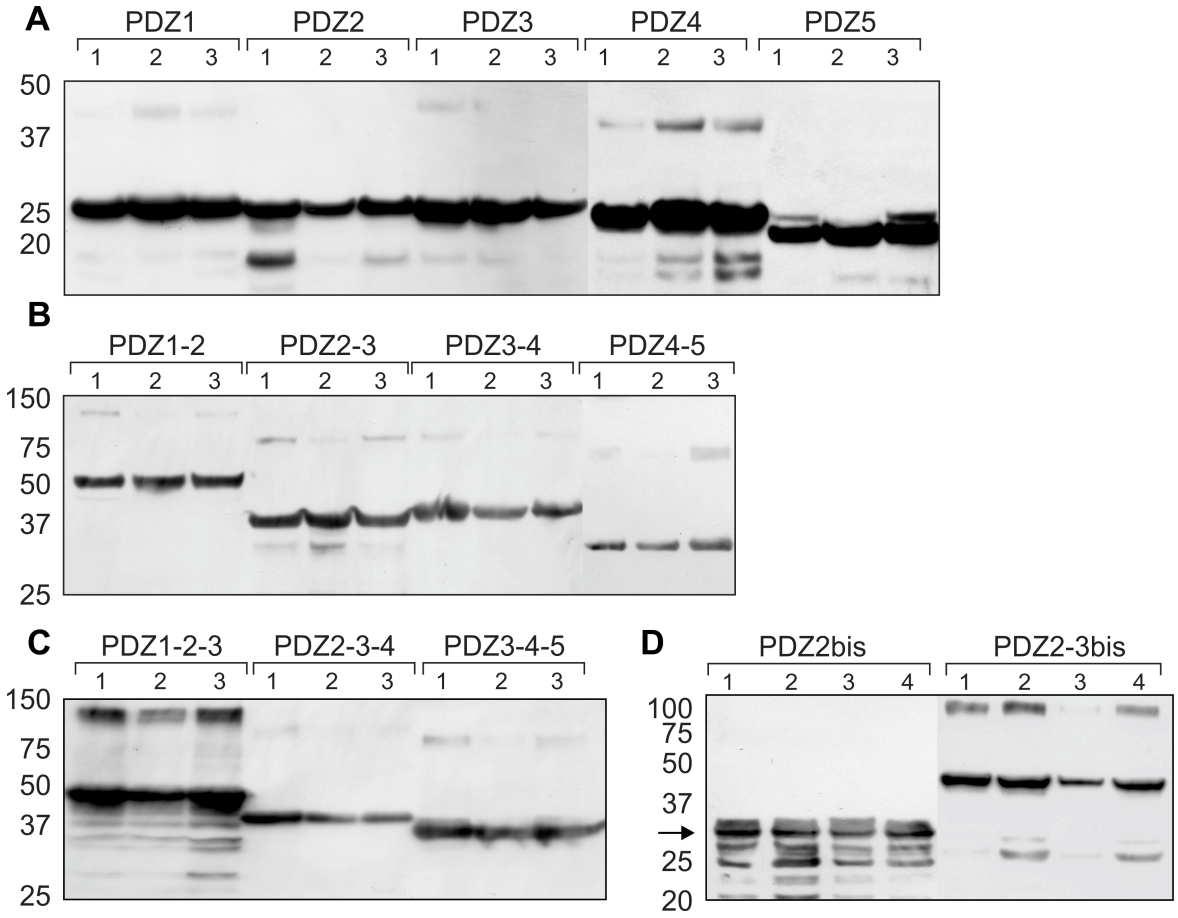


Figure 12: Western Blot analysis of independent yeast clones for prey fusions expressing different combinations of PDZ domains. Protein extracts of three or four independent yeast clones for each prey fusion were probed with anti-HA antibody (SIGMA 1:5000). In table 3, the theoretically calculated molecular masses are reported. A) Single PDZs. The ~20-25 kDa signals indicate that all the fusions are expressed in yeast cells and the absence of interaction in the two hybrid assay cannot be explained by the absence of expression. A band of molecular mass compatible with a dimer is visible in correspondence to PDZ1, PDZ3 and PDZ4. B) Tandem PDZs and C) Three PDZs. All the fusions are correctly expressed in yeast cells and traces of dimerization are visible in all the combinations. D) Extended version of PDZ2 and PDZ2-3 tandem, including the CaM motif upstream the canonical PDZ2 boundary.

In vivo dimerization of PDZ domains has been reported for some PDZ-containing proteins, although no information is available regarding its role. However, for those proteins studied in deeper detail, dimerization of PDZ domains seems not to influence the binding to their partners, as sites different from those involved in dimerization mediate this interaction. Because some PDZ domains need other PDZ domains

connected in tandem to fold properly and interact with their partners [Lee *et al.,* 2010], it was thought that the interaction between dCRY and INAD could require tandem PDZ domains.

In figure 13, the two hybrid assay results are reported. Single INAD PDZ domains did not display any interaction with dCRY. Prey fusions expressing tandems of PDZs linked by their native spacer sequences were still not able to interact with dCRY. An *in silico* analysis performed with CSpritz [Walsh *et al*., 2011] revealed the presence of an α-helical motif upstream the PDZ2 domain, specifically the motif MAKI (aa 235-238), which could form a unique extension of the PDZ domain and is also part of the known Calmodulin binding motif. Based on these assumptions, an “extended” version of the PDZ2-PDZ3 tandem prey fusion was generated. This extended PDZ2-PDZ3 tandem prey fusion, ranging from residue 207 to 448, once implemented in the two-hybrid system, showed high affinity for dCRY. The binding affinity was even significantly stronger than that of the whole INAD protein. This result suggests that the PDZ2-PDZ3 tandem mediates the interaction between INAD and dCRY and that PDZ2 domain needs to be extended upstream, with respect to the canonical PDZ domain boundary.

The length of the fusion sequences was further extended by adding a third domain: three different portions of INAD including PDZ1 to 3 (aa 17-448), PDZ2 to 4 (aa 249-577) and PDZ3 to 5 (aa 364-664), were thus obtained. Only the fusion expressing the N-terminal PDZ1 to PDZ3 domains showed affinity for dCRY confirming the previous data and suggesting that PDZ4 and PDZ5 are not involved in the interaction between dCRY and INAD.

A particular, non-canonical structure of the PDZ2 domain, conferring a higher binding affinity for the dCRY motif, may explain the higher signal for the extended PDZ2-PDZ3 tandem compared to larger INAD fragments. This affinity is likely reduced when PDZ1 is present, due to entropy losses caused by increased structural rigidity. The expression levels of all fusions, analyzed by western-blot on yeast lysate with an anti-HA antibody, were comparable (figure 12).

Figure 13

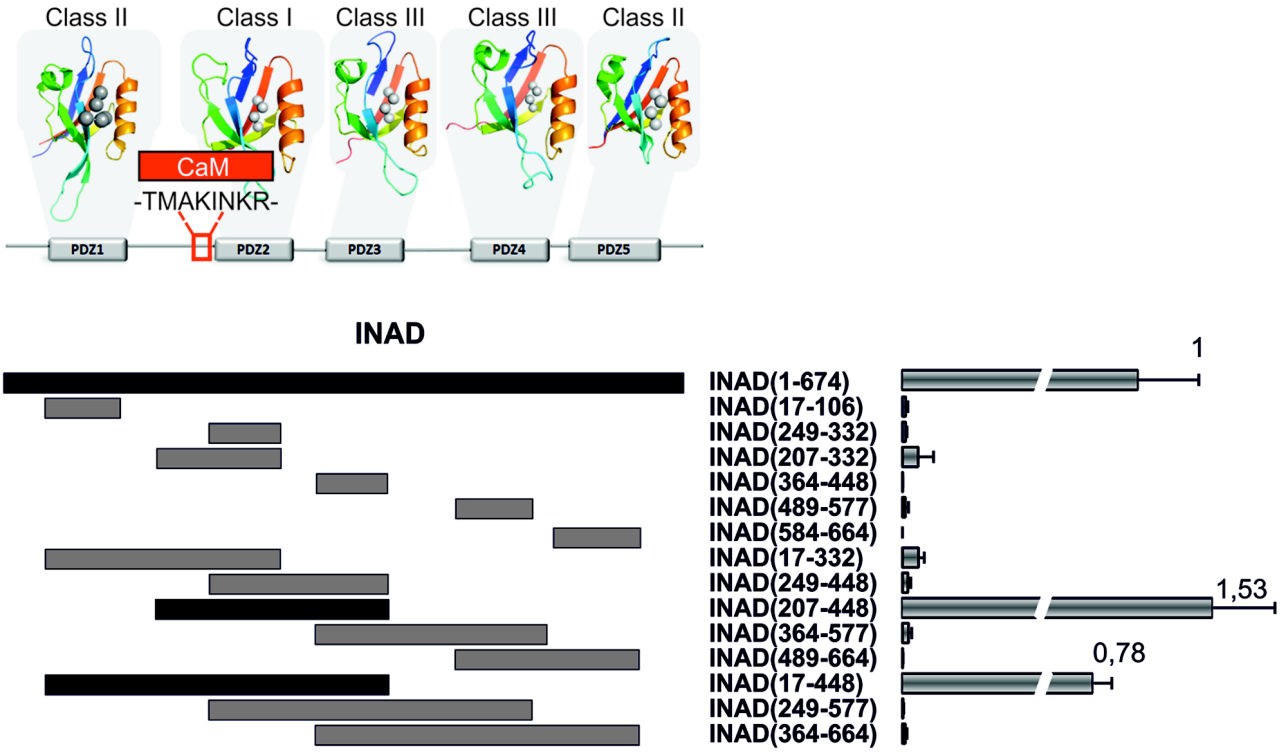


Figure 13: Identification of the interaction domains of dCRY and INAD using the yeast two-hybrid system. At the figure top, the five INAD PDZ domain sequences were structurally modeled and assigned to putative PDZ sub-types depending on the residue types at the linear motif binding site. The relevant sequence motifs are reported within rectangles on the INAD sequence.

In the second, lower part of the figure, the two hybrid assay results are reported in the same way as in figure 11. Different INAD domains were tested for interaction with full-length dCRY in the presence of light. Interacting fusions are shown in black and relative β-galactosidase activity is reported for each fusion setting the interaction signal between full-length partners as unit reference. As in the case of the dCRY test, the data reported are the mean ± SEM of at least 7 independent clones for each fusion, analyzed in triplicates. An extended version of the PDZ2-3 tandem, INAD (207-448) exhibits a significantly stronger affinity for dCRY compared to the whole protein ($F_{14,87}$ = 67.81, P < 0.0001).

2.3 dCRY is related to the Visual Cascade Components

The reported interaction between INAD and NINAC in the formation of the Signalplex [Wes *et al.*, 1999], together with the interaction between INAD and dCRY that was detected in this study, suggested that the interaction between dCRY and NINAC may

be specifically mediated by INAD. To analyze whether dCRY, INAD and NINAC form a ternary protein complex, a three-hybrid system was designed, in which dCRY was used as bait, NINAC as prey and a FLAG tagged form of INAD was selectively just expressed in the yeast nucleus. The expression of all fusions was tested by western-blot on yeast lysate with anti-HA antibody for NINAC and anti-FLAG antibody for the nuclear INAD (figure 14).

Figure 14

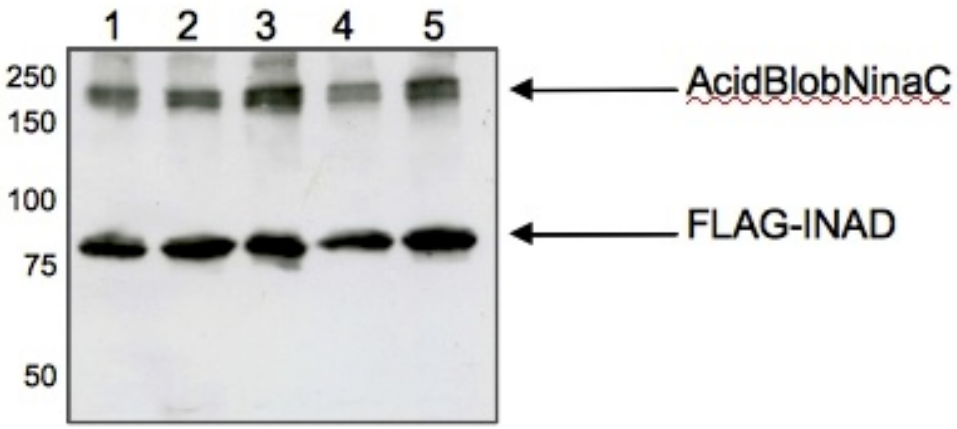


Figure 14: Western-blot analysis of independent yeast clones expressing NINAC as prey fusion and INAD in the nucleus. Independent yeast clones expressing dCRY as bait, NINAC as prey and a FLAG-tagged form of INAD specifically in the yeast nucleus were probed with anti-HA and anti-FLAG antibodies. The signal corresponding to the expressed fusions are shown.

When dCRY and NINAC were challenged in a two-hybrid system as bait and prey, respectively, no direct interaction between the two proteins was observed while expression of INAD in the nucleus, in a three hybrid system, resulted in the expression of the reporter gene, indicating that the formation of a three component complex is necessary to restore the activity of the transcription factor (figure 15).

Figure 15

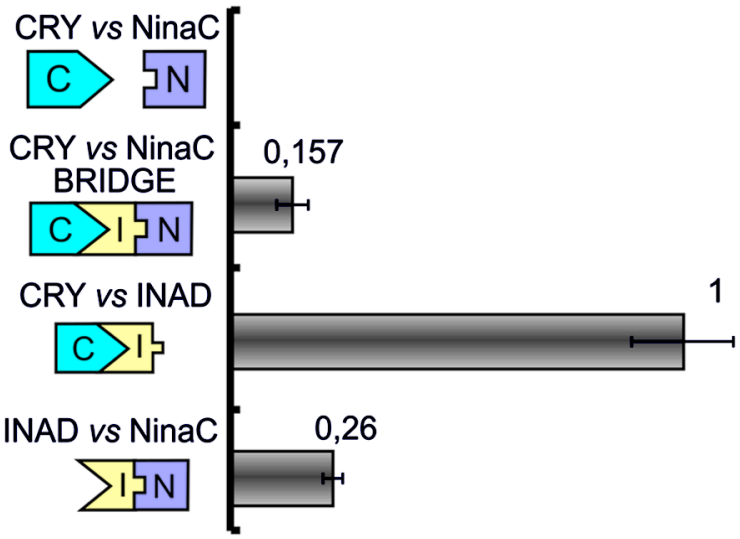


Figure 15: Yeast two- and three-hybrid assays highlighting that the interaction between dCRY and NINAC occurs through in INAD. The scheme shows the different proteins used as bait or prey fusion. C: dCRY, N: NINAC, I: INAD. Relative β-galactosidase activities are reported for each fusion. The interaction of dCRY and INAD was set as reference. The values are the mean ± SEM of at least 6 independent clones for each fusion, analyzed in triplicates. The challenge of dCRY and NINAC in a two-hybrid assay does not result in the activation of the reporter gene while the expression of INAD in the yeast nucleus, obtained through a three-hybrid system, shown β-galactosidase activity. This result implies that INAD acts as structural BRIDGE between the two proteins ($F_{3,24}$ = 57.20, P < 0.0001). To cover the entire spectrum of possibilities, also the results of two-hybrid assays of couples dCRY-INAD and INAD-NINAC are shown and both tests reported an interaction signal.

The surprising association of dCRY with the visual cascade complex could underline a role, direct or indirect, for this photoreceptor in fly vision, which has not been entertained as yet. To investigate a possible involvement of dCRY in the fly eye-mediated light response, the optomotor and phototactic behavior of flies was analyzed. Fly strains were used with dCRY completely knocked out (cry^{01}) [Dolezelova *et* al., 2007] or for which dCRY was lacking the C-terminus tail (cry^{M}) [Busza *et al*., 2004]. The optomotor response was analyzed with two different set-ups. In both set-ups, either cry^{01} or cry^{M} strains displayed impairment in their optomotor turning response (figure 16A and B).

In one case, cry^{01} and cry^{M} exhibited 61.6% and 64.2% of correct turns, respectively, while wild-type controls (Oregon *R,* WT-ALA and the progeny of a CS x w^{1118} cross) achieved 78.2%, 73.2%, and 75.2%, respectively (figure 16A). In the other case, both

cry^{01} and *cry*^{M} mutants showed an optomotor response (OR) of about 48% while control flies (the progeny of CS x *w*^{1118} and WT-ALA x *w*^{1118} crosses) an OR of about 71% and 60%, respectively (figure 16B). Whereas the 50% level in figure 16A denotes random choice behavior (no optomotor response to the moving stripes), the same value of 50% would indicate in figure 16B that the fly completed 50% of the revolutions imposed by the rotating striped drum. Therefore, the mutants' OR levels in figure 16B correspond to about 68%-80% of the wild type OR. Both optomotor experiments concordantly reveal a significant OR reduction in *cry*^{01} and *cry*^{M}. Moreover, in a phototaxis assay using countercurrent distribution in which wild-type flies orient and move toward a light source [Benzer, 1967] *cry*^{01} and *cry*^{M} mutants showed a reduced performance index of 0.41, compared to 0.63 of the progeny of CS x *w*^{1118} cross used as control (figure 16C).

Figure 16

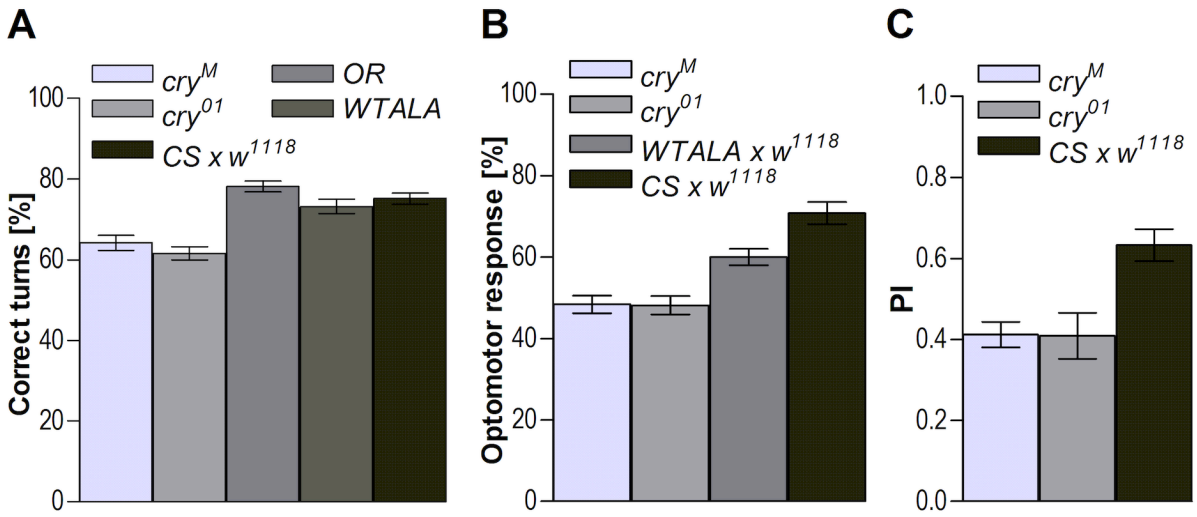


Figure 16: Visual behavior of dCRY mutants. A) Percentage of correct choices with respect to the direction of stripe motion (optomotor stimulus) inside a T-shaped tube (Set-up A, see Materials and Methods). 100 flies (males) for each of the following genotypes were analyzed: *cry*^{M}, *cry*^{01}, Oregon R, WT-ALA and CS x *w*^{1118}. Mean values ± SEM are given. Both *cry*^{01} and *cry*^{M} displayed an impairment in their optomotor turning response with respect to controls ($F_{4,495}$ = 19.53, P < 0.0001). No difference was found between the two cry mutants (P > 0.05). B) Optomotor response for *cry*^{M}, *cry*^{01}, WT-ALA x *w*^{1118} and CS x *w*^{1118} males, with Set-up B (See Materials and Methods). The mean optomotor response of single flies placed in a plexiglas arena is expressed as percentage of fly-revolutions with respect to the number of revolutions of the optomotor stimulus (striped drum). 32 flies for each genotype were analyzed. Mean values ± SEM are given. As in Set-up A, both cry mutant flies showed a reduced optomotor response with respect to the wild type flies ($F_{3,124}$ = 22.35, P < 0.0001). C) Phototaxis response for *cry*^{M}, *cry*^{01}, and CS x *w*^{1118}. The performance index (PI) is expressed as the numbers of times that flies show phototaxis in a 5 cycles test, with 0 meaning no fly showed phototaxis and 1 meaning all flies showed 5

times phototaxis. About 400 flies for each genotype were tested. Either *cry*^{01} or *cry*^{M} flies showed a significant reduction in the phototactic response with respect to the wild type flies ($F_{2,26}$ = 8.2, P = 0.002).

Further studies: Studies on the relation of dCRY with *Drosophila* visual system were carried on after the end of this thesis work.

The possible dCRY involvement in the fly eye-mediated light response was investigated with electroretinogram (ERG) tests. Wild type flies show a diurnal rhythm in visual sensitivity determined by ERG recordings, with maximal sensitivity in the first half of the night [Chen DM *et al.,* 1992]. A comparable rhythm was found in control flies (CS x *w*^{1118}) with a pronounced sensitivity and a maximum in the middle of the night. In contrast, the visual sensitivity of *cry*^{01} mutants was not dependent on the time of day. Optomotor response tests were performed to support ERG analysis. *Cry*^{01} mutants responded less than control flies to visual stimuli throughout the day, but this impairment was most evident during the first half of the night, around the wild-type flies' maximum in optomotor turning response.

To test whether the impaired optomotor response depends on CRY-function in the compound eyes, CRY was selectively rescued in the eyes with the help of the UAS-GAL4 system, driving GAL4 under control of the eye-specific *glass multiple reporter* (*gmrGAL4*) [Freeman M. 1996]. The expression of CRYPTOCHROME selectively in the compound eyes restored the optomotor response of *cry*^{01} mutants to almost wild-type levels [Mazzotta *et al.,* PNAS in revision].

DISCUSSION

The results of this study clearly indicate that the *Drosophila* circadian blue-light photoreceptor dCRY interacts with the visual transduction complex (Signalplex) through the scaffolding protein INAD.

As a general concept, in experimental design when protein-protein interactions are considered, a look beyond individual protein domains must be given due to the supra-modular nature of proteins.

In this study, it is demonstrated that a single structural domain does not mediate the interaction between dCRY and INAD: rather, the interaction occurs through a larger structural region of INAD, which includes the PDZ2-PDZ3 tandem. Furthermore, in this case, the PDZ2-PDZ3 tandem region cannot be defined with the canonical classification parameters, but needs to be extended upstream with respect to the usual PDZ domain boundary to include a stretch of amino acids known to be part of a Calmodulin (Calcium Modulated Protein, CaM) binding motif.

Interactions modulated by multiple INAD PDZ domains have been described before [Liu *et al*., 2011]. Liu and co-workers, demonstrated that the redox potential of INAD PDZ5 is allosterically regulated via a direct conformational coupling with PDZ4. The generation of this PDZ4-5 super-domain blocks PDZ5 in its reduced state and allows the system to associate with the TRP channel into the Signalplex. Long and co-workers [Long *et al*., 2003] demonstrated, for another PDZ-containing protein (PostSynaptic Density protein, PSD-95), that its first two PDZ domains bind with a synergic effect to their multimeric membrane targets. This is an interesting case, with two valid hypotheses arisen to explain the PSD-95 tandem PDZ synergic interaction: a) synergistic interaction might promote dimerization of target molecule monomers by stabilization of the dimers (C-termini cross-link) or b) target dimers have higher priority compared to monomers to interact with PSD-95.

It can be realistically hypothesized that two or three PDZ domains connected in tandem may exhibit different specificity in their target-binding properties compared to single isolated domains. This characteristic might have evolved to modulate interaction affinity with the partners, in this way affecting their availability for signaling or other cellular reactions.

With the data collected in this work, it has also established that the 20 amino acids of

dCRY C-terminal sequence are involved in the binding with INAD. It is well know that PDZ domains preferentially interact with the absolute carboxy-terminal ends of their target proteins. For instance, PostSynaptic Density protein (PSD-95) recognizes the very last C-terminal amino acidic residues of Shacker-type K^+ membrane channels [Kim *et al*., 1995]. Another example is p55, which belongs to the family of MAGUK (Membrane-Associated GUanylate Kinase) proteins and contains one PDZ domain, which binds with the last 22 carboxy-terminal residues of glycophorin C [Hemming *et al*., 1995]. Proteins like hDig, Dsh, LIN-7 and PTPL1/FAP1 are some other cases reported in the literature, of PDZ-containing proteins that recognize linear motifs within the last twenty-some C-terminal residues of their molecular partners [Saras and Helding, 1996]. Furthermore, also the ELM program predicted the presence of either Class III or Class II/IIIPDZ binding motifs in the C-terminus of dCRY that are probably involved in the interaction with INAD PDZ domains.

The last twenty amino acid residues at the very C-terminus of dCRY were shown experimentally to mediate the interaction with INAD. When this short sequence was challenged with full-length INAD in a yeast two-hybrid system, the interaction displayed was light-independent: in both yeast strains grown in light and darkness conditions, the reporter gene was expressed. These data suggest that light influences the interaction of the full-length dCRY through the photolyase related portion of the protein structure. Moreover, the fact that the INAD PDZ 4-5 tandem, known to be regulated by light-dependent conformational changes, is not involved in the interaction observed, is in support of this hypothesis. Since the short dCRY C-terminal tail was detected within the photolyase-like DNA binding pocket during crystallographic studies [Zoltowski *et al*., 2011], without any additional experimental data, it might be hypothesized that, upon light activation, the tail is released and therefore free to be bound by INAD.

The interaction between dCRY and NINAC observed *in vivo* represents quite a novel and unexpected result. In this work, the first experimental connection between dCRY and a cardinal component of the fly visual cascade [Wes *et al*., 1999] is demonstrated. Furthermore, this study proves that the interaction occurs through the scaffolding protein INAD.

A functional importance for the fly vision was found for this interaction. In fact, flies lacking CRYPTOCHROME showed to be significantly impaired in motion vision (optomotor response) and reduced phototaxis. In detail, phototaxis is mediated by all 8

photoreceptors in the compound eyes [Yamaguchi and Heisenberg, 2011], whereas motion detection depends mainly on intact vision in photoreceptors R1-6 with minor contribution of R7 and R8 [Yamaguchi *et al*., 2008 and Wardill *et al*., 2012]. dCRY is expressed throughout the entire cytoplasm of the photoreceptor cells and apparently the highest concentration is close to the rhabdomer membranes, the place in which the visual cascade occurs [Yoshii *et al*., 2008]. Therefore, dCRY may easily interact with INAD and eventually modulate TRPA1 channel opening, in interplay with the other PDZ proteins of the Signalplex. Moreover, it can be hypothesized that even low concentrations of dCRY are sufficient to promote this interaction. One of the optomotor tests and phototaxis assay were performed at the end of the day (ZT11-12), when cryptochrome level reaches its minimum concentration [Emery *et al*., 1998] and this test gave identical response as the others, performed on flies collected in different day slots.

Recently, dCRY was shown to be also involved in the membrane excitability (K^+ channel conductance) of the large ventral Lateral clock Neurons [Fogle *et al*., 2011]. These neurons fire action potentials upon illumination with blue light and this firing is dependent on dCRY. Although the way dCRY regulates l-LN_v firing rate in relation to K^+ channel conductance remains unclear, our results further support an involvement of dCRY in membrane potential modulation. To optimize *Drosophila* vision capability under different regimes of light intensity and controlling the visual coding efficiency, a functional circadian clock within photoreceptor cells is crucial [Barth *et al*., 2010].

In further studies performed in the lab, the expression of dCRY was rescued specifically in the eye of cry^0 flies: these flies showed an optomotor response comparable to wild type, supporting the evidence of the important role of dCRY in the fly vision.

The research on INAD and its interaction with CRYPTOCHROME is currently carried on with recombinant *E. coli* strains expressing the PDZ2-3 tandem sequence. The aim of this study is to purify PDZ2-3 tandem as single polypeptide construct to determine its structure with Nuclear Magnetic Resonance methodologies. The specific molecular interactions occurring between PDZ2-3 tandem and the dCRY C-terminal sequence will be investigated.

MATERIALS & METHODS

1.0 FLY STRAINS

The following Drosophila strains were used in this study:

Control strains

w^{1118}: It is characterized by: *white* (*w*, 1-1.5), recessive allele caused by a large deletion in the *white* locus, which determines the "white eyes" phenotype. The deleted gene is involved in production and distribution of brown (ommochrome) and red (phteridin) pigments present within adult fly eyes

Oregon-R and wt-ALA: Strains Oregon-R [Lewis, 1960] and wt-ALA are natural isolates [Vanin *et al*., 2012].

Canton-S: Canton-S is a common laboratory *Drosophila* strain that was collected in Ohio (U.S.A.) in 1930's.

The progeny of crosses Canton-S X w^{1118} and wt-ALA X w^{1118} were also used as wild-type controls.

Mutated strains

inaD^{1}: this *Drosophila* line has an amber nonsense mutation leading to premature termination of the INAD polypeptide chain. The strain is a completely null INAD mutant [Tsunoda et al., 1997].

ninaC$^{\Delta 174}$: This strain was generated by oligonucleotide directed mutagenesis. In this case the mutation is a T➔G transversion that prevents the RNA splicing of ninaC isoform 174. This *Drosophila* line expresses only ninaC isoform variant 132 and results null for ninaC variant 174 [Porter *et al*., 1992].

cry^{0}: this line is characterized by a knock out of the entire cryptochrome gene. The construct for homologous recombination was designed such that the entire coding sequence of the cryptochrome allele was replaced by mini-*white* [Dolezelova *et al*., 2007].

cry^{M}: *Drosophila cry*^{M} carry a truncated form of cryptochrome that lack of the C-terminal tail for the last 19 amino acid residues. The photolyase domain is left intact [Busza *et al*., 2004].

yw; timGal4: this strain was used to activate the tissue specific transcription of the analyzes genes by the UAS-GAL4 system. The Gal4 sequence is controlled by the promoter of the circadian gene timeless, whose transcription oscillates during the 24 hours with a peak of maximum expression at the beginning of the night. This driver expression in all tissues where are expressed both elements of the central clock (lateral neurons that drive the circadian rhythmicity) and elements of the pheripheral clock [Emery *et al*., 1998].

w; UAS-HAcry 16.1: transgenic line which express the fusion protein HACRY after activation by the UAS-GAL4 system. The yeast upstream activation sequence (UAS) is the binding site for the GAL4 protein that allows the transcription of the construct. The UAS sequence controls the expression of dCRY full-lenght cDNA fused at the n-terminal with the HA (hemoagglutinin) coding sequence [Dissel *et al*., 2004].

All flies were reared on a standard yeast–glucose–agar medium and maintained at 23°C, 70% relative humidity, on a 12h light : 12h dark cycle (LD 12:12).

2.0 *Drosophila* HEAD RNA EXTRACTION & RETRO-TRANSCRIPTION

Drosophila wt-ALA flies were synchronized by light-dark cycles (LD 12:12; 500 Lux : 0 Lux) for at least 3 days at 23°C, then flies were collected at ZT1 and ZT12 (ZT: Zeitgeben time) and frozen in liquid nitrogen.
RNA was extracted using Trizol (Invitrogen) as indicated in manufacturer data sheet instruction. The whole procedure must be carried out in RNAase free conditions.

The procedure is as follow:

RNA extraction

Homogenize 50 fly heads in 1mL Trizol;

Incubate 5', T_{amb};

I. Add 0.2 mL chloroform;
II. Mix for 30";
III. Centrifuge 13200 rpm for 15' at 4°C
IV. The mixture is separated in 3 phases. Transfer the aqueous upper phase in a new vial;
V. Add an iso-volume of isopropanol to the aqueous phase;
VI. Incubate 15', T_{amb};
VII. Centrifuge 13200 rpm for 15' at 4°C;
VIII. Remove the liquid phase and wash the pellet with ethanol/water 70% v/v;
IX. Centrifuge 13200 rpm for 5' at T_{amb};
X. Dry the pellet and resuspend it in RNAase free water.

Retrotranscription

(total reaction volume 20µL)

I. Mix 1µg of RNA with 1µL of dNTPs (10µM) and 1µL oligo dT (10µM);
II. Incubate 5', 65°C;
III. Add 4µL of reaction buffer 5x (Invitrogen), 2µL DTT (0.1M), 1µL RNAse OUT (100 u/mL) and 1µL Superscript II (100 u/mL) (Invitrogen);
IV. Incubate at 42°C for 1h;
V. Incubate at 70°C for 15'.

3.0 CLONING GENERALITIES

The molecular biology techniques applied along this work were based on the current laboratory practice and followed standard protocols [Maniatis and Sambrook, 2001], where not specifically stated otherwise.

3.1 Gene PCR

High fidelity polymerase was used for gene amplification: Phusion® High-Fidelity DNA Polymerase (Finnzymes OY, Finland). This mutated polymerase derives from *Pyrococcus* strains and shows enhanced processivity. The error rate is low ($4.4 \bullet 10^{-7}$ in HF Phusion® buffer) with respect to standard enzymes and between 50 and 6 folds lower than other engineered polymerases such as *Thermus acquaticus* or *Pyrococcus furiosus*.

As consequence of the polymerase employed, to set the PCR thermo-cycling parameters the following indications were followed:

a) peculiar characteristics of the Phusion® polymerase, which requires 98°C as denaturation temperature and 15-30 second per kb at 72°C as elongation parameters.

b) elongation time needs to be in accordance with the length of the nucleotide sequence to be amplified. It this work elongation time ranged from few minutes for ninaC and inaD full gene amplifications from *Drosophila* cDNA to 20 seconds for shortest sequences such as dCRY C-terminal tail.

c) primer annealing temperature needs to be as higher as allowed trying to combine it with a reasonable primer design length. In any case never below 55°C. To calculate the optimal annealing temperature in HF Phusion® buffer as basic rule 3°C were added to the lower primer calculated melting temperature. At www.finnzymes.com an online tool is available for calculating the optimal primer annealing temperature in Phusion® buffers.

3.2 PCR product first cloning step

As normal procedure all PCR products where firstly inserted into pGEM® T Easy vector (Invitrogen) for amplification and sequencing (figure MM1).

Figure MM1

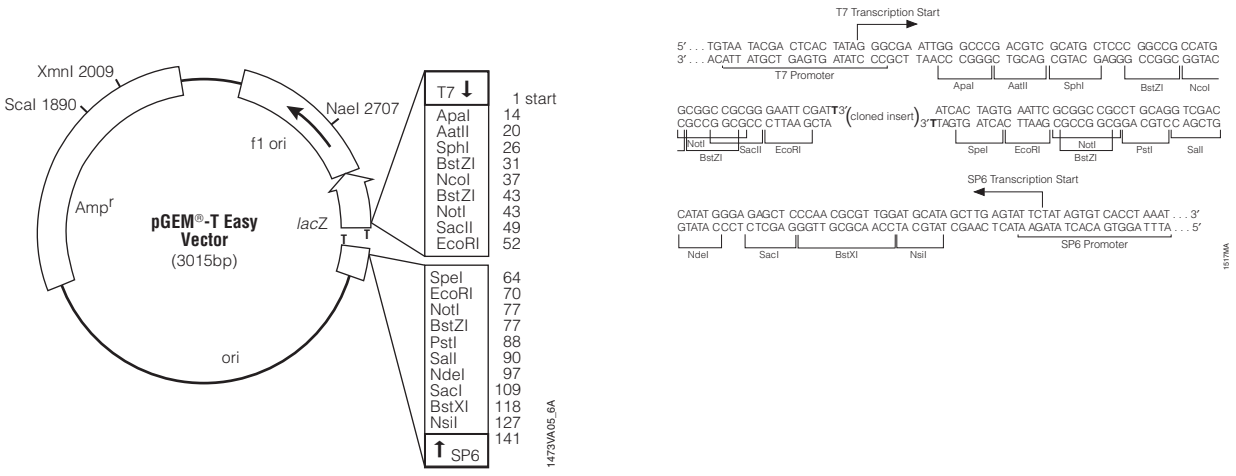


Figure MM1: pGEM® T Easy vector map and mcs scheme from Promega technical manual TM042 6/09

pGEM® T Easy is a linearized vector with a single 3' terminal thymidine at both ends. These two thymidine residues allow an easy insertion for the PCR product. Usually normal polymerases leave an adenine residue hanging at 5' ends. High-fidelity polymerase (as Phusion®), normally do not leave adenine residues at product extremities, so a "tailing step" with a normal polymerase is required prior ligation in pGEM® vectors. This step is simply performed by adding directly to the PCR tube, at the end of the high-fidelity amplification, a standard polymerase and incubate at 72°C for additional 30 minutes.

A preliminary cloning step into a specifically designed vector is very useful because once detected the correct clones the plasmid can be expanded within the bacteria shuttle system in high copy number without compromising sequence accuracy. Furthermore enzymatic cuts are much efficient within nucleotide sequences rather than at extremities.

Blue/White colonies screening, enzymatic digestion and sequencing was the normal procedure applied during this work to obtain the correct constructs in bacterial host.

4.0 YEAST TWO AND THREE HYBRID SYSTEMS

4.1 Introduction

In molecular biology, eukaryotic two-hybrid system allows to identify any interaction between proteins, or fragments of them, by the expression of a reporter gene. The system is based on the fact that the eukaryotic transcription factors are able to function even if the DNA binding domain and the transcription activation domain are located on different proteins. These domains do not have to belong to the same polypeptide to be able to promote the reporter gene expression.

Experimentally, the system provides for the generation of two fusion hybrids in which the two potential partners are respectively fused to the DNA binding domain (BAIT) and the activation domain (PREY) of a transcription factor. Once the two constructs are expressed if a physical interaction occurs, this will lead to the formation of a fully active transcription system and consequently the reporter gene expression.

Three-hybrid systems are set up when it is thought that two proteins may not interact in a direct way, but by means of a third partner, which acts as a structural "bridge" between them.

In this work, yeast *Saccharomices cerevisiae* (strain: EGY48 - MATα, *ura3, trp1, his3,* 3LexA-operator-LEU) was used for all two and three hybrid tests.

4.2 Yeast Two Hybrid System

In this study the Golemis and Brent approach was applied [Golemis and Brent, 1997].

BAIT construct: dCRY, either full-length or fragments, is in fusion to the LexA moiety in the bait vector (vector pEG202).

PREY construct: INAD (full-length or fragments) is in fusion with the “acid-blob” portion of the prey vector (vector pJG4-5)

REPORTER construct: bacterial β-galactosidase is expressed in case of Pray-Bait interaction from the Reporter vector (vector pSH18-34)

In figure MM2 the vector maps, utilized in this study to set up two and three yeast hybrid systems, are depicted.

11.2.1 Experimental construction of the system

The mRNA from heads of w^{1118} flies was extracted and retro-transcribed to obtain the relative cDNA.
Full-length INAD coding sequence was amplified from the cDNA with primers INAD-FL-F and INAD-FL-R (table 2). Those primers add additional *NdeI-EcoRI-AatII* and *XbaI-XhoI-HindIII* restriction sites respectively.
The PCR product underwent a first cloning step, in which it was inserted into pGEM® vector, expanded in bacteria host, purified and sequenced to control for unwanted mutations. Correct construct was digested with *EcoRI* and *XhoI* and directionally cloned in the pJG4-5 vector linearized with the same enzymes. After this passage the final construct was newly sequenced in order to assure the in-frame insertion of the inaD gene.
All the constructs with the different INAD fragments (single PDZ domain, two and three PDZ tandem elements) were obtained with the same strategy. pJG-INAD full length was used as template for all fragment PCRs. The total list of primer used is reported in table 2.

TAG stop codons were incorporated in all reverse primers before the *XbaI* restriction site. The reliable expression of prey fusions in the EGY48 yeast strain transformed with the bait vector were confirmed by immunoblot. Protein extracts were obtained with common procedures [Ausbel *et al*., 1989], subjected to SDS-Page (NuPAGE-Invitrogen®) and probed with a specific anti-HA antibody (SIGMA, 1:5000). Expected molecular weights for the tested fusions are listed in table 1.

4.3 β-galactosidase Assay

Quantification of β-galactosidase activity was performed in liquid culture as in Ausbel *et al*. (1989) and each experiment was repeated at least three times.
The procedure is as follow:

I. Pre-culture: Incubate single yeast colony in 3mL of minimal selective medium for 24 hours at 30°C
II. Transfer 50μL of pre-culture in 3mL of minimal inductive medium and incubate for 48 hours
III. Centrifuge 2mL of culture for 3' at maximum speed and remove the surnatant
IV. Resuspend yeast pellet with 2mL of Z-Buffer and read absorbance at 600nm (OD_{600})
V. Dilute 1:10 the solution IV. in Zbuffer (100μL in 900μL)
VI. Add to solution V. 40μL SDS 0.1% and 80μL chloroform
VII. Vortex for 15"
VIII. Equilibrate for 15' at 30°C
IX. Add 200μL of substrate solution and vortex for 5"
X. Incubate at 30°C untill the solution becomes pale yellow (usually 30', but if the interaction is very strong, and then there is a high expression of β-galactosidase, 2-5' is enough). Keep record of the incubation time.
XI. Stop enzymatic catalysis by addition of 500μL blocking solution.
XII. Centrifuge 5' at 2500rpm
XIII. Read absorbance at 420nm and 550nm (OD_{420} and OD_{550}).
XIV. Apply the following formula to obtain the β-galactosidase activity per minute:

$$U = [1000 \bullet (OD_{420} - (1.75 \bullet OD_{550})] / (t \bullet V \bullet OD_{600})$$

Where: t is the reaction time in minutes
V: is the cell volume of step V. in mL (with this protocol it is 0.1mL)

Solutions:

Minimal selective medium:

Yeast Nitrogen Base ..... 0.67% w/v

Glucose ..... 2% w/v

Leucine 10mM

Minimal inductive medium

Yeast Nitrogen Base	0.67% w/v
Galactose	2% w/v
Raffinose	1% w/v
Leucine	10mM

Zbuffer

$Na_2HPO_4•7H_2O$	60mM
$NaH_2PO_4•H_2O$	40mM
KCl	10mM
$MgSO_4•7H_2O$	1mM
β-mercaptoethanol	50mM

Substrate solution

Ortho-NitroPhenyl-β-Galactoside	0.4% w/v
$NaH_2PO_4•H_2O$	40mM
K_3PO_4 (pH 7.0)	100mM

Blocking solution

Na_2CO_3	1M

4.4 Yeast Three Hybrid System

In this experiment, dCRY full-length has been used as bait and NINAC (p174) as prey. The coding sequence of NINAC (p174) was amplified from cDNA retro-transcribed form w^{1118} flies head mRNA, with primers NINAC-5F and NINAC-PBR (table 2) that add a *SalI* restriction site at both ends. The PCR product was directly digested with *SalI* and cloned into pJG4-5 vector linearized with *XhoI*. *SalI* and *XhoI* generate compatible ends, but once used this cloning method, both restriction sites are destroyed. Clones with the insert in the right orientation have been fully sequenced in order to assess the in-frame insertion of the cDNA and to control for unwanted mutations.
Correct expression of NINAC polypeptide was checked by western blot with the anti-HA antibody.

4.4.1 Third partner construction

In order to obtain a three-hybrid system, expression of INAD in the yeast nucleus was required. This purpose was achieved by cloning the full-length INAD in a modified version of the pDBLeu vector (Invitrogen), where the DNA binding domain was removed by restriction with *HindIII* and *SalI*. The coding sequence of INAD was amplified directly from *Drosophila* head cDNA, with primers inaD-NLS-FLAG_F and inaD-Xho-R.
Primer inaD-NLS-FLAG_F: it is a long primer, which adds a *HindIII* site at 5' end of the PCR product in frame with sequences for a Nuclear

Localization Signal and a FLAG tag necessary to drive and recognize INAD into the cell nucleus.

Primer inaD-Xho-R: it adds *XhoI* site at 3' end of the PCR product.

The PCR fragment was directly digested with *HindIII* and *XhoI* and directionally cloned in pDBLeu *HindIII-SalI*. Positive clones were sequenced in order to check for unwanted mutations. The expression of the nuclear form of INAD was assessed by western blot on protein extracts with a specific anti-FLAG antibody (SIGMA, 1:500).

β-galactosidase activity was quantified as previously described.

Figure MM2

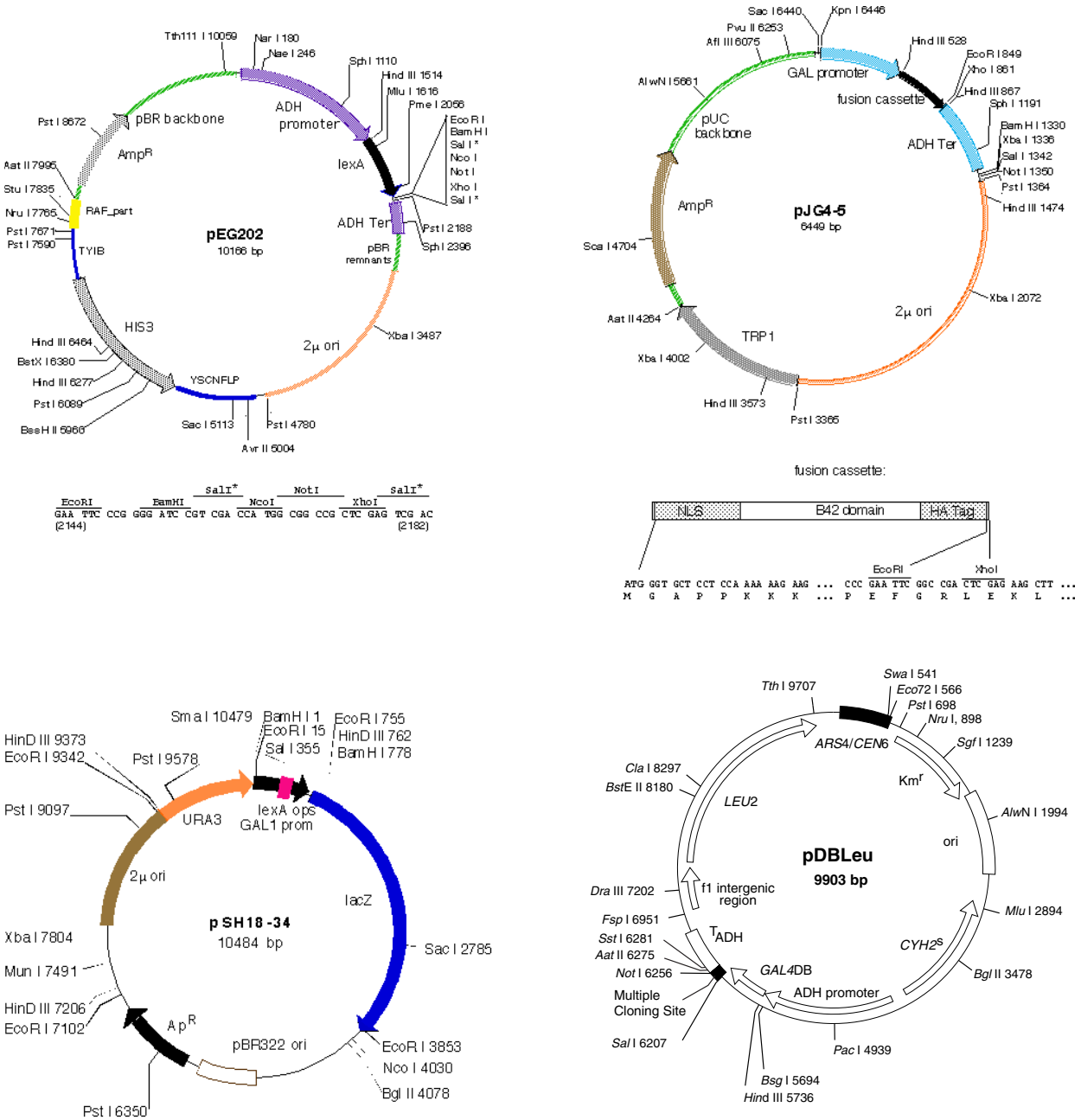


Figure MM2: pEG202 bait vector contains the ADH constitutive promoter, which drives LexA expression. Downstream the LexA coding frame a multiple cloning site is placed to allow the insertion of the polynucleotide sequence of interest. This vector has 2μ yeast origin of replication and complement for the Histidine synthesys (HIS3), which lacks in the yeast strain used. In can be expanded in bacteria by having the pBR origin of replication and ampicillin resistance.

pJG4-5 prey vector harbors a fusion cassette containing a nuclear localization domain of virus SV40 and an acidic domain of transcription activation. Furthermore a hemoagglutinin epitope (HA tag) is present right before the cloning site of the desired protein. This expression cassette is under control of GAL1 promoter (galactose inducible). This vector complements for Tryptophane biosynthesis through gene TRP1, as pEG202 it has a 2μ origin of replication in yeast. To be expanded in bacteria it carries ampicilline resistance and the pUC origin of replication.

pSH18-34 LacZ reporter vector carries the β-galactosidase gene under control of 8 LexA binding sites in tandem and complements through gene URA3 to uracil biosynthesis deficiency in the yeast strain.

pDBLeu is a commercial vector from Invitrogen. In this work, vector pDBLeu was modified by removing the DNA binding domain sequence by restriction with *HindIII* and *SalI*.

Table 2

Primer	Lenght (bp)	Position	Direction	Sequence (5'→3')
InaD_FL_F	36	1–18	F	CATATGGAATTCGACGTCATGGTTCAGTTCCTGGGC
InaD_FL_R	36	2007-2025	R	TCTAGACTCGAGAAGCTTCTAGGCCTTGGGTGCCTC
InaD_PDZ1_F	36	48-66	F	CATATGGAATTCGACGTCATGGTGACCCTGGACAAG
InaD_PDZ1_R	42	297-318	R	TCTAGACTCGAGAAGCTTCTAGTCGAAGGTCTGAATCTCCAG
InaD_PDZ2_F	36	744-762	F	CATATGGAATTCGACGTCAGGATCGAGGTCCAGAGG
InaD_PDZ2bis_F	42	559-582	F	CATATGGAATTCGACGTCGACGAGGACACCCGGGACATGACC
InaD_PDZ2_R	39	978-996	R	TCTAGACTCGAGAAGCTTCTAGCGTCGCGAGGTGATCAT
InaD_PDZ3_F	36	1080-1098	F	CATATGGAATTCGACGTCTTTCCCAAGGCGCGCACG
InaD_PDZ3_R	45	1311-1335	R	TCTAGACTCGAGAAGCTTCTACAATAGAATCATGGTCACTACGCC
InaD_PDZ4_F	42	1464-1488	F	CATATGGAATTCGACGTCCTCATTGAGTTGAAGGTGGAAAAG
InaD_PDZ4_R	39	1713-1731	R	TCTAGACTCGAGAAGCTTCTAAGGATCAGCGCGGAAGAC
InaD_PDZ5_F	41	1749-1772	F	CATATGGAATTCGACGTCAACGTTGACCTTATGAAAAAAGC
InaD_PDZ5_R	40	1973-1992	R	TCTAGACTCGAGAAGCTTCTACTTGGGTCGTGTCACTTCC
CRYdeltaF	29	1561-1581	F	CCGAATTCCCGCATTGCCGACCATCCAAC
CRYR	33	1604-1629	R	CCCTCGAGTCAAACCACCACGTCGGCCAGCCAG
InaDNLSFLAG_F	112	1–31	F	CCAAGCTTGAATTCATGGATTACAAGGATGACGACGATAAGGGTGCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGGTATCAATAAAGTTCAGTTCCTGGGCAAACAGGGCACCG
InaDXhoR	31	2006-2025	R	GGTCGACTCGAGCTAGGCCTTGGGTGCCTCC
Nina5F-Sal	32	1–24	F	CCGTCGACATGATGTATTTACCGTACGCGCAA
NinaPB3-Sal	31	4484-4506	R	GGGTCGACTTAGATATCGACGGCATAGCCTG

Position reflects nucleotide location in FLYBASE: Fbgn0001263 (INAD), Fbpp0079064 (NINAC), Fbpp0083150 (dCRY).

5.0 *Drosophila* HEAD PROTEIN EXTRACTION AND CO-IMMUNOPRECIPITATION

5.1 *Drosophila* Head Proteins Extraction

3-5 days old flies overexpressing HA-dCRY (*yw*; *tim-GAL4/+; UAS-Hacry/+*) were collected at ZT 24 (ZT 0 lights-on and ZT 12 lights-off in a 12:12 light-dark cycle) and after a 15 min light pulse given at the same time point. Heads were homogenized in extraction buffer [20 mM Hepes pH 7.5, 100 mM KCl, 2.5 mM EDTA pH 8.0, 5% Glycerol, 0.5% Triton X-100, 1 mM DTT, complete protease inhibitors (Roche)], centrifuged at maximum speed for 10 min and the supernatant precleared with protein-G agarose beads (SIGMA) for 20 min.

5.2 *Co-Immunoprecipitation*

The extract was then incubated with anti-HA (1:1000, SIGMA) for 2 h at 4°C before the addition of 30 µl of protein G agarose beads (1:1 slurry) for 1 h. The beads were precipitated by centrifugation at 2000 g and then washed 3 times with 1 ml of extraction buffer and once with 1 ml of 20 mM Hepes, pH 7.5.
For electrophoresis proteins have been detached from the beads by the addition of loading buffer (LDS - Invitrogen®) and heating at 70°C for 10 min and analyzed by SDS-PAGE on 4-12% NuPAGE® Novex® Bis-Tris Gels (Invitrogen®).

6.0 WESTERN-BLOT

Following transfer onto nitrocellulose filters, proteins were analyzed by western blotting using the following antibodies: rabbit polyclonal anti-INAD [Wes et al., 1999], (1:500), rabbit polyclonal anti NINACp174 [Porter et al., 1992] (1:500), mouse anti-HA (SIGMA, 1:5000), mouse anti-FLAG (1:5000).

7.0 OPTOMOTOR ACTIVITY IN MUTANT VS CONTROL FLIES

7.1 Set-up A

The walking optomotor test was performed as in Zordan *et al*., (2006). In particular, 3-8 days old flies (entrained in a 12:12 LD cycle) were placed in a T-shaped tube with the longer arm painted black, placed in the center of an arena located inside a rotating

drum and tested between ZT1 and ZT4. The internal walls of the drum were painted with alternating black and white stripes and the apparatus was illuminated from above with a white light (2500 lux). Attracted by the light, tested flies exited the darkened arm of the T tube and were then exposed to the black and white rotating drum. Normal flies tend to move in the same direction as the rotating environment. The test was repeated ten times for each fly: five with clockwise and five with counterclockwise rotations. Each fly was thus scored for the number of correct turns taken in the ten trials.

7.2 Set-up B

5-6 days old flies were starved for 3 hours before the experiments in order to increase the general activity level. Between ZT11 and ZT12 single flies were put in a plexiglass arena (Ø 3 cm, h 1,5cm) and placed in the middle of an upright cylinder (Ø 8 cm, h 4,5 cm). This cylinder shows vertical stripes in black and white, which can rotate around the arena and therefore gives the stimulus for the optomotor behavior. The illumination is provided by a ring of white LEDs around the upright cylinder. For the experiment the flies were dark adapted for 10 minutes. Then the cylinder rotated for 5 minutes clockwise with a speed of 10 turns per minute. After 30 seconds of darkness the cylinder rotated 5 minutes counterclockwise with the same speed. Wildtype flies follow the stripes and therefore move clockwise in circles in the first 5 minutes, and counterclockwise in the second interval. For quantification the number of fully absolved rounds into the right direction were counted. The given values are the number of turns divided by the number of possible turns (2 x 5 minutes with a speed of 10 turns per minute gives 100 possible turns). For each genotype 32 flies were tested.

8.0 PHOTOTAXIS

The experiments for phototaxis have been done as described in Benzer 1967. The light source for the experiment was a fluorescent lamp (intensity of the light at the apparatus: approximately 3000 lux. The experiment consisted of 5 cycles, whereby the flies were able to run from one tube into another for 15 sec. At the end the flies were distributed within 6 tubes. By counting the number of flies in the different tubes a performance index (PI) was calculated with 0 meaning no fly showed phototaxis and 1 meaning all flies showed 5 times phototaxis. For each genotype using about 400 flies have been tested.

9.0 STATISTICAL ANALYSIS

All the results were expressed as means ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) and Unpaired Student's t-test to determine significant differences. P values <0.05 were considered statistically significant. Bonferroni's Multiple Comparison tests was applied for post hoc comparison.

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