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Evaluation of the steric impact of flavin adenine dinucleotide in *Drosophila melanogaster* cryptochrome function

Alessandro Masiero [a], Simona Aufiero [a], Giovanni Minervini [a], Stefano Moro [b], Rodolfo Costa [c], Silvio C.E. Tosatto [a,*]

[a] Dept. of Biomedical Sciences, University of Padova, Padova, Italy
[b] Dept. of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy
[c] Dept. of Biology, University of Padova, Padova, Italy

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ABSTRACT

Photoreceptors are crucial components for circadian rhythm entrainment in animals, plants, fungi and cyanobacteria. Cryptochromes (CRYs) are flavin adenine dinucleotide (FAD) containing photoreceptors, and FAD is responsible for signal transduction, in contrast to photolyases where it promotes DNA-damage repair. In this work, we investigated an alternative role for FAD in CRY. We analyzed the *Drosophila melanogaster* CRY crystal structure by means of molecular dynamics, elucidating how this large co-factor within the receptor could be crucial for CRY structural stability. The co-factor appears indeed to improve receptor motility, providing steric hindrance. Moreover, multiple sequence alignments revealed that conserved motifs in the C-terminal tail could be necessary for functional stability.

1. Introduction

Light sensing is a fundamental task of living organisms, which have evolved in a rhythmic environment characterized by endless light–dark cycles. Light sensing is present in all kingdoms of life, due to several evolutionary strategies, which have provided organisms with this capability. One of these is light sensing by means of a protein termed photoreceptor [1]. Cryptochromes (CRYs) are blue-light sensing receptors, first identified in *Arabidopsis thaliana* in 1993 [2]. The hidden nature of their co-factor, which remained unknown for a long time, gave name to this particular class of proteins [1]. They are classified as close relatives to photolyases (PLs), with whom they share a major structural part termed photolyase domain and the presence of a FAD co-factor, which is contained in an alpha-helix domain in both PLs and CRYs. PLs work by capturing electrons and providing them to reverse DNA damage, particularly removing pyrimidinic base dimerizations. In the receptor region, where PLs interact with DNA, CRYs show a longer C-Terminal Tail (CTT) [3]. In spite of their structural and sequence similarity to photolyases, CRYs show a different kind of activity [4,5]. Indeed, they are involved in Insects in circadian rhythm entrainment and magnetosensitivity while in mammals they act as transcription repressors in the main negative feedback loop at the core of the clock. Moreover they lack DNA-repair activity in animals [6]. CRYs are therefore widely present in eukaryotic organisms, with a flavin-adenine-dinucleotide (FAD) co-factor [1] providing blue light sensing activity. FAD works as an antenna, capturing photons and transducing them in a chemical reduction response. Unlike mammals, CRY has only one ortholog *Drosophila melanogaster* (dCRY). dCRY is rhythmically expressed in both *Drosophila* clock neurons and the compound eye, responding to light/dark variations [7]. During light exposure, dCRY gets activated and opens its CTT [3,5], thus binding to TIMELESS (TIM) and promoting its proteasomal degradation, a process which the F-Box protein Jetlag (Jet) [8,9]. In darkness, TIM forms a dimer with PERIOD (PER). The TIM–PER heterodimer enters the nucleus, inhibiting the CLOCK and CYCLE protein interaction [7,10]. On the other hand, when dCRY is binding TIM, it inhibits formation of the TIM-PER heterodimer, allowing CLOCK and CYCLE to interact with a specific E-box DNA segment promoting transcription of clock genes [7,10]. This pathway is located at the core of the circadian clock in *D. melanogaster*, and is responsible for its synchronization to the natural light–dark cycles characterized by a 24 h period through dCRY light sensing [7]. At a molecular level, receptor activation passes through absorption of a photon by FAD, causing its reduction with electrons towards a radical semiquinone state. FAD chemical reduction appears to play a role in receptor C-terminal tail (CTT) opening. The CTT seems to be involved in protein–protein interactions under light exposure, as it contains

* Corresponding author. Fax: +39 049 8276260.
E-mail address: silvio.tosatto@unipd.it (S.C.E. Tosatto).

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plenty of known linear motifs [6], in particular PDZ motifs. One crucial interaction between dCRY CTT and the CTT-coupled motif appears to be mediated by a motif involving the residues FFW on the CTT, as demonstrated in [3]. Flavin ring reduction after light exposure and subsequent CTT opening allows TIM, which contains the FFWL motif as well, to interact with dCRY, putatively in the CTT-coupled motif [3]. In 2011, Zoltowski and co-workers solved a 2.30 Å resolution X-ray dCRY crystal structure in the dark state (PDB identifier: 4GU5, former 3TVS, Fig. 1) [11]. This provided a basis for deeper understanding of the photoreceptor structure and function. FAD is required for receptor activation, as it works like an antenna. However, its structural role in the receptor has not been widely investigated so far.

To explain the role of the co-factor and of the close Mg^{2+} ion contained in the dCRY FAD binding pocket and their impact on the protein structure, we investigated the time dependent behavior of the holoprotein (HdCRY), the apoprotein (AdCRY) and the system containing only Mg^{2+} (MdCRY) with molecular dynamics (MD) simulations. Moreover, a multiple sequence alignment was performed, adding value and providing new clues on dCRY activation and mechanism. The results of this work suggest that FAD presence has not only a functional meaning, but it may be required to provide an overall increase in fluctuation, decreasing the amount of necessary light input energy to activate the photoreceptor.

2. Methods

2.1. Molecular dynamics simulations

The crystal structure of dCRY containing two 539 residues long chains was used as starting model for all simulations (PDB code: 4GU5, Fig. 1) [11]. The structure contains 30 α-helices (267 residues, 49% of the sequence) and 10 β-strands (37 residues, 6%) and is characterized by the presence of one FAD molecule in bent conformation [12] and one Mg^{2+} ion in the binding pocket. All simulations were carried out with NAMD v 2.9 [13], using the CHARMM-27 force field [14] on a standard x86 Linux workstation. A phosphorylation on S536 was added through the available NAMD stream files for phosphoserine, which were added to both the topology and parameter files. We used an explicit TIP3p solvent model with cubic boxes of 100 Å × 94 Å × 81 Å, containing ~22,500 water molecules. Periodic boundaries were set at 10 Å from the most external protein atom in the corresponding cartesian axis. All boxes were placed in particle-mesh Ewalds (PME) grids and the overall system charge was neutralized in a 0.150 M NaCl medium. Solvent ions were added with VMD [15], which was also used to generate topology files. The co-factor was kept in its quinonic form in order to avoid any activation-prone movement. The bent conformation [16] was kept during the entire simulations. Oxidized FAD parameters were derived from [17] and implemented in the CHARMM-27 [18]. All simulation runs consisted of 100 conjugate gradient minimization steps, 100 ps in NVT conditions, 100 ps in NPT conditions and 20 ns of classical molecular dynamics simulation. In all simulations, the temperature was kept at 300 K and pressure at 1.01325 bar, excluding NVT pre-simulation steps. The integration timestep was 2 fs and the integrator was based on the Verlet method [13]. The system was analyzed by four different MD simulations, consisting in the holoprotein crystal structure simulation, a simulation containing a phosphorylation in Ser526 previously reported by Hemsley et al. [6], a simulation containing only the Mg^{2+} ion and a last one without FAD, Mg^{2+} and containing no PTMs. The four runs were then compared in terms of root mean square deviation (RMSD) and root mean square fluctuation (RMSF) to explain the role of each different system component. RMSF indexes are reported as both plots and structure coloring and thickening.

2.2. Trajectory analysis

All obtained trajectories first went through visual inspection with Chimera [19], cleaning and analysis with Carma [20] and EUCB [21]. The first step consisted in water, ion and co-factor removal and system recentering with Carma, obtaining trajectory files containing only the protein structure and a constant fitted center of mass. The second step consisted in backbone RMSD and RMSF index calculation and plot generation with EUCB. In order to obtain information about the flexibility of protein regions in the structures, images were generated coloring and thickening residue ribbons proportionally to their RMSF values with Chimera. To obtain these images, the occupancy column of the structure files was replaced by the residue RMSF value with an *ad hoc* script. All colored structures refer to the last frame of the respective run.

2.3. Structure analysis

Both dCRY [11] and (6–4)-Photolyase (PL) [22] structures went through RING [23] and BLUUES [24] analysis. Ring generates a network with residues depicted as nodes and interactions depicted as edges, and provides information on conservation and residue interactions. RING found 33 sequences, and calculated overall residue conservation among them. BLUUES provides an electrostatic potential analysis.

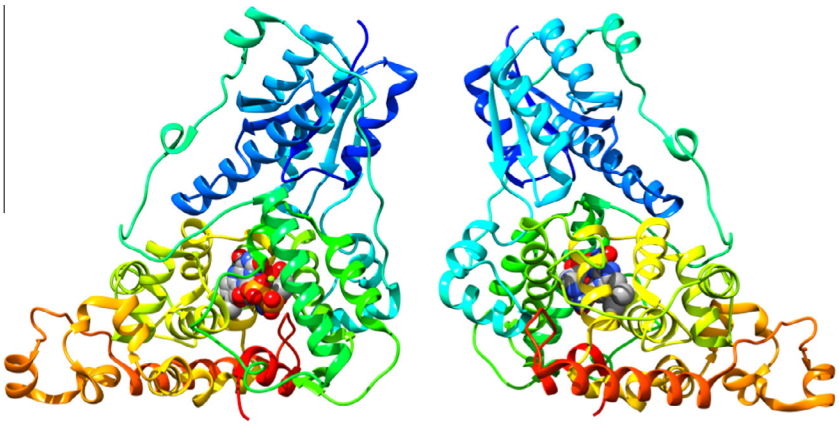

Fig. 1. *Drosophila melanogaster* cryptochrome structure, PDB identifier: 4GU5. FAD co-factor is represented in spheres, while the receptor is depicted in ribbons.

2.4. Multiple sequence and structure alignments

The previously considered Drosophila-like-CRY sequences [4] were retrieved by Blast search using dCRY as a query. TIM sequences were collected from the same species with the same approach. The multiple sequence alignment was constructed with T-Coffee [25] using a BLOSUM62 matrix. The number of extracted sequences is 36 of which 18 are available for both dCRY and TIM. A sequence logo was built with Weblogo [26] from this data and overlaid with the experimental dCRY peptide structure (PDB ID: 4GU5). Intrinsic disorder for dCRY CTT and TIM sequences and secondary structures for TIM was predicted with CSpritz [27]. The structures (PDB IDs: 4GU5 and 2WB2) were superposed with Chimera, using the Needleman-Wunsch algorithm with the BLOSUM62 matrix. The RMSD between the protein structures was 0.963 Å.

3. Results

3.1. Structural impact of the co-factor

The rationale for this work was first the clarification of FAD impact on structure stability by better investigating the behavior of its surrounding. To have a comparison between two FAD containing systems and to better highlight its impact on the structure, we performed a comparison between the holoreceptor, kept as a blank, and the holoreceptor containing a previously reported phosphorylation in S526 (PdCRY), which is a known occurring event [6]). In order to investigate the co-factor impact on its surrounding protein elements, MD simulation outputs were evaluated in terms of RMSD and RMSF. *A priori*, we hypothesized that FAD presence would improve the overall receptor stability through intermolecular interactions and its removal from the binding pocket would yield receptor misfolding. To evaluate the structural role of the co-factor, the first index used was RMSD from trajectory files obtained by 20 ns MD simulations. RMSD evaluates the mean receptor backbone distance between the first and other frames generated for a given trajectory. RMSD plots of the systems showed how the more stable conformations are those not containing the co-factor (see SM 2). Very recently, Vaidya and co-workers demonstrated *in vitro* that the structural elements surrounding the CTT are exposed to Trypsin cleavage under light conditions, suggesting disorder [3]. They also report a general photoreceptor structure involvement after light exposure. Our study focuses on the effect of FAD itself before the reaction, considering its effect on the overall structure. HdCRY RMSD plot reveals a mean plateau value of 4.5 Å, and the phosphorylated system an even higher peak of almost 5.0 Å. In the latter system, the RMSD slope is much higher compared to other systems (see SM 2), highlighting the strong impact of the post-translational modification on the system. RMSD values of the other two systems containing no FAD and neither FAD nor Mg^{2+} are lower, suggesting more rigid behaviors. The second output analysis was RMSF, measuring the mean backbone RMSD

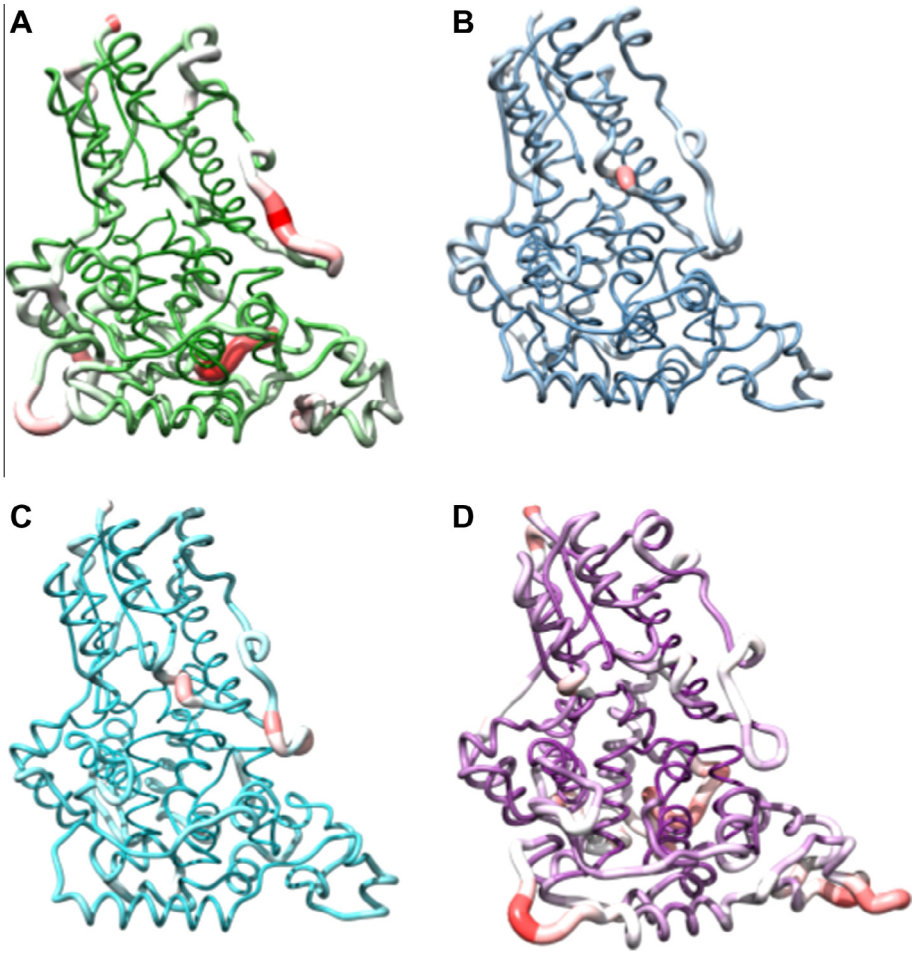

Fig. 2. RMSF thickening and coloring for (A) holoprotein, (B) cryptochrome without FAD, (C) cryptochrome without both FAD and Mg^{2+}, (D) cryptochrome with phosphorylated S526.

value for each residue during an entire run, distinguishing more fluctuating and more rigid regions of a system. The RMSF plots showed that the overall structures not containing the FAD co-factor are more rigid and the fluctuations minimized (see SM 3). The regions more affected by FAD presence, according to RMSF plots, are residues 200–300 and the CTT region. The first corresponds to the FAD phosphate binding pocket surface moiety boundaries, a solvent exposed segment and the protrusion motif (see SM 1). A comparison between HdCRY, AdCRY and MdCRY dynamics clearly shows that the holoreceptor region between residues 200 and 300 is fluctuating with peaks reaching 4.0 Å, while in AdCRY and MdCRY these values are lower at 3.5 Å and 2.5 Å respectively (see SM 3). Another region deeply biased by FAD presence/absence is the so-called CTT base loop (residues 154–160) [3]. The highest motility was found in the system containing a phosphorylation in S526, the Mg^{2+} ion and the FAD co-factor. The phosphoserine forms hydrogen bonds with both the Phe428 and Glu429 backbone (C-Terminal lid) for almost all analyzed frames. The RMSF plot of this system shows slightly lower peaks in the 200–300 region, with values reaching 3.5 Å, but an improved CTT motility compared to all other RMSF plots (see SM 3). These results show that FAD presence in the receptor improves overall motility, as well as the kinetic energy of the system. AdCRY and MdCRY were definitely more stable and rigid (see SM 3 and Fig. 2). This can be explained with steric effects and is clearly noticeable by RMSD plot comparison (see SM 2). The slope of the RMSD plot for systems without FAD is dramatically lower.

Interestingly, RING network analysis revealed that residues with more than 80% conservation (87 residues) are mostly kept in the hydrophobic protein core (see SM 1). Of those 87 residues, 16 are in close contact with the cofactor. This analysis was complemented with a multiple sequence alignment, which also revealed high conservation also for some CTT residues. The conservation rate decreases proportionally to Phylum distance. BLUUES electrostatic potential map comparisons between dCRY and (6–4)-PL revealed a positive potential in both dCRY and (6–4)-PL, (see SM 4). In this region, (6–4)-PL contains positively charged residues interacting with DNA. In dCRY, this region corresponds to the CTT lid, which remained rigid during the simulation. This result, in addition to structural superposition, could suggest a DNA mimicking role of this region.

3.2. The FFW motif and S526 phosphorylation effect

S526 superposes to a DNA phosphate moiety in the *D. melanogaster* PL crystal structure. The same DNA phosphate moiety was found to partially superpose with dCRY E530 as well, suggesting a DNA-mimicking role of the CTT. Furthermore, the mainly aromatic ring nature of the residues of the FFWL motif is suggesting some similarity with nucleic acid base rings. The importance of these motif residues can find partial confirmation in the multiple sequence alignment, which revealed a high conservation of both the SNEEE and FFW motifs through all analyzed Drosophila-like-CRYs (see Fig. 3). S526 is 100% conserved, while the FFW motif the residues are 100%, 88%, 100% conserved, respectively (see Fig. 3). The sequence logo shows how the CTT linear motifs are located in a segment with strong sequence conservation, in a putative α-helix inside an intrinsically disordered region (see Fig. 3).

3.3. TIM sequence analysis

Starting from the reported FFW motif correspondence between TIM and dCRY CTT [3], we extended the analysis to TIM sequences belonging to the same species. We found that TIM keeps the FFW

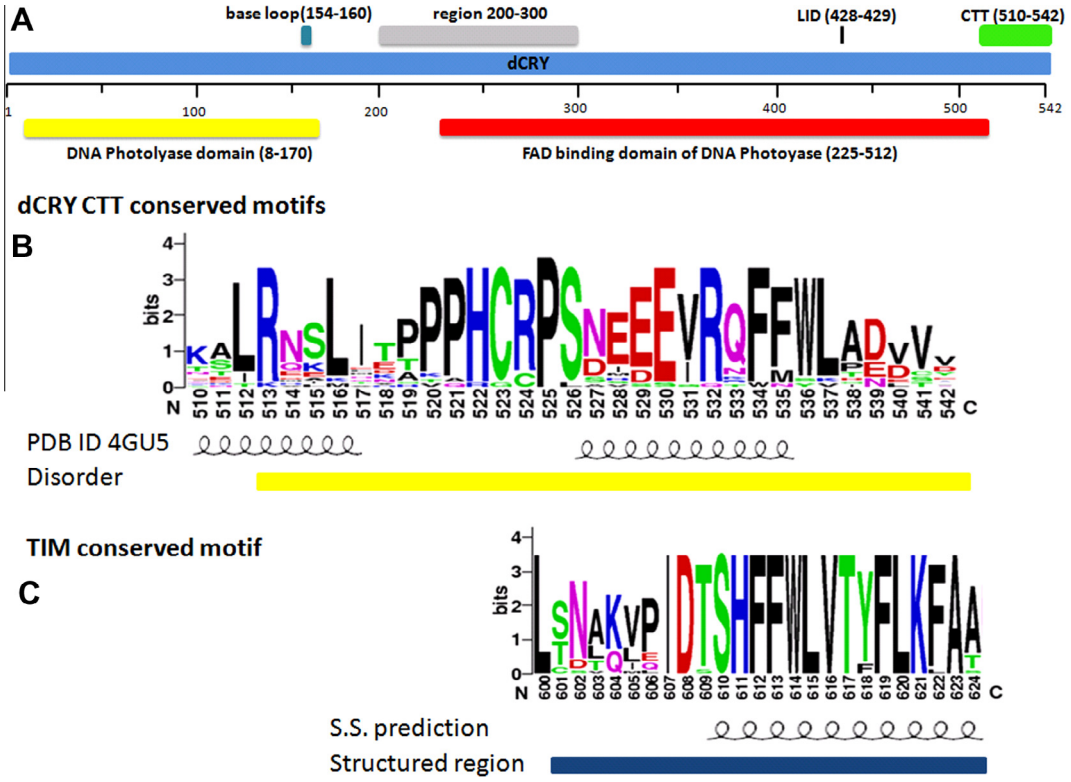

Fig. 3. (A) Schematic representation of dCRY, below the regions Photolyase-like, above the most important regions influenced by presence/absence of FAD co-factor. (B) Overview of the dCRY CTT features. dCRY CTT sequence logo shows the conserved motifs above, features from the crystallographic dCRY structure (PDB identifier 4GU5) and predicted disorder are showed below. The secondary structure is shown as helix. Predicted disorder is shown as a yellow line. (C) TIM sequence logo shows the FFW conserved motif suggested to interact with dCRY receptor region, secondary structures (S.S.) prediction and structured region are showed below. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

motif highly conserved (100%) among all explored species (Fig. 3). This suggests that the conserved tryptophan of TIM and the dCRY CTT FFW motif could interact in the same manner with dCRY. Conversely, neither motif was found among *D. melanogaster* Photolyase orthologs.

4. Discussion

Light signal transduction into chemical responses is frequent in nature. Here we investigated whether the presence of FAD could have not only a light sensing role within photoreceptors, but could be also required to provide a more suitable overall structure to maximize receptor reactivity towards light impulses. The overall fluctuations of the holoreceptor and phosphorylated system, in absence of light, suggest that the steric impact of the co-factor inside its binding pocket could be a feature required for receptor activation. Interestingly, the receptor is globally less rigid when the co-factor is contained in its pocket as reported by the RMSF and RMSD plots shown. Thus, we cannot exclude FAD from also having a role due to its molecular structure and hindrance, rather than only being involved in photo reduction and receptor activation itself. This could be explained considering the more fluctuation-prone HdCRY behavior, likely requiring less input energy to go towards a state transition and activation. The RMSF value of the CTT preceding region is reinforcing this hypothesis. All systems containing FAD have higher RMSF values towards the CTT between residues 450 and 525. Whenever FAD is lacking, this region is more rigid and less ready to move. Given that receptor activation appears to be dependent on this residue segment, we can speculate that FAD could also have a prominent role before receptor activation unconnected to its intrinsic light exposure reactivity. Analysis of the phosphorylated system (P-dCRY) revealed an even higher overall fluctuation compared to all other systems. The S526 phosphorylation site is close to the CTT, and provides higher fluctuations in this region. Our results suggest phosphorylation could occur before receptor activation, further facilitating receptor activation. This could explain the crucially close CTT position, suggesting a DNA mimicking role for CTT and TIM. Furthermore, dCRY E530 and S526 are close to a PL phosphate DNA moiety after superposition (0.98 Å and 2.25 Å, respectively for the closest atoms), thus reinforcing what previously demonstrated in [28]. On the other hand, multiple sequence and structural alignments also revealed that the CTT could mimic the damaged DNA Photolyase-DNA interaction and establish contacts with the FAD binding pocket. In particular, the aromatic rings of the FFW motif residues show similarity with nucleic acid base rings and are conserved in the main dCRY interactor TIM [3]. In the end, our data suggest that even if chemical reactivity of the co-factor is the main reason behind receptor activation, steric hindrance of the molecule could be a required feature as well. The multiple sequence alignment also showed that the SNEEE and FFW motifs are conserved in CRY ortholog CTTs. These conserved species-specific motifs, which are not present in the Photolyase family, could suggest a functional role in the photoreceptor. Light exposure induces dCRY activation, opening its CTT [3,6] and allowing binding to TIM, which it is subsequently degraded by the proteasome [8,9]. Further *in vitro* validation of the obtained results should be carried out in order to shed light on these issues, due to the *in silico* origin of data. Our results nevertheless suggest that presence of the conserved FFW motif in both TIM and dCRY and SNEEE, once phosphorylated, and FFW motifs in dCRY could mimic DNA interaction with the CTT coupled motif. Finally, these findings are suggesting an additional prominent structural role of the co-factor in the receptor activation process, providing another small piece to the enormous light stimuli response mechanism jigsaw.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.038.

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