

## Title

### A marine cryptochrome with an inverse photo-oligomerization mechanism

## Authors

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## Abstract

Cryptochromes (CRYs) are a structurally conserved but functionally diverse family of proteins that can confer unique sensory properties to organisms. In the marine bristle worm *Platynereis dumerilii*, its light receptive cryptochrome L-CRY (*PdLCry*) allows the animal to discriminate between sunlight and moonlight, an important requirement for synchronizing its lunar cycle-dependent mass spawning. Using cryo-electron microscopy, we show that in the dark, *PdLCry* adopts a dimer arrangement observed neither in plant nor insect CRYs. Intense illumination disassembles the dimer into monomers. Structural and functional data suggest a mechanistic coupling between the light-sensing flavin adenine dinucleotide chromophore, the dimer interface, and the C-terminal tail helix, with a likely involvement of the phosphate binding loop. Taken together, our work establishes *PdLCry* as a CRY protein with inverse photo-oligomerization with respect to plant CRYs, and provides molecular insights into how this protein might help discriminating the different light intensities associated with sunlight and moonlight.

## Introduction

Cryptochromes (CRYs) are found in both animals and plants and have evolved from DNA repairing photolyases (PLs)<sup>1</sup>. Animal CRYs can be subdivided into light-sensitive CRYs, namely type I (*Drosophila*-like) and type IV CRYs, and light-insensitive CRYs, namely type II (mammalian-like), CRYs<sup>2</sup>. In contrast, plant CRYs appear to be exclusively light-sensitive<sup>3</sup>.

While animal and plant CRYs have evolved from different ancestral PL backgrounds<sup>4</sup>, they share a highly conserved PL homology region (PHR). Unlike PLs, the CRY PHR is modified by a specific C-terminal expansion (CTE), and sometimes an N-terminal expansion (NTE), postulated to dictate its biochemical function<sup>1,5,6</sup>. The PHR can be structurally sub-divided into two regions, namely an N-terminal  $\alpha/\beta$ -region and a C-terminal  $\alpha$ -helical region connected by an extended connector region. The  $\alpha$ -helical region contains the binding pocket for the major chromophore, flavin adenine dinucleotide (FAD), which is required for the light response. In the dark resting state of type I CRYs, FAD is non-covalently bound in its oxidized form (FAD<sup>OX</sup>)<sup>7</sup>. Absorption of a blue light photon induces a photoexcited state FAD<sup>OX\*</sup>, which in turn abstracts

an electron from a nearby tryptophan (TrpH) residue forming a radical pair between a now one-electron reduced anionic semiquinone (ASQ,  $FAD^{\bullet-}$ ) and a cationic tryptophanyl radical ( $TrpH^{\bullet+}$ ), which decays back to the ground state within minutes in the dark<sup>7</sup>.

The molecular mechanism behind the conversion of redox changes in FAD into an actual physiological signaling state in CRYs is still under debate. Since CRYs have no known enzymatic activity, a signaling state will most likely be defined by one or more conformational changes. The highly divergent CTE is a likely candidate for these<sup>1,4,6</sup>. Proteolytic experiments have suggested a conformational change involving the CTE upon blue light excitation for type I<sup>8-10</sup>, type IV<sup>11</sup>, and plant CRYs<sup>12</sup>. For the *Drosophila melanogaster* CRY (*DmCry*) this has been corroborated by time-resolved x-ray solution scattering experiments and molecular dynamics (MD) simulations<sup>13</sup>. Indeed, due to its unusually short CTE, forming only a short C-terminal tail helix (CTT, helix  $\alpha 22$ ), *DmCry* has been a focal point of attention in attempts to unravel the molecular coupling between FAD photoreduction and CTE conformational changes. FAD is not known to undergo a conformational change during photoactivation, but the photoinduced electron transfer results in a net negative charge at the flavin isoalloxazine ring. Structural data have revealed that *DmCry* His378 is positioned between the isoalloxazine ring and a so-called FFW motif at the C-terminal end of the CTT<sup>9,14</sup>, similar to an active site histidine that is essential for PL DNA repair<sup>15</sup>. MD simulations suggest that the hydrogen-bonding network connecting His378, isoalloxazine ring and FFW motif is indeed sensitive to an additional negative charge on the isoalloxazine ring<sup>16</sup>, which was later confirmed by further experimental data<sup>17</sup>. The recent surge in structural data of CRYs from other species has suggested that this proposed coupling mechanism may be conserved in the CRY superfamily, albeit with lineage-specific fine-tuning, particularly for plant CRYs, since His378 is not conserved in the plant lineage and their most stable FAD form is a one-electron reduced neutral semiquinone. (NSQ,  $FADH^{\bullet}$ )<sup>7</sup>. Interestingly, while it is widely accepted that blue light activated plant CRYs oligomerize and that this is important for downstream signaling<sup>18</sup>, signaling by non-plant CRYs is largely thought to depend on the conformation of the CTE in the CRY monomer<sup>19</sup>. There are currently two known exceptions to this dogma: Firstly, dark state dimers have been reported for the animal-like CRY

from the algae *Chlamydomonas reinhardtii* (CraCry)<sup>20</sup>, although this could not be reproduced in later experiments<sup>21</sup>. Secondly, for the photoactive CRY from the bristle worm *Platynereis dumerilii* (PdLCry), dimers were observed in *in vitro* assays using SEC-MALS and proposed to be of physiological importance<sup>22</sup>.

PdLCry is one of two light-sensitive CRYs identified in this marine invertebrate, which is an important model system for both genetic and molecular studies<sup>23</sup>. Genetic and behavioral experiments have identified PdLCry as an important timekeeper of its lunar-circle dependent spawning behavior<sup>22,24</sup>. Both *in vivo* and *in vitro* data revealed a peculiar light-sensitivity that allows PdLCry to respond to dim moonlight<sup>22,24</sup>.

We have solved the structure of both dark state and blue light illuminated PdLCry using cryo-electron microscopy (cryo-EM) in combination with single-particle reconstruction techniques. Our dark state structure reveals a previously unknown dimerization interface centered on helix  $\alpha 8$ , while the blue light illuminated structure shows that PdLCry monomerizes upon intense illumination. Furthermore, our structural analysis together with functional data suggests the phosphate binding loop (PBL) as a central element for the molecular coupling between the chromophore, the CTT and the dimer interface. Together, our study uncovers a novel mechanism of light-induced inverse oligomerization, which has important implications for our understanding of the ability of PdLCry to discriminate moon- from sunlight.

## Results

PdLCry features subtle but distinct sequence differences separating it from archetypical type I CRYs.

As a first step to decipher why PdLCry is able to dimerize, we performed an evolutionary analysis of its sequence. This analysis confirmed the previous association of PdLCry with type I CRYs<sup>25</sup>. However, we find that PdLCry is distinctly separated from classical members of this lineage (Fig. 1a). Indeed, a detailed alignment of PdLCry with the only type I CRY with a known

structure to date, *DmCry*<sup>9,14</sup>, reveals subtle but distinct sequence differences in regions reported to be important for the molecular properties of *DmCry*: *PdLCry* has a shortened protrusion loop, an FCW sequence instead of an FFW sequence at the CTT, and a tyrosine in the sulfur loop (Fig. 1b, Supplementary Fig. 1, Supplementary Table 1). However, even a close comparison of their sequences did not provide a molecular explanation for the different oligomeric states of the two proteins. Therefore, we set out to elucidate the molecular architecture of *PdLCry* using molecular cryo-electron microscopy (cryo-EM).

### *PdLCry* forms a dimer in the dark state featuring a novel dimer interface

*PdLCry* was heterologously expressed in Sf9 insect cells and purified under far-red light conditions (Supplementary Fig. 6a) to ensure that FAD remained in its oxidized ground state (FAD<sup>OX</sup>) as previously described<sup>22</sup>. As for *Poehn et al.*, *PdLCry* purified as a homodimer under these conditions with an absorbance ratio of 450 nm to 370 nm of 1.06 (Fig. 2a), indicating an FAD<sup>OX</sup> loaded protein<sup>26</sup>. The purified protein was vitrified in liquid ethane under far-red light conditions to produce cryo-EM grids of the dark state. Data acquisition and unsupervised 2D classification indeed revealed predominantly dimeric particles with a small fraction of putative monomers (Fig. 2b). We were able to refine the subset of particles composed of two subunits to obtain a dimeric structure with the *PdLCry* subunits arranged in parallel. As we did not observe any asymmetry during the reconstruction procedure, we enforced C2 symmetry in later reconstruction stages, yielding a final cryo-EM density map at 2.6 Å resolution (Fig. 2c, Supplementary Fig. 2). The exquisite resolution of the final, C2 symmetric 3D map allowed us to build an almost complete atomic model of *PdLCry* (Fig. 2d, Supplementary Table 2). We observe a near stoichiometric occupancy of the ligand FAD, but no density for additional antenna chromophores or other nucleotides. Our map shows an overall fold reminiscent of that observed in other type I CRYs, with an N-terminal  $\alpha/\beta$  region (31-161) and a C-terminal  $\alpha$ -helical region (243-524) connected by a largely well-resolved connector (162-242). While we do not observe density for the *PdLCry* specific NTE (1-30), the *PdLCry* specific CTE (526-565) is largely well-resolved and features a well-resolved C-terminal tail helix (CTT, 537-544), which resembles the helix observed in *DmCry*<sup>9,14</sup>. The density for the last nine C-terminal residues

(557-565) was strongly fragmented, suggesting considerable flexibility. The arrangement of the subunits in the dimer does not resemble either that of plant CRYs, nor that of the crystal dimer arrangement of *DmCry* (Supplementary Fig. 3a, b). Instead, the dimer interface in *PdLCry* is predominantly formed by the N-terminal part of the  $\alpha$ -helical region with some contribution from the connector region. Molecular dynamics simulations show that the solvent accessible surface area buried in the interface is comparable to that of the biologically relevant *AtCry2* dimer, despite its different arrangement (Supplementary Fig. 3c).

The main dimer interface is formed between helices  $\alpha 8$  of both subunits, which run antiparallel at an angle of 42 degrees, flanked by interactions mediated by the connector region and the PBL (Fig. 3a, b). While helix  $\alpha 8$  and the flanking regions adopt a fold that overlays well with available structures of *DmCry*, the archetypical type I CRY, several key residues differ (Fig. 3c): the positions of Thr253 and Thr260 in *PdLCry* are occupied by bulkier amino acids in *DmCry*, namely Glu236 and His243, which would likely clash in a similar dimer arrangement. Similarly, the position of the central Met256 is occupied by a bulkier lysine (Lys239), and the position of Glu249 is occupied by a non-polar leucine residue (Leu232) in *DmCry*. Apart from the differences in helix  $\alpha 8$ , we note unique dimer interactions involving Tyr266 of the PBL, especially with Pro279 (PBL) and Asn238 (connector) from the other subunit. These interactions are not possible in *DmCry* because Tyr266 is replaced by a phenylalanine (Phe249), which appears to be conserved in other animal and animal-like CRYs (Fig. 3c). In addition, the position of Asn238 is occupied by a non-polar isoleucine (Ile221) in *DmCry*. Indeed, MD simulations confirm the importance of Asn238, Glu249, and Tyr266 as key residues that, due to their polar side chains, stabilize the dimer interface through hydrogen bond formation. (Supplementary Fig. 3d).

#### Structural elements highlight a molecular connection between the chromophore FAD, the CTT, and the dimer interface

A chain of four tryptophan residues, comprising Trp<sub>A</sub>428, Trp<sub>B</sub>405, Trp<sub>C</sub>351, and Trp<sub>D</sub>402, lines the path from the FAD to the protein surface and occupies identical positions as observed in the

archetypical type I CRY *DmCry* (Fig. 4a, b). However, *PdLCry* has an additional surface-exposed Tyr352 that occupies a position similar to the terminal tyrosine in the type IV CRY *CiCry4*<sup>11</sup> (Fig. 4b). In *CiCry4*, this terminal tyrosine (Tyr319) has been shown to increase the lifetime of the radical state<sup>11</sup>, as also observed for the terminal tyrosine residue that replaces Trp<sub>D</sub> in *CraCry*<sup>20</sup>.

In the dark state, the CTE is anchored in the vicinity of the FAD binding site by a CTT comprising residues 537 to 545. This CTT occupies an identical PL-DNA substrate binding pocket as in *DmCry*<sup>9</sup> (Fig. 4c). Interestingly, Cys544 of the FCW motif in *PdLCry* adopts a conformation similar to Phe535 of the FFW motif in *DmCry* and, despite being less bulky, binds into an almost identical hydrophobic pocket delineated by Val431, Leu440, Val540 and Phe446, the latter occupying a similar position to *DmCry* Val437 (Fig. 4c). Notably, the smaller size of the cysteine side chain compared to the phenylalanine side chain allows His390 of helix  $\alpha$ 14 to adopt a different conformation compared to the corresponding Asn382 in *DmCry* (Supplementary Fig. 4a). Avian type IV CRYs<sup>11</sup> and the animal-like *CraCry*<sup>27</sup> also have a histidine at this position instead of an asparagine, but in a rotamer superimposed on Asn382 in *DmCry* (Supplementary Fig. 4a). Compared to *PdLCry*, this different histidine rotamer could possibly be due to the absence of a CTE in the truncated constructs used for crystallography.

As for *DmCry*, Trp545 in *PdLCry* is positioned at the end of the CTT. His386 (corresponding to *DmCry* His378) and the PBL residues Pro274 (corresponding to *DmCry* Pro257) and Gln271 delineate the binding pocket of Trp545. Thereby, the FCW motif in *PdLCry* is coupled to the dimer interface through the PBL. Interestingly, Gln271 is residing in a similar position as *DmCry* Arg298 from the protrusion loop, which is considerably shorter in *PdLCry* compared to *DmCry* (Fig. 4c). In *DmCry* Arg298 has been implicated in stabilizing the dark state by anchoring *DmCry* Trp536<sup>9,28</sup>, suggesting that Gln271 could play a similar role in *PdLCry*.

Phe543 occupies an identical position to *DmCry* Phe534 and is similarly delineated by Trp430 (*DmCry* Trp422) and His386 (*DmCry* His378). Unlike in *DmCry*, the CTE is additionally stabilized by a cation- $\pi$  interaction between Trp530 from the CTE and Arg174 from the connector region (Supplementary Fig. 4b), which is neither possible in *DmCry* nor *CiCry4* as the

corresponding residues are Pro521 and Ile153, and Arg497 and Ser146 respectively. C-terminal to the CTT, the remaining CTE passes through a cleft defined by both subunits of the dimer, although the more fragmented density in this region implies conformational flexibility.

The FAD chromophore bound adjacent to the CTT is stabilized by a binding pocket defined primarily by residues from helices  $\alpha 9$ ,  $\alpha 14$ , and  $\alpha 16$ , the PBL and the  $\alpha 15$ - $\alpha 16$  loop (Fig. 4c, d). As in other type I CRYs, the N5-interacting residue is a cysteine, namely Cys424 (*DmCry*416), which has been proposed to disfavor the conversion to the two-electron reduced NSQ state of the chromophore FAD<sup>9,14,29</sup>. Solvent access to the FAD binding site<sup>7</sup> could be blocked by Tyr413, which occupies an equivalent position as Leu405 in *DmCry*. As reported for *DmCry*<sup>16,17</sup>, His386 (*DmCry* His378) bridges the FAD chromophore to the FCW motif of the CTT and is thus in a suitable position to allow the electronic state of the isoalloxazine ring to be transmitted to the CTE.

Interestingly, in addition to the Gln271-mediated coupling between helix  $\alpha 8$  and the CTT, the dimer interface helix  $\alpha 8$  is also directly coupled to the FAD via Arg254, which is located at the center of helix  $\alpha 8$  (Fig. 4d). We therefore asked ourselves whether there is a mechanistic coupling between the redox state of FAD, the CTE and the oligomeric state of *PdLCry*.

#### Activation of *PdLCry* by intense blue light disrupts the dimer.

To test whether *PdLCry* does indeed respond to blue light by changing its oligomeric state, we performed size-exclusion chromatography (SEC) under continuous and intense blue light irradiation. In comparison with the elution pattern observed under far-red light conditions, we observe a shifted elution peak, likely corresponding to monomeric *PdLCry*, with an absorbance ratio of 450nm to 370 nm of 0.28, suggesting an ASQ loaded protein (Fig. 5a). *PdLCry* had previously been shown to revert to the ground state within minutes<sup>22</sup>. Therefore, cryo-EM samples were vitrified in liquid ethane within less than 5 seconds after intense blue light irradiation. Data acquisition and unsupervised 2D classification indeed revealed almost exclusively monomeric particles (Fig. 5b). Unfortunately, the low molecular mass combined with a strong preferential orientation of the particles on the grid precluded us from obtaining a high-

resolution reconstruction (Supplementary Fig. 5). Due to the limited resolution and strong anisotropy of the 3D cryo-EM map, we refrained from building an atomic model for the activated state. Nevertheless, rigid body fitting of a single subunit from our dark state atomic model into the monomeric 3D map reveals a general fit (Fig. 5c), and implies several structural changes at the level of secondary structure elements. Importantly, helix  $\alpha 22$  and the CTT appear disordered in the photoexcited state, as density for these structural elements is largely absent (Fig. 5d). Interestingly, helix  $\alpha 22$  had already been observed to become more mobile upon blue light activation in the animal-like *CraCry*<sup>21</sup>. In addition, we observe changes in the N-terminal part of the connector region, suggesting a rearrangement of helices  $\alpha 5$  and  $\alpha 6$  and the connecting loop.

#### Mutagenesis highlights the role of helix $\alpha 8$ , the PBL and the CTT in *PdLCry* dimer formation.

To identify the structural elements that control the oligomeric state of *PdLCry*, we introduced selected amino acid mutations and investigated the oligomeric state of the mutant proteins by performing SEC under far-red light conditions. First, we investigated direct mutations of the dimer interface helix  $\alpha 8$ . To study the effect of steric clashes, we introduced a bulky side chain into helix  $\alpha 8$  by replacing Thr253 of helix  $\alpha 8$  with an arginine. Indeed, this shifts the elution volume of *PdLCry*<sup>T253R</sup> towards lower mass (Fig. 6a, Supplementary Fig. 6b). Similarly, introducing a charge reversal by replacing Glu249 of helix  $\alpha 8$  with an arginine shifts the elution volume of *PdLCry*<sup>E249R</sup> towards lower mass ( Fig. 6a, Supplementary Fig. 6c). Combining both T253R and E249R as a double mutation in *PdLCry*<sup>E249R,T253R</sup> shifts the SEC elution volume to the same volume as observed for the *PdLCry*<sup>E249R</sup> and *PdLCry*<sup>T253R</sup> proteins (Fig. 7a, Supplementary Fig. 6d). Comparing the elution volumes of *PdLCry*<sup>T253R</sup>, *PdLCry*<sup>E249R</sup> and *PdLCry*<sup>E249R,T253R</sup> with the elution profile of wildtype *PdLCry*, we note that the elution peak of the mutations coincides with a shoulder in the elution profile of wildtype *PdLCry*. Since we observed a subpopulation of monomeric particles in the dark state cryo-EM dataset (Fig. 2b), we assume

that this shoulder peak corresponds to these monomeric particles, further corroborating that the direct interface mutations lead to a monomerization even in the dark state.

Next, based on the hypothesis that CTT undocking is a downstream effect of photoexcitation in light sensitive CRY proteins, we investigated whether there is a functional coupling between the CTT and the oligomeric state in *PdLCry*. To this end, we individually mutated Phe543 and Trp545 of the FCW motif, which anchor the CTT to the PL-DNA substrate binding pocket, to alanine (Supplementary Fig. 6e, f). Indeed, both *PdLCry*<sup>F543A</sup> and *PdLCry*<sup>W545A</sup> elute at volumes implying a monomer (Fig. 6b), suggesting a crosstalk of the CTT conformation to the dimer interface in *PdLCry*.

Our cryo-EM derived atomic model highlights the PBL as an important structural element that is directly involved in dimer formation, via hydrogen bonds mediated by Tyr266, and connects the dimer interface to the CTT, via an interaction between Gln271 and Trp545 (Fig. 4c, d). While *PdLCry*<sup>Y266A</sup>, in which Tyr266 of the PBL has been replaced by an alanine that cannot participate in side-chain hydrogen bonding, elutes with a volume clearly indicative of a monomer, the mutation of Gln271 to alanine has a less clear effect, and *PdLCry*<sup>Q271A</sup> shows an elution volume between that expected for dimer and monomer (Fig. 6c, Supplementary Fig. 6g, h).

#### The oligomeric state of *PdLCry* affects the kinetics of dark recovery.

Given the unique sensitivity of *PdLCry* compared to the archetypal monomeric type 1 CRY *DmCry*<sup>22,24</sup>, and the observation that *PdLCry* responds to intense illumination with monomerization, we asked whether the oligomeric state of *PdLCry* has an influence on the properties of its photoreaction. To ensure constitutive monomer formation throughout the spectroscopic measurements, we used the combined dimer interface mutation *PdLCry*<sup>E249R,T253R</sup> for these experiments. Based on an absorbance ratio of 450 nm to 370 nm of 1.14, which is comparable to that of the wildtype protein, *PdLCry*<sup>E249R,T253R</sup> eluted as a monomer loaded with FAD<sup>OX</sup> under far-red light conditions (Fig. 7a). Therefore, the double mutation does not appear

to affect the ground state of the FAD chromophore. Under continuous and intense blue light illumination, both wildtype *PdLCry* and *PdLCry*<sup>E249R,T253R</sup> elute with almost identical volumes, further corroborating that the double mutation is indeed a monomer. Under these conditions, the absorbance ratio of 450 nm to 370 nm drops to 0.28 (Fig. 7b) for both wildtype *PdLCry* and *PdLCry*<sup>E249R,T253R</sup>, suggesting that while the double mutation disrupts the dimer, it does not affect the overall ability of the chromophore in *PdLCry* to be photoreduced to the FAD<sup>ASQ</sup>-state by blue light photons.

To analyze whether the oligomeric state affects the kinetics of *PdLCry* photoreduction or dark recovery, we performed time-resolved UV/Vis spectroscopy. UV/Vis spectroscopic analyses confirmed that in monomeric *PdLCry*<sup>E249R,T253R</sup> FAD is completely photoreduced to FAD<sup>ASQ</sup> after 2 min of illumination with intense blue light (41 W/m<sup>2</sup> at the sample), as also observed for wildtype *PdLCry* (Supplementary Fig. 7a). To gain insight into the photoactivation of monomeric *PdLCry* under sunlight conditions, we also analyzed FAD photoreduction upon illumination with a sunlight source mimicking naturalistic sunlight 5 m below the water surface (the natural habitat of *P. dumerillii*),<sup>22</sup> which has a ~9-fold lower intensity (4.6 W/m<sup>2</sup> at sample) than our blue light source. We found that monomeric *PdLCry*<sup>E249R,T253R</sup> - like wildtype *PdLCry* - showed slower FAD photoreduction kinetics under sunlight compared to blue light, reaching the sunlight state with fully photoreduced FAD<sup>ASQ</sup> within approximately 20 min (Figure 7c, d). Moreover, the monomeric mutant showed a wildtype-like sunlight photoreduction rate (average  $t_{1/2}$  wildtype = 22.7 +/- 0.4 s;  $t_{1/2}$  mutant = 22.6 +/- 0.8 s). Thus, dimer formation is not essential for efficient FAD photoreduction under naturalistic sunlight conditions. This would allow sunlight photoreduction to continue to completion, even if sunlight-induced dimer dissociation should occur early in the photoreduction process. However, wildtype *PdLCry* had a slightly slower dark recovery rate than monomeric *PdLCry*<sup>E249R,T253R</sup> (average  $t_{1/2}$  wildtype = 185.2 +/- 8.8 s;  $t_{1/2}$  mutant = 156.7 +/- 1.1 s) (Fig. 7e, f).

## Discussion

Our work confirms that *PdLCry* from the marine bristle worm *Platynereis dumerilii* is indeed able to form dimers, as previously proposed<sup>22,24</sup>, and reveals a novel dimerization interface centered on helix  $\alpha 8$  (Fig. 3), which has not been observed in CRY proteins before (Supplementary Fig. 3). While all CRY and PL proteins structurally characterized so far share the same fold and a high degree of structural similarity, it has been shown for plant CRYs that oligomerization interfaces can depend on few surface residues<sup>30</sup>, complicating predictions solely based on CRY sequences. For *PdLCry*, we could show that dimerization depends on the presence of specific amino acids, namely E249 and Thr253, within helix  $\alpha 8$  (Fig. 6a). Moreover, we found that the unique Tyr266 of the PBL, which in other animal and animal-like CRYs is usually replaced by a phenylalanine, is important for stabilizing the dimer (Fig. 6c). The importance of both Glu249 and Tyr266 is further highlighted by our MD simulation of hydrogen bonds stabilizing the dimer interface (Supplementary Fig. 3d). The unique dimer architecture of *PdLCry* places the PBL at a focal point connecting the chromophore, the dimer interface helix  $\alpha 8$ , and the CTT (Fig. 4c, d). The PBL has been identified by proteolysis experiments as a structural element affected by illumination of the chromophore in both type I<sup>10</sup> and type IV CRY<sup>11</sup> proteins. For the type IV CRY *C/Cry4*, molecular dynamics simulations have confirmed that the PBL exhibits stronger mobility upon excitation of the chromophore<sup>31</sup>. In the context of our structural data, it is easy to imagine that a higher mobility of the PBL would affect the positioning of the dimer interface residue Tyr266, which is part of this loop. Furthermore, in the dark state, we observe that Pro274 and Gln271 of the PBL interact with Trp545 of the CTT, likely stabilizing this helix in its binding pocket (Fig. 4d). Indeed, our mutagenesis experiments strongly suggest that the PBL is functionally linked to both the dimer interface and the CTT (Fig. 6b, c). In addition, our *PdLCry* structure reveals that the FFW motif of the CTT is not invariant in type I CRYs as previously hypothesized<sup>14</sup>, with *PdLCry* having a cysteine instead of a phenylalanine as the middle residue without affecting positioning or anchoring of the CTT (Fig. 4c, d).

Due to its role as a light-signal gatekeeper for the circalunar clock that controls the spawning behavior of *Platynereis dumerilii*<sup>24,32</sup>, *PdLCry* must be able to respond to dim light conditions. Indeed, *in vitro* experiments confirmed that *PdLCry*, but not its ortholog *DmCry*, is photoreduced

by a light source that mimics underwater moonlight illumination<sup>24</sup>. Our molecular structure reveals that, unlike *DmCry*, *PdLCry* has an extended electron transport chain with Tyr352 as a fifth, highly surface exposed, member (Fig. 4b). In the avian CRY protein *C/Cry4* the corresponding Tyr319 has been shown to confer an unusually high quantum yield to the protein<sup>11</sup>. Interestingly, sunlight and moonlight illumination lead to different photoreduced states of *PdLCry* in *in vitro* experiments<sup>22</sup>, suggesting that the protein is able to distinguish between intensive and dim illumination as required for its *in vivo* activity<sup>24</sup>. Our current work confirms that *PdLCry* can indeed form a stable dimer (Fig. 2, Supplementary Fig. 3c) at dark conditions with the chromophore FAD in the resting oxidated state FAD<sup>OX</sup>. After conversion of both chromophores of the dimer to the reduced form FAD<sup>ASQ</sup> by intense blue light illumination, we observe that *PdLCry* is quantitatively disassembled into single monomers (Fig. 5). Based on the intense illumination conditions employed, we hypothesize that this monomeric state corresponds to the sunlight activated state rather than to the moonlight state.

*In vitro* experiments have shown that moonlight-mimicking low photon flux rates are only able to activate half of the FAD present in an *PdLCry* sample, even after hours of illumination, while higher flux rates lead to a full conversion of FAD within minutes<sup>22</sup>. Our data suggests that structural elements linking the chromophore to the dimer interface, such as the PBL, could hypothetically allow the relay of information between both chromophores, inducing an asymmetry in *PdLCry*. If thus activation of one chromophore leads to the dampening of the second chromophore, a stably half-activated moonlight state would be easy to reconcile at low photon flux rates. High flux rates however would overcome the dampening of the second chromophore, resulting in the formation of monomeric *PdLCry* as observed in our experiments (Fig. 5). Such an asymmetric, activation-rate dependent input differentiation has also been observed in the rod photoactivation cascade, allowing the dimeric photoreceptor phosphodiesterase 6 (PDE6) to differentiate thermal noise from actual photon-detection<sup>33</sup>.

Moreover, functional assays showed that while the moonlight-induced state can be converted to the sunlight-induced state, the sunlight-induced state cannot be directly converted to the moonlight-induced state<sup>22</sup>. Our finding that *PdLCry* features an inverse photo-oligomerization

mechanism rationalizes this observation: For conversion to the sunlight state, a partially activated moonlight state of *PdLCry*, possibly an asymmetric dimer, simply requires higher photon flux rates to activate both subunits simultaneously, leading to the dissociation of the dimer in the sunlight state. Reassembly of the dimeric state, which we propose to be necessary for the transition from the monomeric sunlight state to the half-reduced moonlight state, may only be possible after FAD has returned to the resting state FAD<sup>OX</sup>, available only by transition through the dark state (Fig. 8). Our proposed activation scheme would also explain the different dark recovery rates observed for wildtype *PdLCry* and the monomeric mutant *PdLCry*<sup>E249R/T253R</sup> in our time-resolved spectroscopy experiments (Fig. 7): while we monitored the sunlight state to dark state transition - thus from monomer to dimer - for the wildtype protein, the recovery measured for *PdLCry*<sup>E249R/T253R</sup> was from the monomeric sunlight state to an artificial monomeric dark state.

The shift from a dimeric to a monomeric protein population only at high photon flux rates could also explain the different cellular localization observed for *PdLCry* depending on the illumination conditions<sup>24</sup>, assuming that only the monomer is targeted for nuclear export. Monomerization, nuclear export and subsequent cytosolic degradation could thus prevent erroneous activation of downstream signaling pathways by sunlight and allow input signal differentiation on the physiological level.

## Methods

### Sequence alignment and evolutionary analysis

The evolutionary analysis of the cryptochrome family was based on a maximum likelihood approach implemented in MEGA11<sup>34</sup>. For this the evolutionary history was inferred by using the maximum likelihood method and the Jones-Taylor-Thornton (JTT) matrix-based model<sup>35</sup>. The bootstrap consensus tree inferred from 100 replicates<sup>36</sup> was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial trees for the heuristic search were obtained

automatically by applying Neighbour-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8230)). This analysis involved 38 amino acid sequences, and there were a total of 1112 positions in the final dataset. For the list of used sequences see Supplementary Table 1.

Detailed pair-wise sequence alignments were calculated in Jalview 2.11<sup>37</sup>, relying on the JABAWS 2.2 web service<sup>38</sup> to run the Clustal Omega alignment method<sup>39</sup>.

### Cloning, expression and purification of PdLCry

Full length N-terminally His6-tagged *Platynereis dumerilii* PdLCry (GenBank UUF95169) was heterologously expressed in *Spodoptera frugiperda* (Sf9) insect cells using the Bac-to-Bac baculovirus expression system with the pCoofy27 expression vector, as described in Poehn *et al.*<sup>22</sup>. Sf9 cells were grown as suspension cultures in Sf-900 SFM III media (Thermo Fisher Scientific) at 27°C, 90 RPM. 1 L of  $1 \times 10^6$  Sf9 cells/mL were transfected with P1 virus stock and incubated at 27°C for 72 h. Cells were harvested by centrifugation at 7000 rpm for 20 min and stored at -80°C until purification. All purification steps were carried out in dark or dim red light conditions. Columns were wrapped with aluminum foil to avoid light-activation of PdLCry. The cell pellets were resuspended in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 20 mM imidazole, 5% glycerol, 5 mM  $\beta$ -mercaptoethanol) and lysed using a Branson sonifier (Analog Sonifier 450-CE). The lysate was centrifuged at 27000 rpm for 45 min. The clarified supernatant was loaded onto a HisTrap FF crude 5 mL column (Cytiva), and PdLCry was eluted in a 20 mM to 1000 mM imidazole gradient. Elution fractions containing PdLCry were concentrated, diluted with no-salt buffer (50 mM Tris pH 7.5, 5% glycerol, 1mM DTT) and loaded onto a 5 mL Hitrap Q HP anion exchange column (Cytiva). A gradient from 0 % to 100 % high salt buffer (50 mM Tris pH 7.5, 1 M NaCl, 5% glycerol, 1mM DTT) was applied. PdLCry containing fractions were pooled, concentrated and loaded onto a Superdex S200 10/300 GL or a HiLoad S200 16/60 SEC column (buffer 25 mM Bis-tris propane pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM TCEP).

Fractions containing pure *PdLCry* were pooled, concentrated to 10 mg/mL and snap frozen in liquid nitrogen for storage at -80°C.

#### Site-directed mutagenesis, expression and purification of *PdLCry* mutants

*PdLCry* mutants were generated by QuikChange (Agilent) site-directed mutagenesis and verified by sequencing. The mutant constructs were expressed and purified essentially as described for wildtype *PdLCry*.

#### Cryo-EM sample preparation and data collection

All samples for cryo-EM were prepared in a dark lab under far-red light illumination (Osram OSOLON SSL 120, GF CSSPM1.24). Concentrated samples of dark-adapted *PdLCry* were diluted to a concentration of 0.7 mg/mL using glycerol-free SEC buffer (25 mM Bis-tris propane pH 8.0, 150 mM NaCl, 1 mM TCEP). Dark-adapted samples were additionally supplemented with 0.01% fluorinated octyl maltoside (fOM). 4.5  $\mu$ L of the mixture were then applied on either a glow-discharged UltrAUfoil R1.2/1.3 Au300 or a Quantifoil R1.2/1.3 Cu300 grid (Quantifoil Micro Tools GmbH), blotted for 3 sec and flash-frozen in liquid ethane using a Vitrobot Mark IV device (Thermo Fisher Scientific) set to 100% humidity at 21 °C. Grids were stored under liquid nitrogen conditions until usage. For blue light activation, grids were illuminated for 30 secs in the humidifier chamber of the freeze-plunger using a 455 nm blue light LED (Thorlabs M455L4, operated at 1000 mA). To ensure optimal illumination, a liquid-light guide (Thorlabs LLG03-4H) was used to bring the light source within 3 mm of the grid surface. Cryo-EM data was acquired using a Titan Krios G3i (Thermo Fisher Scientific) electron microscope operated at 300 kV. Images were collected automatically using EPU 2.12 (Thermo Fisher Scientific) on a Falcon III direct electron detector with a calibrated pixel size of 0.862 Å/px. For the dark state, movies were either acquired in integration mode using with a total dose of 66  $e^-/\text{Å}^2$  distributed among 19 frames, or in counting mode with a total dose of 27  $e^-/\text{Å}^2$  distributed among either 24 or 48 frames. For the blue light state, movies were acquired in counting mode with a total dose of 30  $e^-/\text{Å}^2$  distributed among 36 frames. Defocus values ranged from -0.3 to -2.0  $\mu$ m.

#### Image processing and model building

Image processing was performed using cryoSPARC 3.3.2<sup>40</sup>. Movie stacks were first corrected for drift and beam-induction motion, and then used to determine defocus and other CTF-related values. Only high-quality micrographs with low drift metrics, low astigmatism and good agreement between experimental and calculated CTFs were further processed. On these high-quality micrographs putative particles were automatically picked and subjected to reference-free 2D and subsequent 3D classification using C1 symmetry. Particles showing protein-like density features, especially features resembling secondary structure elements, were further refined using the non-uniform refinement strategy. For the dark state sample, C2 symmetry was enforced yielding a map at a global resolution of 2.6 Å. For the blue light activated sample C1 refinement yielded a map at a nominal global resolution of 3.5 Å that was strongly anisotropic and thus filtered to 8 Å for further interpretation. For further details see Supplementary Fig. 2 and 5, and Supplementary Table 2.

The atomic model was build starting from the *DmCry* crystal structure (PDB ID 4JZY) and the *PdLCry* amino acid sequence (GenBank UUF951691). First, Coot 0.9.4.7<sup>41</sup> was used to semi-manually fit amino acids into a single subunit of the EM density map. Next, Phenix 1.20<sup>42</sup> was used to generate a non-crystallographic symmetry (NCS)-related second subunit and both subunits were then simultaneously refined against the 3D density map. This process was iterated until the fit to the density map and geometric parameters converged. The final atomic model accounts for residues 31 to 556. For further details and statistics see Supplementary Table 2. Molecular visualization and analysis was done using UCSF ChimeraX<sup>43</sup>.

### MD simulations

The *PdLCry* structure used for simulations was from this study. The structures for *Arabidopsis thaliana* Cry2 (*AtCry2*, PDB ID: 6K8I) and *Drosophila melanogaster* Cry (*DmCry*, PDB ID: 4JZY) were taken from PDB database. The experimentally unresolved parts of *AtCry2* were reconstructed using AlphaFold<sup>44,45</sup>, where the *AtCry2* sequence was used as an input. An RMSD comparison of the backbone atoms between the AlphaFold generated *AtCry2* monomeric structure and the PDB structure showed a difference of 1.8 Å.

Altogether 4 molecular dynamics (MD) simulations were performed using NAMD<sup>46–48</sup> interfaced through the VIKING platform<sup>49</sup>. Two replica simulations of *PdLCry* were carried out for 190 ns, while *AtCry2* and *DmCry* were simulated once for 190 ns. All simulations initially included 10,000 conjugate gradient minimization steps. The simulations were then split into four distinct stages, first three used to equilibrate the system, and the fourth stage being used for analysis. CHARMM36 force field with CMAP corrections was used in all simulations<sup>50–55</sup>. The investigated *PdLCry* was set to be in the inactive state that is usually present in dark conditions, where the FAD cofactor is fully oxidized. Simulation parameters for the FAD cofactor were adopted from earlier studies<sup>31,56–60</sup>. NaCl was used to neutralize the total charge of the system and assumed at a concentration of 0.15 mol/L in all simulations. Van der Waals and electrostatic interactions were treated with the cut-off distances of 12 Å, while the particle-mesh Ewald (PME) summation method was used to treat the long-range electrostatic interactions<sup>61</sup>. The size of the simulation box for *AtCry2* was 104 Å × 139 Å × 42 Å and contained 201,877 atoms, for *DmCry* the simulation box was 137 Å × 107 Å × 138 Å with 199,362 atoms, and for *PdLCry* the simulation box was 114 Å × 123 Å × 112 Å, containing 148,535 atoms.

To analyze the buried area of the dimeric cryptochrome structures, the difference between the solvent accessible surface area (SASA) was computed as:

$$S_0 = (S_1 + S_2) - S_d. \quad (\text{Eq 1})$$

Here  $S_0$  is the interaction surface (or the buried area) between the monomers in a dimer,  $S_1$  and  $S_2$  are the SASAs of the two monomers and  $S_d$  is the SASA value of the whole dimer. The SASA maximal speed molecular surfaces (MSMS) algorithm<sup>62</sup> in VMD<sup>63</sup> was used to compute the values in Eq. (1), assuming each atom having a radius of 1.4 Å. Restricted selections were used in the calculations to avoid protein pockets affecting the results. Hydrogen bond analysis was performed using the HBonds plugin in VMD<sup>63</sup>.

#### Analytical SEC and inline UV/Vis spectroscopy

For inline UV/Vis spectroscopy analytical SEC runs were performed in a dark lab under far-red light illumination (Osram OSOLON SSL 120, GF CSSPM1.24) using a Superdex 200 Increase 5/150 GL (Cytiva) SEC column connected to an bioinert AZURA HPLC system (Knauer) equipped with an autosampler and a TIR multiwavelength detector set to measure absorption at 280 nm, 370 nm, 404 nm and 450 nm. The flow-rate for all runs was 0.16 mL/min. Concentrated samples of dark-adapted *PdLCry* were diluted to a concentration of 5 mg/mL using a buffer containing 25 mM Bis-tris propane (pH 8.0), 150 mM NaCl, and 1 mM TCEP, prior to injection of 7  $\mu$ L per run. For dark condition runs, the chromatography column was wrapped in aluminium foil. For blue light condition runs, samples were pre-activated for 60 sec using a 455 nm blue light LED (Thorlabs M455L4, operated at 1000 mA) at a distance of 1 cm. During the run, the chromatography column was continuously illuminated by a 451 nm blue light LED array (24x Osram OSOLON SSL 120, GF CSSPM1.14) installed at a distance of 10 cm in parallel to the column and reflected by mirror elements.

Analytical SEC runs without inline UV/Vis spectroscopy were carried out under far-red light conditions using a Superdex 200 10/300 GL column wrapped in aluminium foil connected to a Biorad NGC Quest10 Plus Chromatography System. For each SEC run, 100  $\mu$ L sample diluted to a concentration of 2 mg/mL concentration was injected.

#### UV/Vis spectroscopic analyses of blue light and sunlight photoreduction and dark recovery

UV/Visible absorption spectra of the purified *PdLCry* proteins in final SEC purification buffer (25 mM Bis-tris propane pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM TCEP), supplemented with 1mM DTT, were recorded on a Tecan Spark 20M plate reader. A light state spectrum of *PdLCry* with fully photoreduced FAD<sup>ASQ</sup> was collected after illuminating dark-adapted *PdLCry* for 2 min with a 450 nm blue LED (41 W/m<sup>2</sup> at sample). To analyze sunlight dependent FAD photoreduction kinetics, dark-adapted *PdLCry* was illuminated with naturalistic sunlight (Marine Breeding Systems GmbH<sup>22</sup>) with 4.6 W/m<sup>2</sup> intensity at the sample for up to 20 min (protein kept on ice) and complete UV-VIS spectra (300 – 700 nm) were collected after different time intervals. Sunlight photoreduction kinetics (on ice) were determined based on changes of 450 nm absorbance values, that were extracted from these UV/VIS spectra after normalization to their

isosbestic point at 416 nm. Normalization was required to correct for baseline shifts, because two additional samples had to be measured to determine FAD photoreduction after 10 s and 20 s sunlight illumination. Dark recovery kinetics (FAD reoxidation) after photoreduction by 20 min sunlight illumination were determined at 18 °C by recording absorbance changes at 450 nm.

Absorption spectra and kinetics were analyzed using GraphPad PRISM. The half-life time values ( $t_{1/2}$  [s]) for dark recovery and photoreduction kinetics were calculated by fitting a single exponential curve to the experimental data. The single exponential model is described as  $Y = (Y_0 - Y_{\text{plateau}}) * \exp(-K * X) + Y_{\text{plateau}}$ , where  $Y_0$  and  $Y_{\text{plateau}}$  correspond to the 450 nm absorbance values at the start- and end points of the measurement,  $K$  represents the rate constant [1/s] and the half-life time  $t_{1/2} = \ln(2)/K$  [s]. The standard error of the mean (SEM) was used to describe the error of the measurements and of the  $t_{1/2}$  values.  $SEM = \frac{SD}{\sqrt{n}}$  where SD is the standard deviation and  $n$  is the number of replicates.

### **Data availability**

The EM map for the dark state and blue light activated state have been deposited in the EMDB under accession codes EMD-17429 and EMD-17553. Atomic coordinates for dark state *PdLCry* have been deposited in the Protein Data Bank under the accession code PDB 8P4X. Other data used to generate tables and figures has been provided as source data with this paper.

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## **Author contributions**

H.H.V., S.K. and C.H. prepared protein samples; H.H. V. carried out protein mutagenesis; H.H. V., H.B., D.D. and C.H. carried out SEC experiments; H.H.V. carried out time-resolved spectroscopy experiments; H.B. carried out sequence analysis; H.B., M.G. and G.J. prepared cryo-EM samples; M.G. and G.J. acquired cryo-EM data; H.B. and E.B. performed cryo-EM data analysis; H.B. prepared atomic models with input from E.W. and E.B.; M.H. carried out MD experiments; H.H.V., H.B., M.H., I.A.S., E.W. and E.B. interpreted data; E.B. and E.W. conceived the project; I.A.S., E.W. and E.B. supervised the project; E.B. wrote the manuscript with input from all authors.

## **Competing Interests statement**

None of the funding bodies was involved in the design of the study, the collection, analysis, and interpretation of data or in writing of the manuscript. The authors declare no competing interests.

## Figure legends:

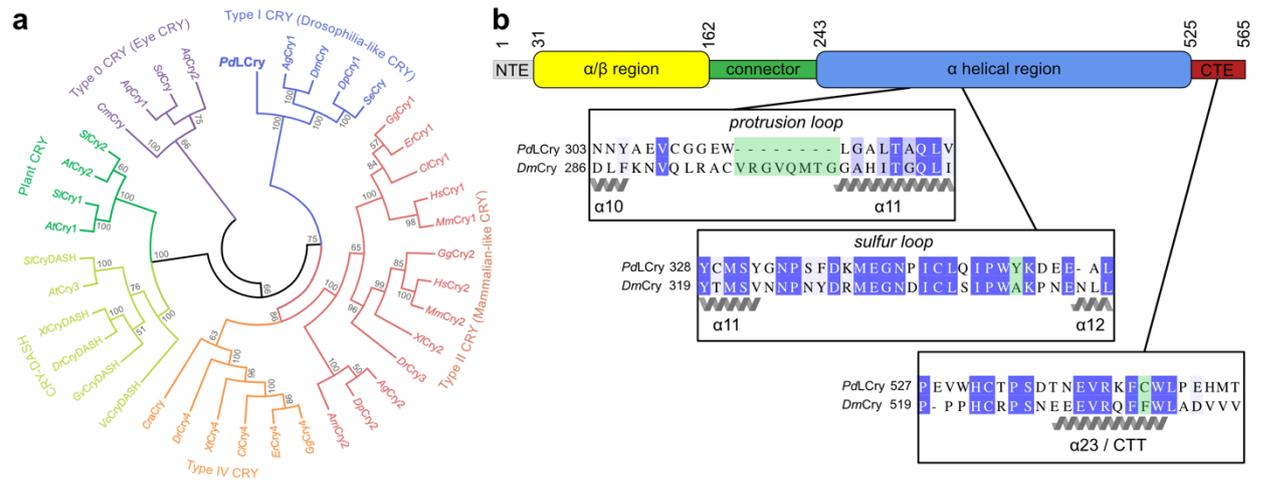


Figure 1: *PdLCry* belongs to the type I CRY lineage but has unique sequence features.

**a** Evolutionary analysis of the CRY family using a maximum likelihood approach shows that *PdLCry* belongs to the type I CRY lineage (blue), but forms a subbranch of this lineage. Small numbers indicate the bootstrap supports of the branch association. Full species names and protein sequences used are listed in Supplementary Table 1. **b** Cartoon representation of the domain composition of *PdLCry*. Small numbers indicate the residue at the region boundary. Inserts show excerpts from a sequence alignment to *DmCry*, highlighting differences (marked green) in the protrusion loop, the sulfur loop and the C-terminal tail helix (CTT) FFW motif. See Supplementary Fig. 1 for the complete sequence alignment. Secondary structure annotation ( $\alpha$ -helix in gray) is based on Czarna *et al.*<sup>1</sup>.

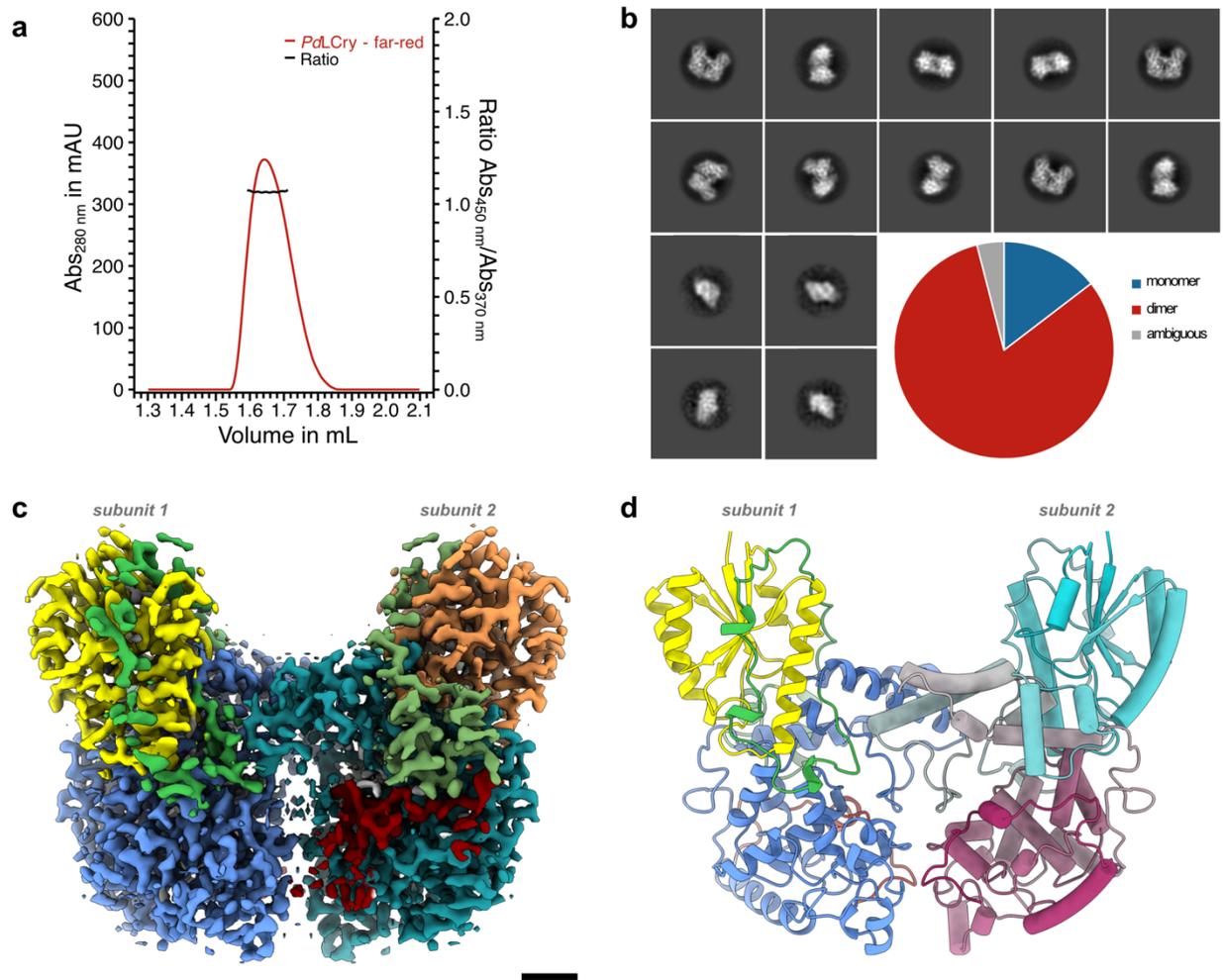


Figure 2: FAD<sup>OX</sup>-bound PdLCry forms a unique dimer in the dark state.

**a** Analytical size exclusion chromatography of PdLCry using a Superdex 200 Increase 5/150 GL column under far-red light conditions shows a slightly tailing elution peak following absorption at 280 nm (red curve), likely corresponding to a dimeric state of the protein. The nearly equal absorbance at 370 nm and 450 nm (black line;  $Abs_{450nm}/Abs_{370nm} = 1.06$ ) confirms that PdLCry was successfully maintained in the FAD<sup>OX</sup> bound dark state<sup>2</sup>. **b** Representative 2D class averages of an unsupervised 2D classification of the dark state PdLCry cryo-EM sample show predominantly dimeric particles, with few monomeric particles. Quantification of all protein-shaped particles suggests that approximately 80% of the protein is present as a dimer after vitrification under far-red light conditions. **c** Surface representation of the cryo-EM density map of PdLCry shows a butterfly-shaped dimer, with a large interaction interface formed by the  $\alpha$ -helical region (blue) with contributions from the connector region (green). Subunit 2 is shown in

darker shades. The CTE is only visible for subunit 2 (dark red). Scale bar corresponds to 10 Å.

**d** Atomic model built into the cryo-EM density map shows that *PdLCry* adopts the typical PL/CRY fold and that both subunits are arranged in parallel. Subunit 1 is shown in cartoon style using a coloring scheme identical to that used for subunit 1 in panel C. Subunit 2 is shown in a pipes-and-planks style using a color gradient from cyan (N-terminus) to maroon (C-terminus).

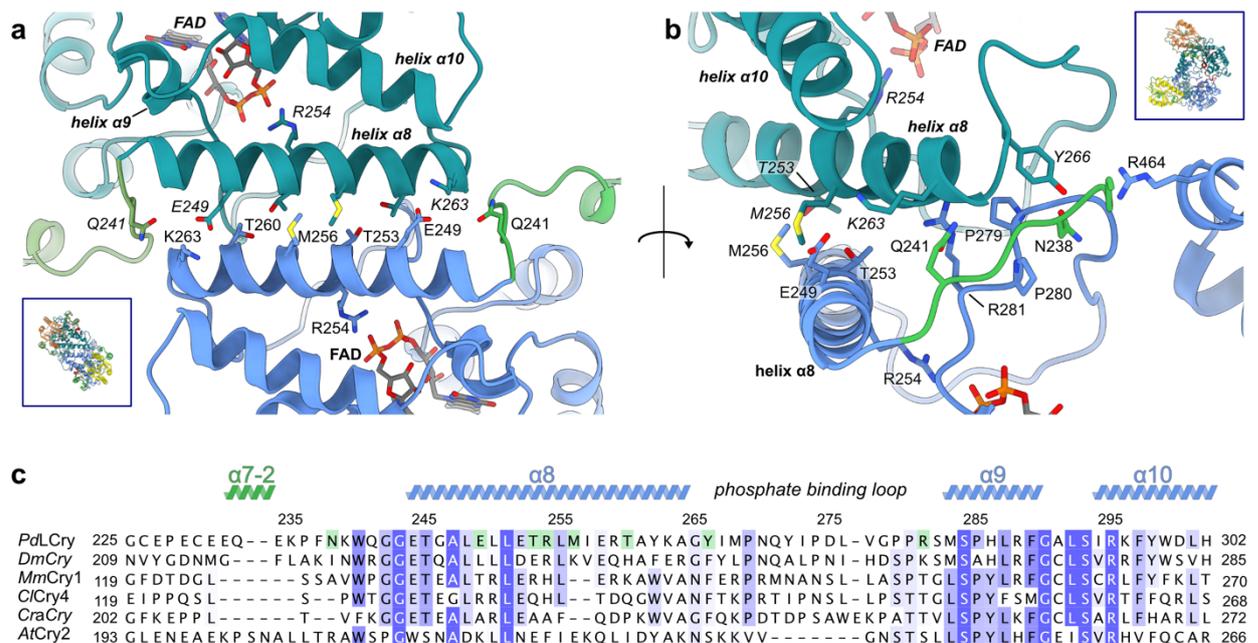


Figure 3: Helix  $\alpha 8$  and its flanking regions are central to the dimer interface.

**a** Close-up view of the dimer interface and **b** 90-degree rotated view highlight the importance of helix  $\alpha 8$  for the dark state dimer. Helix  $\alpha 8$  of subunit 1 (using the same color code as in Fig. 2) runs antiparallel to helix  $\alpha 8$  of subunit 2 (colored in darker shades as in Fig. 2), at an angle of 42 degrees. The contact point between the two helices is at the central Met256, flanked by Thr253 and Thr260. Glu249 and Arg281 appear to lock the helices in place, with possible additional stabilization by interactions centered around Tyr266 of the phosphate binding loop. Arg254 of helix  $\alpha 8$  contacts the FAD chromophore. Inserts show the respective orientation of the dimer in panels a and b. **c** Sequence alignment of *PdLCry* with plant, animal and animal-like CRYs of known structure indicates that the observed dimer interface is unique to *PdLCry*. Residues discussed in the text are highlighted in green. Secondary structure elements and amino acid

numbering of *PdLCry* are shown above the sequences. Full species names and protein sequences used are listed in Supplementary Table 1.

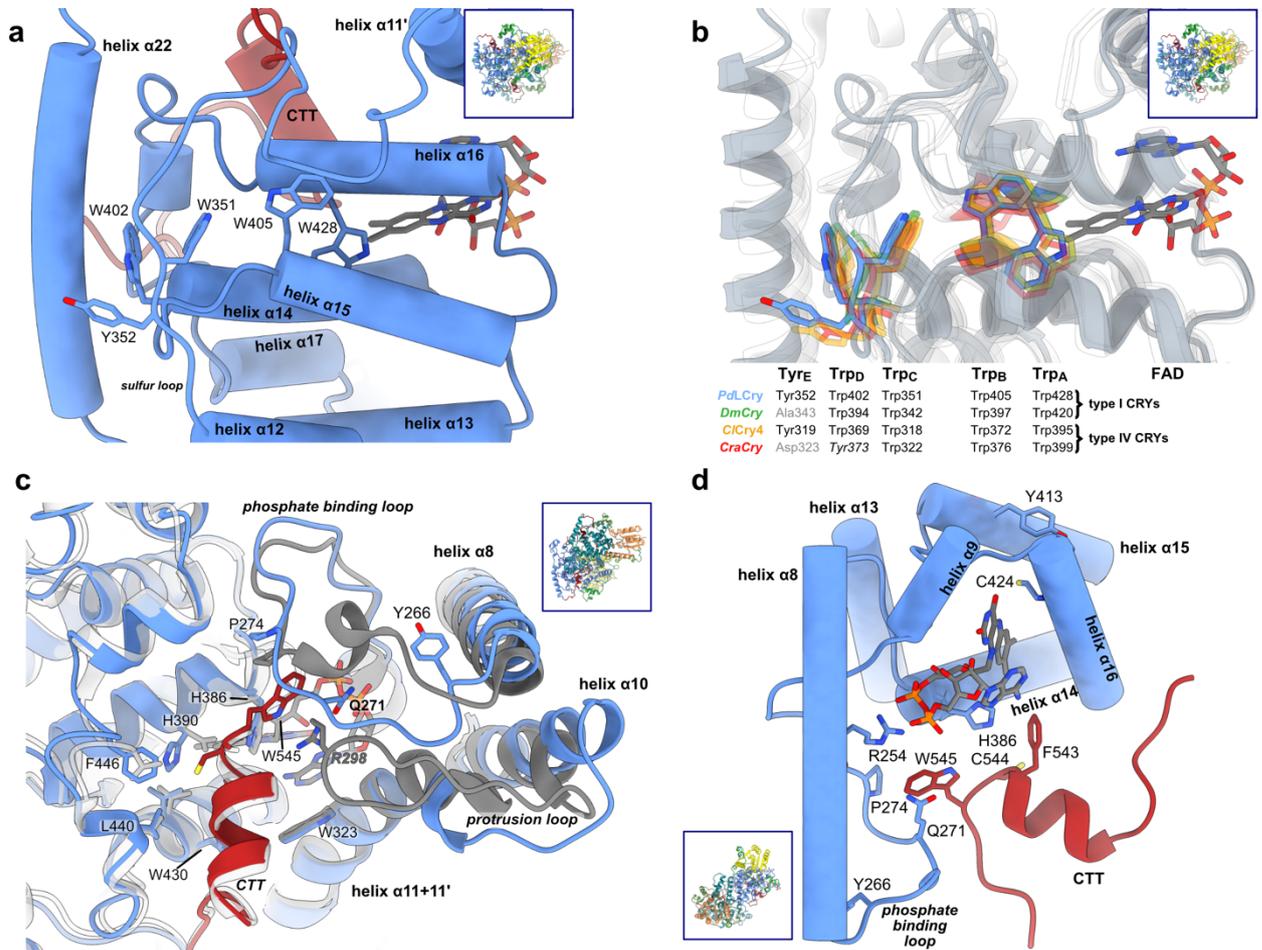


Figure 4: Structural data suggest that the PBL links chromophore activation, dimerization and the CTT release.

**a** *PdLCry* has five aromatic residues potentially involved in the photoinduced electron transfer, namely Trp428 (Trp<sub>A</sub>), Trp405 (Trp<sub>B</sub>), Trp351 (Trp<sub>C</sub>), Trp402 (Trp<sub>D</sub>) and Tyr352 (Tyr<sub>E</sub>). **b** The core Trp-triad consisting of Trp<sub>A</sub>, Trp<sub>B</sub>, and Trp<sub>C</sub> is conserved in the light-responsive type I and IV CRYs whose structure has been solved to date: *PdLCry* (blue), *DmCry* (green; PDB 4JZY), *C/Cry4* (orange; PDB 6PU0), and *CraCry* (red; PDB 6ZM0). The fourth aromatic residue, which extends this to a Trp-tetrad, is conserved except for *CraCry*, which features a tyrosine at this position. Only *PdLCry* and the type IV CRY *C/Cry4* further extend the electron transport chain by a fifth member, which is a tyrosine in both proteins. *PdLCry* is shown in transparent gray, the other CRY structures are shown in transparent white. **c** An overlay of the *PdLCry* (colored) and

the *DmCry* (gray and white; PDB 6WTB) structures shows that both share a CTT that is anchored in the DNA substrate binding pocket of PLs. However, instead of an FFW motif, the *PdLCry* CTT is anchored by an FCW motif. The smaller Cys544 interacts with His390, which occupies a different position compared to *DmCry* Asn382. Importantly, the *PdLCry* protrusion loop is considerably shorter than its wild-type *DmCry* counterpart (dark gray). This results in a different contact to the FCW motif: whereas in *DmCry* Arg298 (gray) from the protrusion loop contacts the CTT tryptophan residue, in *PdLCry* this stabilizing contact is mediated by Gln271 from the phosphate binding loop, which also harbors Tyr266. Therefore, in *PdLCry* the CTT is structurally linked with the dimer interface helix  $\alpha 8$ , rather than with the helices  $\alpha 10$  and  $\alpha 11$  as observed in *DmCry*. **d** FAD is bound in a typical type I CRY FAD-binding site, with Cys424 contacting the N5 position of the isoalloxazine ring of FAD, and Tyr413 blocking solvent access to FAD. Our dark state structure reveals a molecular network surrounding FAD and connecting it to both the CTT and the dimer interface helix  $\alpha 8$ . His386 is positioned between FAD and the FCW motif, while Gln271 from the phosphate binding loop connects helix  $\alpha 8$  to Trp545 of the CTT-anchoring FCW motif, and Arg254 connects this helix to the chromophore. Inserts show the orientation of the dimer for each panel.

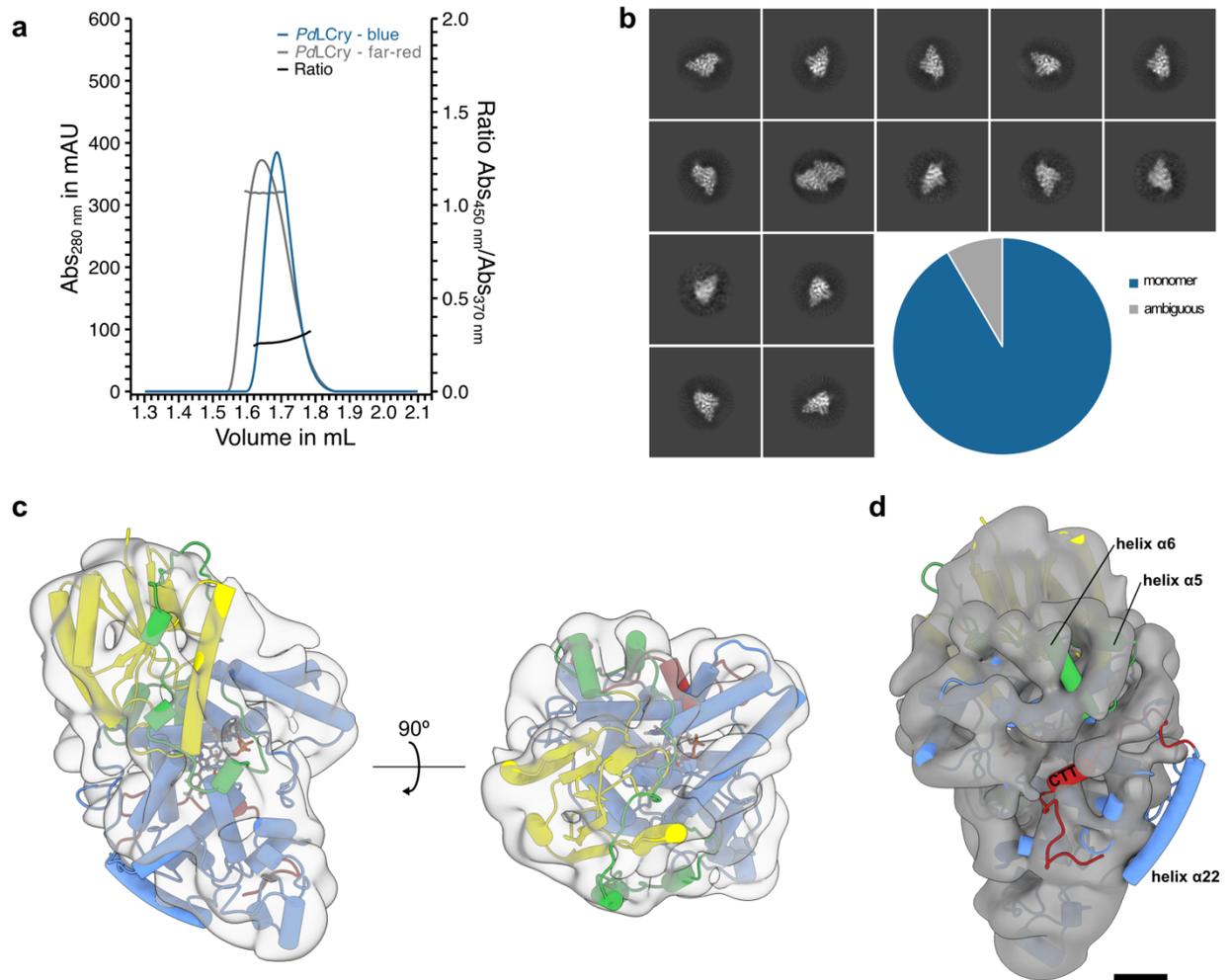


Figure 5: Under intense illumination  $FAD^{ASQ}$ -bound *PdLCry* disassembles into a monomer.

**a** Analytical size exclusion chromatography using a Superdex 200 Increase 5/150 GL column of *PdLCry* under continuous blue light conditions shows an elution peak following absorption at 280 nm (blue curve), which, based on comparison with the elution profile of the dark state SEC (gray curve), likely corresponds to a monomeric state of the protein. The dominant absorption at 370 nm compared to 450 nm (black line;  $Abs_{450nm}/Abs_{370nm} = 0.28$ ) suggests that *PdLCry* was maintained in the  $FAD^{ASQ}$ -bound photoactivated state, in which the anionic semiquinone radical shifts the spectral properties of  $FAD^2$ . **b** Representative 2D class averages of an unsupervised 2D classification of the photoactivated *PdLCry* cryo-EM sample confirm the absence of dimeric particles after illumination. Quantification of all protein-shaped particles implies that at least 90% of the protein is monomeric. Only a few classes are ambiguous, showing two subunits but in an unusual arrangement, possibly due to the close packing of the particles on the cryo-EM grid. **c**

Surface representation of the cryo-EM density map of photoactivated *PdLCry* low-pass filtered to 8 Å shows a classical PL/CRY-shaped monomer. Due to the limited data quality, we refrained from building an atomic model. However, a single subunit of the dark state *PdLCry* structure (using the same colors as in Fig. 2) fits well with the observed density. **d** Apart from helix  $\alpha 22$ , for which there is no clear density, parts of the connector region, especially helices  $\alpha 5$  and  $\alpha 6$ , appear to adopt a different conformation, and the density for the CTT and C-terminal parts of the C-terminal expansion is largely fragmented. Scale bar corresponds to 10 Å.

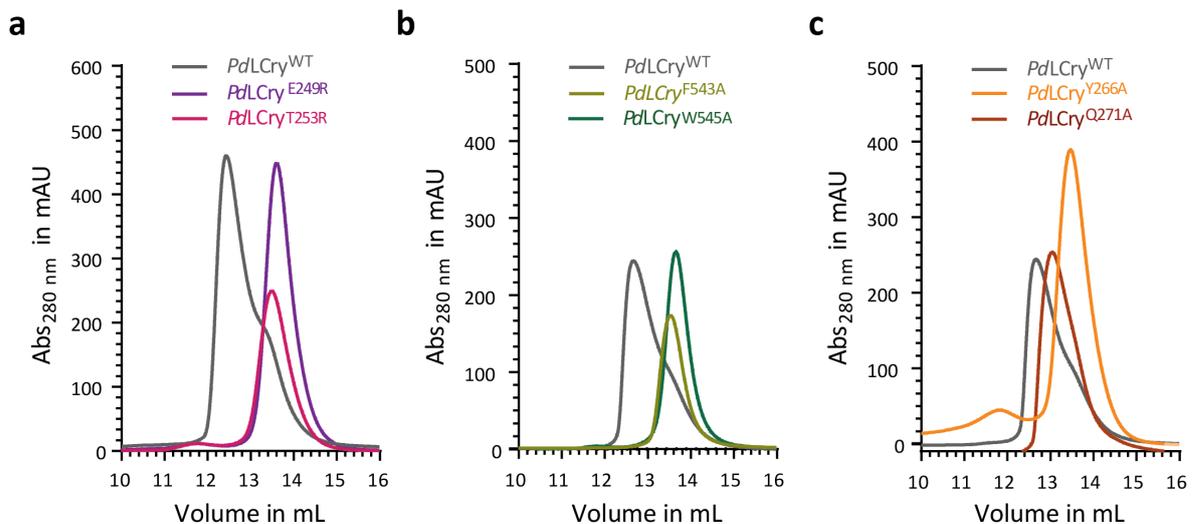


Figure 6: Helix  $\alpha 8$ , the PBL, and the CTT are involved in *PdLCry* dimer formation.

**a** Analytical size exclusion chromatography of wildtype (wt) *PdLCry* (grey) using a Superdex 200 10/300 GL column under far-red light conditions shows a main elution peak at 12.5 mL with a significant shoulder at 13.3 mL. This shoulder suggests a partial dimer dissociation under far-red light conditions as also observed during cryo-EM (compare Fig. 2b). Using the same SEC conditions, *PdLCry*<sup>E249R</sup> (purple) and *PdLCry*<sup>T253R</sup> (pink), with single mutations in the dimer helix  $\alpha 8$ , show a main elution peak coinciding with the shoulder of wildtype *PdLCry* (grey), suggesting that these mutations disrupt the dimer. **b** Analytical size exclusion chromatography of *PdLCry*<sup>F543A</sup> (light green) and *PdLCry*<sup>W545A</sup> (dark green) under far-red light conditions, with single mutations in the FCW motif anchoring the CTT, show a main elution peak coinciding with the shoulder of wildtype *PdLCry*, suggesting that uncoupling the CTT from its binding pocket disrupts the dimer. **c** Analytical size exclusion chromatography of *PdLCry*<sup>Y266A</sup> (orange) and

*PdLCry*<sup>Q271A</sup> (brown) under far-red light conditions, with single mutations in the PBL, also shows an effect on the elution profile. *PdLCry*<sup>Y266A</sup> (orange) shows a main elution peak coinciding with the shoulder of wildtype *PdLCry*, consistent with a disruption of the dimer. *PdLCry*<sup>Q271A</sup> (brown) shows an elution profile with the main peak shifted to 13 mL and a shoulder at 13.5 mL, which could suggest a partial disruption of the dimer. See Supplementary Fig. S6 for final SEC purification step of wildtype and mutant *PdLCry* proteins with SDS-PAGE quality control.

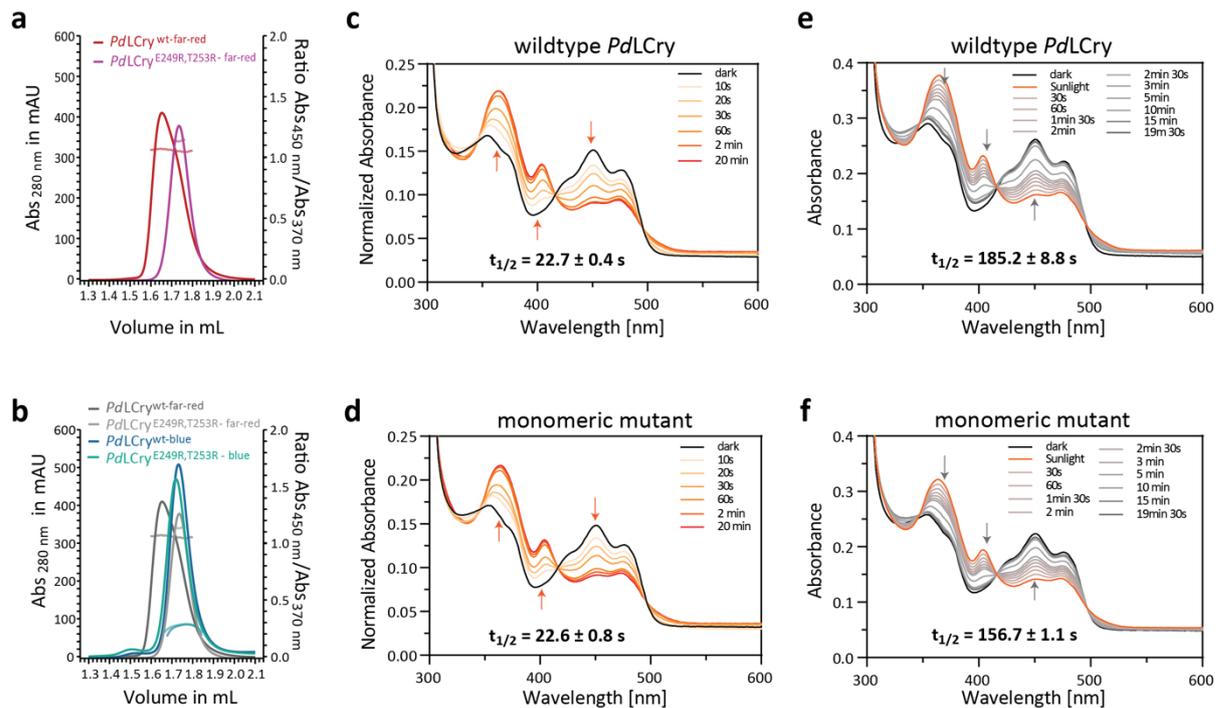


Figure 7: The oligomeric state of *PdLCry* affects its dark recovery kinetics.

**a** Analytical size exclusion chromatography (SEC) using a Superdex 200 Increase 5/150 GL column of *PdLCry* (red) and *PdLCry*<sup>E249R,T253R</sup> (magenta) under far-red light conditions. The chromatography trace shows that the double mutation in the interface helix  $\alpha 8$  quantitatively converts the dimer into a smaller species, without affecting the ground state of the chromophore that is kept in the FAD<sup>OX</sup> state as indicated by the nearly equal absorbance at 370 nm and 450 nm for both wildtype and the double mutation (*PdLCry* red, Abs<sub>450nm</sub>/Abs<sub>370nm</sub> = 1.06; *PdLCry*<sup>E249R,T253R</sup> magenta, Abs<sub>450nm</sub>/Abs<sub>370nm</sub> = 1.14). **b** Upon analytical SEC under continuous blue light conditions, wildtype *PdLCry* and *PdLCry*<sup>E249R,T253R</sup> (dark and light blue) elute at the same volume, which coincides with the smaller species observed under far-red light conditions

(light grey) for  $PdLCry^{E249R,T253R}$ , supporting the constitutively monomeric state of  $PdLCry^{E249R,T253R}$ . The dominant absorption at 370 nm compared to 450 nm ( $PdLCry$  blue,  $Abs_{450nm}/Abs_{370nm} = 0.28$ ;  $PdLCry^{E249R,T253R}$  cyan,  $Abs_{450nm}/Abs_{370nm} = 0.28$ ) highlights that the double mutation does not impact the ability of  $PdLCry$  to be converted into an  $FAD^{ASQ}$ -bound photoactivated state by prolonged illumination. **c,d** Normalized absorption spectra for sunlight photoreduction on ice show that **c** wildtype  $PdLCry$  and **d** monomeric  $PdLCry^{E249R,T253R}$  have similar sunlight photoreduction rates of  $t_{1/2}$  22.7 and 22.6 s. Arrows indicate absorbance changes at 370 nm, 404 nm and 450 nm upon photoreduction of dark-state  $FAD^{OX}$  (black) to the anionic  $FAD^{ASQ}$  radical (dark orange). **e,f** Absorption spectra for dark recovery at 18 °C after full (20 min) sunlight photoreduction show that compared to **e** wildtype  $PdLCry$ , the **f** monomeric  $PdLCry^{E249R,T253R}$  mutant has a faster dark recovery with an average  $t_{1/2}$  of 157 s instead of 185 s. Arrows indicate absorbance changes at 370 nm, 404 nm and 450 nm upon dark recovery of the anionic  $FAD^{ASQ}$  radical (orange) to  $FAD^{OX}$  (black). The full spectra show a representative of three independent replicates. The  $t_{1/2}$  values are the means of three independent measurements with standard error of the mean (SEM). See Supplementary Fig. 7b, c for average data plots.

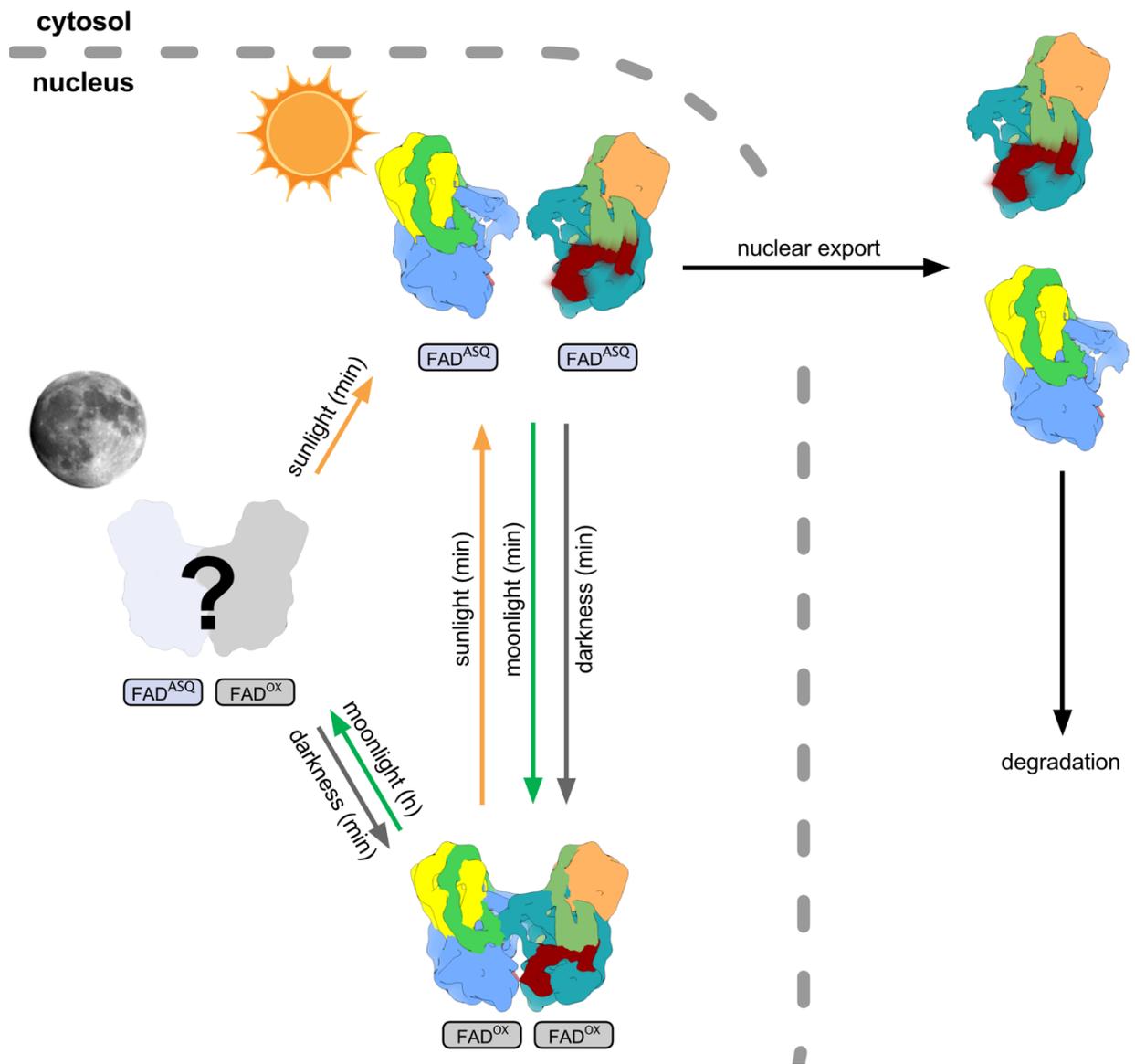


Figure 8: The blue light stimulated dimer-to-monomer transition rationalizes previous *in vitro* and *in vivo* data.

Schematic overview of putative activation pathways at either low photon flux rates (moonlight) leading to a partial activation of the dimer within hours, or at high photon flux rates (sunlight) leading to a monomerization of the sample. *In vitro* assays have shown that the sunlight activated state cannot be directly converted to the moonlight activated state<sup>3</sup>, but is only accessible via the dimeric dark state with FAD<sup>ox</sup> bound in both subunits. The monomeric FAD<sup>asq</sup>-bound state could be transported from the nucleus to the cytoplasm and subsequently targeted by a degradation pathway<sup>4</sup>.

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