



Comparative properties and functions of type 2 and type 4 pigeon cryptochromes

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Abstract

Two types of vertebrate cryptochromes (Crys) are currently recognized. Type 2 Crys function in the molecular circadian clock as light-independent transcriptional repressors. Type 4 Crys are a newly discovered group with unknown function, although they are flavoproteins, and therefore, may function as photoreceptors. It has been postulated that Crys function in light-dependent magnetoreception, which is thought to contribute towards homing and migratory behaviors. Here we have cloned and annotated the full-length pigeon ClCry1, ClCry2, and ClCry4 genes, and characterized the full-length proteins and several site-directed mutants to investigate the roles of these proteins. ClCry1 and ClCry2 are phylogenetically grouped as Type 2 Crys and thus are expected to be core components of the pigeon circadian clock. Interestingly, we find that ClCry4 is properly annotated as a Type 4 Cry. It appears that many birds possess a Type 4 Cry which, as in pigeon, is misannotated. Like the Type 2 Crys, ClCry4 is widespread in pigeon tissues. However, unlike the Type 2 Crys, ClCry4 is cytosolic, and purified ClCry4 possesses FAD cofactor, which confers characteristic UV–Vis spectra as well as two photochemical activities. We find that ClCry4 undergoes light-dependent conformational change, which is a property of insect Type 1 Crys involved in the insect-specific pathway of photoentrainment of the biological clock. ClCry4 can also be photochemically reduced by a mechanism common to all FAD-containing Cry family members, and this mechanism is postulated to be influenced by the geomagnetic field. Thus pigeon Crys control circadian behavior and may also have photosensory function.

Keywords Cryptochrome · FAD · Photoreduction · Magnetoreception · Pigeon · Circadian

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Introduction

The cryptochrome/photolyase (Cry/PL) family consists of homologous and structurally related proteins that exhibit a variety of functions (reviewed in [1–4]). Photolyases reverse far UV-induced DNA damage in a blue light-dependent reaction in which an FAD cofactor plays an essential role in catalysis [5]. Crys are larger than PLs and consist of a photolyase homology region (PHR) plus an additional C-terminus with various functions [6, 7]. This C-terminal sequence varies by species and by the type of Cry, of which there are three in animals. The Type 2 animal Crys do not appear to possess FAD [8], and function as essential transcription factors and integral components of the transcription–translation feedback loop that constitutes the circadian clock in both vertebrates and invertebrates [9–12]. Type 1 Crys do possess FAD [13, 14] and function to entrain the circadian clock to the light–dark cycle in some insects [15–18]. Type 4 Crys have been shown to possess FAD [19, 20], but have

no known function. With a cytosolic localization and an inability to repress transcription [21–23], they are the most recently discovered Cry type and have been identified in various non-mammalian vertebrates, including birds, fish, and frogs [24–26].

An interesting attribute of Cry/PL family members that have the FAD cofactor is the redox state. Following purification of Cry/PL proteins, the FAD is commonly in fully oxidized form [1, 13, 19]. However, photolyase is photocatalytically active only in fully reduced form and it is thought that PLs exist only in reduced form *in vivo* [1, 5]. On the other hand, the light-induced conformational change that is central to the role of the Type 1 *Drosophila* Cry occurs *in vitro* with FAD in either oxidized or reduced form [27]. Unfortunately, the redox state of animal Crys *in vivo* is unknown.

It is well established that the oxidized FAD of purified Cry/PL proteins can be photoreduced *in vitro* by exposure to blue light [1, 19]. In Type 1 Crys and PLs, reduction of FAD initially involves transfer of an electron from an adjacent tryptophan (trp) residue, the first of 3 residues of a “trp triad” electron transport chain that constitutes a conduit extending from the FAD binding pocket to the enzyme surface [14, 28, 29]. During electron transfer from the adjacent trp to FAD, a transient radical pair exists. The lifetime of the pair is sufficiently long that theoretically and experimentally *in vitro*, the geomagnetic field could alter the electronic spin state so as to influence a reaction in which the protein was engaged [30–32]. On this basis it has been suggested that Crys may participate in an as-yet undefined biochemical reaction that is influenced by the magnetic field, in a light-dependent manner, and thereby have a role in magnetoreception and thus migratory and homing behaviors (reviewed in [4]).

As an interesting research subject whose activity requires navigation over a large distance, some birds have been shown to detect and make use of the geomagnetic compass in their daily life (reviewed in [33] and [34]). Moreover, avian Crys are located in the retina of birds which is a seemingly ideal place for photosensing [21, 35], and in this respect avian Crys are considered candidate magnetoreceptors which partially direct bird migration and homing in a light-dependent way (reviewed in [36]). Among a series of birds navigating a large distance, pigeons are recognized for their homing ability which is guided partially by magnetoreception [37–39], and we are interested in characterizing the Crys from pigeons in relation to this and other behaviors. In this study we have characterized the three pigeon genes annotated as ClCrys in Genbank, and we report here their complete sequences and proper annotations as ClCry1, ClCry2 (both Type 2 Crys), and ClCry4 (a Type 4 Cry). The properties of ClCry1 and ClCry2 are consistent with their putative role as transcriptional repressors in the circadian clock. ClCry4 was found to be located in many pigeon

tissues principally in the cytosol, and to purify with stoichiometric FAD in the oxidized state. Cry4 was photoreduced by a mechanism similar to other Cry/PL family members via trp triad and underwent a light-dependent conformational change as described for Type 1 Crys. These results clarify the likely role of two Crys in pigeon circadian behavior and are consistent with Cry4 having a possible role in photoreception and/or magnetoreception, although precise physiological underpinnings and biochemical activities for such a role of Cry4 remain undefined.

Materials and methods

Gene cloning

In light of our interest in the pigeon, we searched Genbank for the presence of Cry/PL family genes. We found three ClCry homologues. Two were annotated as Cry1, one was annotated as Cry2, and there was no PL. As described in Results, two of the Crys were re-annotated by us resulting in a Cry1, a Cry2, and a Cry4.

Pigeons used in the study were obtained from Changsha, Hunan Province, China. Total RNA of retina was extracted using the RNAsimple Total RNA kit (Tiangen Biotech) following manufacturer's instructions. RNA concentration was measured using a Qubit 2.0 Fluorometer (Life Technologies), and the integrity of the RNA was monitored on agarose gels.

To clone the ClCry genes, first strand cDNAs were synthesized from retinal RNA with a transcriptor high-fidelity cDNA synthesis kit (Roche) using an oligo (dT) primer following the instructions of the manufacturer. The second step of RT-PCR included 30 cycles of denaturation at 98 °C for 10 s, annealing at 64 °C for 15 s and extension at 72 °C for 2 min using the high-fidelity polymerase Primestart (Takara). The primers used are listed in Online Resource 1; they were designed according to the sequences as annotated by Genbank. Then we sought to extend the 5' ends and clone the presumptive full-length Cry genes by using the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) following the manufacturer's instructions. The gene specific primers (GSPs) used in RACE are listed in Table 1 of Online Resource 1. The resulting plasmids bearing the full-length Cry genes in the pMD19T T-vector are listed in Table 2 of Online Resource 1. Full-length sequences of ClCry1/2/4 cDNAs have been uploaded to NCBI with accession numbers MG839692, MG839693 and MG839694, respectively.

Phylogenetic analysis

The phylogenetic tree was made using the software Mega 5.0. Protein sequences and NCBI accession

numbers used included XIcCry1 [NP_001081129.1], XIcCry2 s [NP_001083936.1] and XtCry4 [NP_001123706.1] from frog (*Xenopus laevis*, Xl and *Xenopus tropicalis*, Xt), DrCry1 [NP_001070765.2], DrCry2 [NP_571861.2] and DrCry4 [NP_571862.1] from zebrafish (*Danio rerio*, Dr), DpCry1 [AAX58599.1] and DpCry2 [ABA62409.1] from monarch butterfly (*Danaus plexippus*, Dp), DmCry from *Drosophila melanogaster* (Dm), BiCry2 [NP_001267051.1] from bee (*Bombus impatiens*, Bi) and CIcCry1, CIcCry2 and CIcCry4 from pigeon. N-terminal and C-terminal ends of all sequences were removed and only sequences homologous with DpCry2 (from amino acids 27 to 540) were used.

Tissue distribution and expression pattern

Pigeons were housed in a 12:12 h light:dark (LD) cycle for at least 1 week, with white light on from zeitgeber time (ZT) 0 to ZT 12 and off from ZT 12 to ZT 24. Fresh tissues of pigeons, including cerebrum, midbrain, cerebellum, heart, liver, skeletal muscle, retina, kidney, lung, intestine, and skin were acquired every 6 h at ZT 3, ZT 9, ZT 15 and ZT 21, then frozen in liquid nitrogen and stored in -80°C . A second independent repeat used pigeon retina obtained at ZT1, ZT7, ZT13 and ZT19.

We used RT-PCR to characterize gene expression in different tissues. The method of RNA extraction and cDNA synthesis was the same as described in the gene cloning section. For the tissue distribution experiment, cDNAs generated from RNA isolated at ZT 9 were used. Specific primers (shown in Table 1 of Online Resource 1) were designed using Primer premier 5.0 and checked with the NCBI primer blast webpage server (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The PCRs were performed using Dreamtaq (Thermo), including pre-incubation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 20 s, with a final incubation at 72°C for 10 min.

To reveal the circadian expression patterns of CIcCry1/2 in LD cycles, real-time qPCRs were performed using the instrument LightCycler 480 II (Roche). The housekeeping gene encoding pigeon beta actin (β -actin, GenBank AB980793.1) was selected as reference for the calculation of relative expression levels of target genes. PCRs were performed with Light Cycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. The reaction consisted of pre-incubation at 50°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 10 s and extension at 72°C for 10 s. Each cDNA sample was amplified in triplicate. Each data point represents the average result obtained from three individual animals.

Subcellular localization

NIH-3T3 cells were seeded on a chamber slide in DMEM-H medium (Gibco) with 10% FBS (Sigma) and incubated at 37°C in 5% CO_2 . CIcCry1 and CIcCry4 were cloned into pEGFPN1 to create plasmids CIcCry1_pEGFPN1 and CIcCry4_pEGFPN1 (Table 2 of Online Resource 1). These plasmids express the Crys as EGFP fusion proteins. Plasmids were transfected into NIH-3T3 cells using Lipofectamine 3000 (Gibco) following the manufacturer's instructions. After 48 h, we removed the medium from the slide and washed cells once with 0.5 mL PBS. Then cells were fixed in 0.5 mL ice cold methanol on ice for 10 min. Then we washed the cells with PBS three times and stained cells with 0.5 mL of 0.2% propidium iodide in PBS with 10 mg/mL RNase A for 15 min to reveal the cell nucleus. After cells were washed once with 0.5 mL PBS, fluoromount (Sigma) was added, and slides were stored at 4°C and always kept in the dark. Slides were viewed with a fluorescence microscope (Olympus IX81).

Protein expression and purification

Wildtype (WT) CIcCry1 and CIcCry4 genes were cloned into a modified pFastBac1 plasmid to introduce an N-terminal FLAG tag (Table 2 of Online Resource 1), and then were expressed using the Bac-to-Bac baculovirus expression system (Invitrogen). Mutant constructs were acquired using the Q5 Site-Directed Mutagenesis Kit (NEB) following manufacturer's instructions. Passage 4 high titer baculovirus at 1:100 ratio was added to Sf21 cells ($1.2\text{--}1.5 \times 10^6$ per mL), and infected cells were grown for 72 h in the dark in Grace's insect medium with 10% FBS at 27°C . Cells were then harvested by centrifugation, washed with ice cold PBS, and cells from 1 L were resuspended in 20 mL of lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40 and 0.5% Triton-X 100. Resuspended cells were then sonicated 10 times for 10 s each time on ice. The cell lysate was cleared by centrifugation at $17,000 \times g$ for at least 1 h. 400 μL (bed) of anti-FLAG M2-agarose beads (Sigma) was incubated with the cell lysate at 4°C with rotation for 2 h and then washed three times with 30 mL of $1 \times \text{TBS}$ (20 mM Tris-HCl pH 7.5, 150 mM NaCl). 400 μL $1 \times \text{TBS}$ containing 150 $\mu\text{g}/\text{mL}$ FLAG peptide (Sigma) was used to elute target protein. Then proteins were dialyzed overnight against storage buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM DTT and 55% glycerol. Proteins were kept at -20°C and used within a week. All purification steps were performed in dim yellow light.

Photoreduction, reoxidation, spectroscopy, and denaturation

WT and mutant ClCry4 proteins in storage buffer were placed in a 24-well plate on ice and exposed to blue light using a F15T8-BLB black light (General Electric, principal emission 366 nm) as an energy source. In the reduction process, different fluence rates were used. For exposures below 13.5 mJ/cm², 0.1 mW/cm² was used. For irradiation (IR) between 13.5 mJ/cm² and 121.5 mJ/cm², 0.5 mW/cm² was used. For IR above 121.5 mJ/cm², 1.5 mW/cm² was used. A heat-reflecting glass (Precision Glass and Optics) was located between the light source and the samples to prevent heating. Immediately after exposure, the UV-Vis spectrum was measured with a Shimadzu UV-1601 spectrophotometer. After photoreduction, samples were placed in 1.5 mL tubes without caps on ice in a 4 °C cold room with dim yellow light and in aerobic condition to allow reoxidation. Difference absorption spectra were acquired for successive IR doses by taking the absorption spectrum after each specific IR dose and subtracting the spectra obtained using the former lower dose of IR. To denature the protein and free the cofactor, apoprotein was precipitated with 7.2% trichloroacetic acid (final concentration) on ice for 20 min, and then the pH of the solution was adjusted to 7.0 using 3 M NaOH.

Partial proteolysis and western blot

The trypsin proteolysis experiment was performed following previous studies [27] with some modifications. 5 µg of WT FLAG-ClCry4 was aliquoted into 200 µL PCR tubes containing 1×PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄) pH 7.4 in a total volume of 50 µL, and reactions were initiated with the addition of 5 µL of sequencing grade trypsin (Promega) at final trypsin: protein ratios of 1:100, 1:200 and 1:300 (W:W). For low IR and high IR, tubes were exposed to blue light at 0.3 mW/cm² and 1.2 mW/cm², respectively. For dark controls, samples were wrapped with aluminum foil. Reactions continued for 30 min at 25 °C, and then were stopped by adding LDS loading buffer (Invitrogen). Then, 35 µL of each reaction was resolved with a 4–12% pre-cast SDS-PAGE gel (Invitrogen). After proteins were transferred onto a nitrocellulose membrane, western blot was performed with anti-FLAG antibody (Sigma). An exposure for 40 s was used for high exposure and 10 s was used for low exposure.

Results

Cloning, sequences and phylogeny of ClCrys

Initial searches for Cry/PL family genes in the pigeon genome available in Genbank revealed two Cry1 homologues (protein accession numbers XP_005507667.2 and XP_005510394.1) and one Cry2 homologue (XP_013222407.1). Inspection of the gene sequences indicated that one of the ClCry1 sequences (XP_005507667.2) and the ClCry2 sequence were missing their 5' termini. We obtained clones for the three ClCry genes and used 5' RACE to obtain the 5' ends of the two truncated genes. As a result we obtained apparently full-length clones of these two genes. Each has an initiation codon and they encode a 1863 bp ClCry1 and a 1752 bp ClCry2. The amino acid sequences of these two ClCry genes align well with other vertebrate Cry1s (Fig. 1 in Online Resource 1) and Cry2s (Fig. 2 in Online Resource 1). The vertebrate Cry1s and Cry2s are each highly homologous, with some species-specific variability in the C-terminal extensions. Overall, the alignments indicate that the sequences which we obtained are full-length cDNAs properly annotated as Cry1 and Cry2.

We initially considered that the second annotated ClCry1 (XP_005510394.1) found in Genbank could represent another homologue or isoform of ClCry1. However, the second ClCry1 had a relatively short C-terminal extension, as shown in Fig. 3 of Online Resource 1. Therefore, we aligned the second ClCry1 with vertebrate Cry4 sequences which tend to exhibit abbreviated C-termini. The results in Fig. 4 of Online Resource 1 show high homology of the ClCry with the Cry4s especially with the GgCry4 of chicken. Based upon these results and the properties of this Cry described below, we conclude that this cryptochrome is properly annotated as ClCry4. We extended our homology search as shown in Fig. 5 of Online Resource 1 and found that interestingly, Cry4 genes exist in almost all kinds of birds, including migratory and non-migratory birds. These Cry4 genes are correctly annotated in several birds [19, 21, 40], however, most are presently annotated as Cry1-like genes or Cry1 isoforms.

We used our sequence and homology results to construct a phylogenetic tree, in which several kinds of typical and well studied Crys are included. The tree is shown in Fig. 1a. At the first node, invertebrate Cry1s, which are classified as Type 1 Crys, separate from other Crys, most of which are vertebrate. The Type 1 Crys mainly function as circadian photoreceptors in vivo [16, 41]. Some studies suggest that they also work as photo-induced magnetoreceptors [42]. On the other phylogenetic branch, a node separates Type 2 from Type 4 Crys. The Type 2 Cry

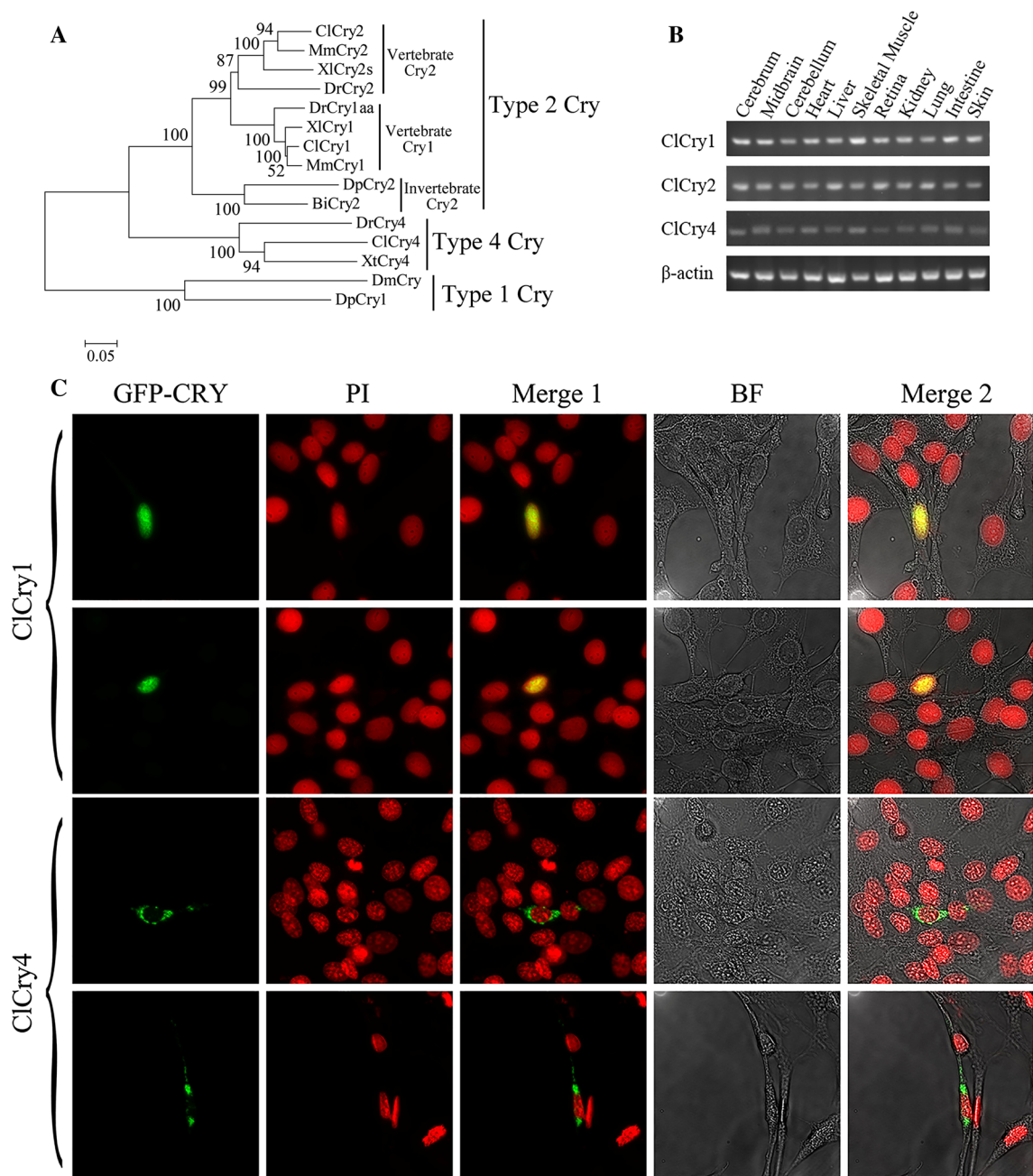


Fig. 1 Phylogenetic analysis, tissue distribution and subcellular localization of ClCrys. **a** Phylogenetic analysis of animal Cry proteins. Bootstrap probabilities are represented by values near the nodes. Several examples of animal Cry proteins are included as Type 1 Cry proteins, Type 2 Cry proteins and Type 4 Cry proteins. Names of species and accession numbers of sequences can be acquired from the “Materials and methods” section. **b** Expression of ClCry1, ClCry2, ClCry4 and β -actin RNA in the 10 indi-

cated pigeon tissues detected by RT-PCR. **c** Subcellular localization of ClCry1 and ClCry4 is revealed by expressing EGFP-ClCry fusion protein in cells. The five columns left to right show EGFP-Cry fusion protein (GFP-CRY), propidium iodide staining (PI), Merge 1 (Merging 1st and 2nd columns), bright field (BF), and Merge 2 (Merging 3rd and 4th columns). The top two rows illustrate GFP-ClCry1, and the bottom two rows illustrate GFP-ClCry4

include vertebrate Cry1s, vertebrate Cry2s, and invertebrate Cry2s. These function as direct transcriptional repressors controlling the circadian rhythm of animals [9, 11]. The Type 4 Cry proteins are the most recently identified and classified group, and Cry4s have been discovered only in

vertebrates but not mammals and as yet have no known function. Overall, the phylogenetic analysis suggests that ClCry1 and ClCry2 are Type 2 Cry proteins that likely mediate pigeon circadian rhythms, and ClCry4 is a Type 4 Cry protein with unknown function. Besides the pigeon Cry proteins in this study,

several other Type 2 and Type 4 avian Crys have also been annotated, including those in chicken, quail and European robins [21, 25, 43, 44].

Circadian expression patterns, tissue distributions and subcellular localizations

The tissue distribution of the three ClCrys was monitored by RT-PCR. The results in Fig. 1b show expression of all three RNAs in all tissues examined. This wide distribution is expected for ClCry1 and ClCry2, since they are presumed to be transcription factors involved in circadian rhythmicity throughout the body. For ClCry4, this distribution is informative.

The function of Crys in the circadian clock is associated with their rhythmic expression in birds, mice and other organisms [21, 45–47]. Therefore, we examined whether ClCry1 and ClCry2 exhibited rhythmicity in pigeon tissues. Results in Fig. 6 of Online Resource 1 demonstrate, under our conditions, possible weak rhythmicity in cerebellum, heart, and liver, especially for ClCry1. Our second repeat in retina shows a mild, distinguishable circadian expression pattern of ClCry1, but not of ClCry4 (Fig. 7 of Online Resource). The mRNA peak of ClCry1 usually appears from the afternoon to early evening, which is similar to the results in the retina of European robin [21]. However, relatively large errors were obtained in some of our results. These may obscure rhythmicity and may arise from animal-to-animal variability associated with the lack of a defined pigeon model system.

Subcellular distributions of ClCry1 and ClCry4, expressed as GFP-fusion proteins in NIH-3T3 cells, are shown in Fig. 1c. Due to the low expression levels of the fusion proteins, here we can only show some scattered cells

in each field. The expression of the ClCrys is displayed in green (first column) and the nucleus is shown in red (second column). In GFP-ClCry1 transfected cells, most cells (77%, 75 cells counted) express ClCry1 only in the nucleus. In GFP-ClCry4 transfected cells, however, most cells (89%, 96 cells total) express ClCry4 only in the cytosol. Recently, the cytosolic localization of robin Cry4 in the double cones and long-wavelength single cones of the retina was reported, in agreement with our results. As a transcription factor and a Type 2 Cry, ClCry1 is expected to be transported to the nucleus; furthermore, ClCry1 shares a nuclear localization signal with other Cry1s due to their high sequence homology (nuclear localization signals of Type 2 Crys were studied in [48]). In contrast, most ClCry4 does not enter the nucleus, revealing that it likely has nothing to do directly with transcriptional regulation.

Analysis of purified ClCry1 and ClCry4

To further characterize pigeon Crys, we wished to purify the proteins and characterize their properties directly. To this end, we prepared baculovirus expression vectors for expression of FLAG-tagged ClCry1 and ClCry4 in Sf21 insect cells. Insect cells were infected for 3 days, and then tagged proteins were purified from cell extracts using anti-FLAG antibody affinity resin. Approximately 300 μ g ClCry1 (~72 kDa) and 400 μ g of ClCry4 (~62 kDa) were obtained (Fig. 2a), each from 1 L of cells. Absorption spectra of the native proteins in Fig. 2b show that ClCry4 contains FAD chromophore, which was found to be nearly stoichiometric. In contrast, ClCry1 has no detectable flavin. Interestingly, the ClCry4 spectrum shows two major absorption regions, one (doublet or triplet) at 447 nm, and the other (doublet) at 359 nm and 375 nm, revealing that ClCry4 mainly contains

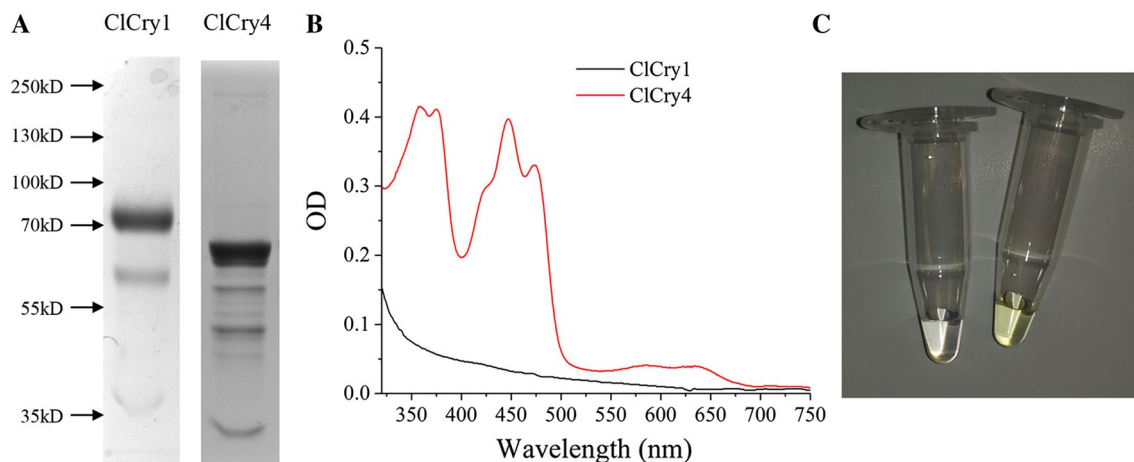


Fig. 2 Purity and UV-visible absorption spectra of ClCry1 and ClCry4. **a** SDS-PAGE of recombinant ClCry1 and ClCry4 with Coomassie staining. **b** Absorption spectra of ClCry1 (black) and ClCry4 (red). **c** Photograph of ClCry1 (left) and ClCry4 (right) taken under white light

oxidized FAD_{ox} in vitro. Moreover, a broad low absorption region from ~ 576 to ~ 644 nm indicates that it also contains a small amount of semireduced FADH^{\cdot} [49]. The photograph of ClCry1 and ClCry4 in Fig. 2c shows that in white light, ClCry1 is colorless and ClCry4 has an obvious light yellow color. Using the concentration of proteins measured by the Bradford Assay (data not shown) and the molar absorption coefficient of FAD_{ox} [50], we calculated the FAD content of the proteins. Recombinant ClCry4 contains no less than 95% FAD, while FAD is not detected in ClCry1.

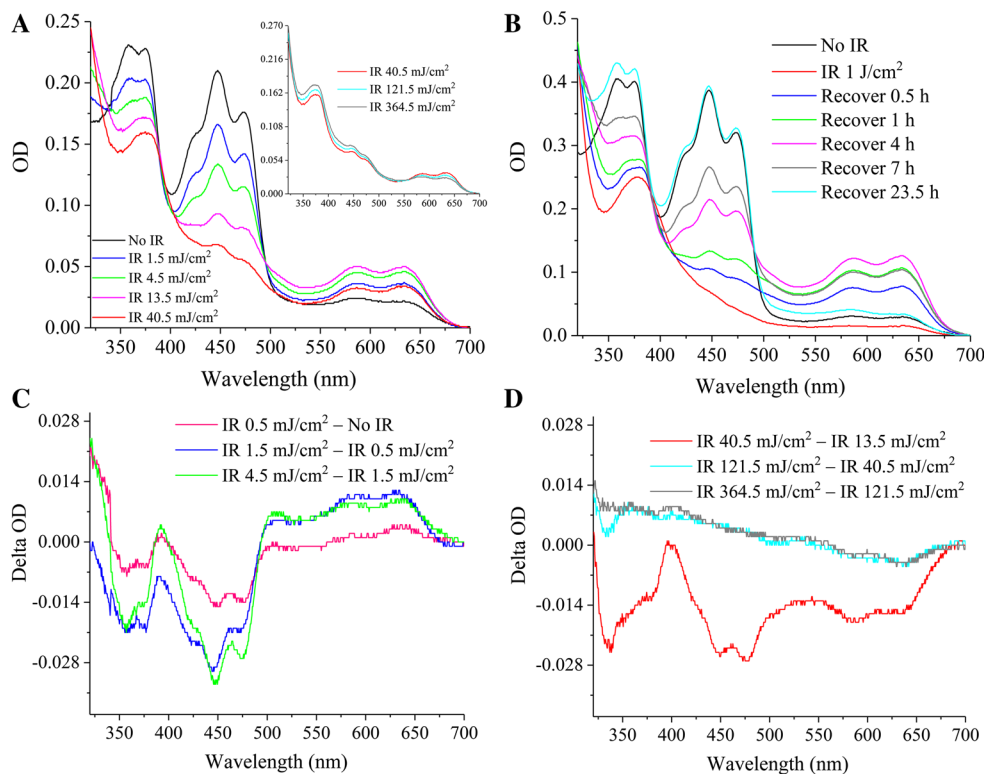
Photoreduction and reoxidation of ClCry4

The FAD cofactor in PLs and Crys exists in various transient or stable oxidation states. PLs are presumed to exist in reduced form in vivo [1, 5], and function only in reduced form. The oxidation state of the Crys in vivo is unknown. PLs are oxidized during purification, and purified Crys are also in oxidized form [1]. Interestingly, oxidized, purified PLs and Crys may be chemically or photochemically reduced. We examined the photoreduction of purified ClCry4 since this property has been suggested to be relevant to its function. We find that the FAD_{ox} in ClCry4 is photoreduced to FADH^- via semireduced FADH^{\cdot} radical. This stepwise reduction can be seen in Fig. 3a. At low doses of blue light (0 – 13.5 mJ/cm^2), the decrease in the 359 nm and 447 nm peaks together with an increase in the broad peak from 576 to 644 nm indicate the loss of the oxidized

FAD (447 nm peak) and creation of the semireduced FADH^{\cdot} (576 – 644 nm peak). With higher doses of blue light (40.5 – 364.5 mJ/cm^2 , inset), the decrease in absorbance of the broad 576–644 nm peak together with the increase in absorbance at 375 nm indicates the transformation from semireduced FADH^{\cdot} to fully reduced FADH^- . This interpretation is supported by analysis of difference absorption spectra. In low energy light (Fig. 3c), there are two troughs of decreased FAD_{ox} at 447 nm and 359 nm, and a broad peak emerges from 576 to 664 nm, indicating FADH^{\cdot} formation. After 40.5 mJ/cm^2 IR (Fig. 3d), semireduced FADH^{\cdot} begins to change to fully reduced FADH^- , with continued negative absorption peaks at 340 nm (mixed absorption of FAD_{ox} and FADH^{\cdot}) and at 447/478 nm (absorption of FAD_{ox}), and then a negative absorption from 576 to 644 nm (loss of absorption by FADH^{\cdot}). Difference spectra after higher energy IR (121.5 mJ/cm^2 and 364.5 mJ/cm^2) are little different from zero indicating that the enzyme is nearly fully reduced, with the remaining FADH^{\cdot} (negative absorption at ~ 620 nm) transforming into FADH^- (absorption at ~ 370 nm). Overall, during photoreduction, the FAD_{ox} in ClCry4 transforms to semireduced FADH^{\cdot} at low energy IR, and both FAD_{ox} and FADH^{\cdot} are reduced to FADH^- with higher energy IR.

In an aerobic environment, photoreduced PLs and Crys spontaneously reoxidize. Figure 3b illustrates the time course of ClCry4 reoxidation in the dark after complete photoreduction with 1 J/cm^2 . Reoxidation of the FADH^- proceeds through the semireduced FADH^{\cdot} intermediate, as indicated

Fig. 3 Absorption and difference absorption spectra showing photoreduction and reoxidation of WT ClCry4. **a** Absorption spectra showing photoreduction of ClCry4 with different doses of blue light IR. **b** Absorption spectra showing the time course of ClCry4 reoxidation following complete reduction with 1 J/cm^2 IR. **c** Difference absorption spectra (Delta OD) of ClCry4 after low energy IR. **d** Difference absorption spectra of ClCry4 after high energy IR. Difference absorption spectra in **c** and **d** are calculated as indicated in the plots; for each interval, the spectral values for the lower energy dose are subtracted from the spectral values for the higher energy dose



by an increase from 0.5 to 4 h in absorbance at 576–664 nm, followed by decreased absorption in this region at 7 and 23.5 h. At the same time, the continuous increase in absorbance at 447 nm from 0 to 23.5 h indicates the accumulation of fully oxidized FAD_{ox}.

FAD photoreduction and electron transfer of ClCry4 via the conserved trp triad

Photoreduction of PLs and Type 1 Crys occurs via a “trp triad”, three conserved trp residues oriented in the protein structure so as to provide a conduit for photoexcited FAD, which is “buried” within the protein, to essentially abstract an electron from the surrounding solvent [14, 28]. To check the role of the trp triad in photoreduction of Type 4 Crys, we aligned the sequence of ClCry4 with the sequences of well characterized Type 1 Crys (Fig. 8 in Online Resource 1) to identify the putative trp triad residues in ClCry4. To investigate the triad, we expressed and purified the putative triad mutants W318F, W372F, and W395F as well as a control protein W350F bearing a mutation in a conserved, but non-triad trp residue (Fig. 8 in Online Resource 1). Figure 9 and Fig. 10 in Online Resource 1 illustrate the purity and absorption spectra of these proteins. The control mutant W350F behaved as wild-type (WT) ClCry4; 300 µg was obtained from 1 L of infected cells, and flavin was present in stoichiometric amount. Unfortunately the triad mutants

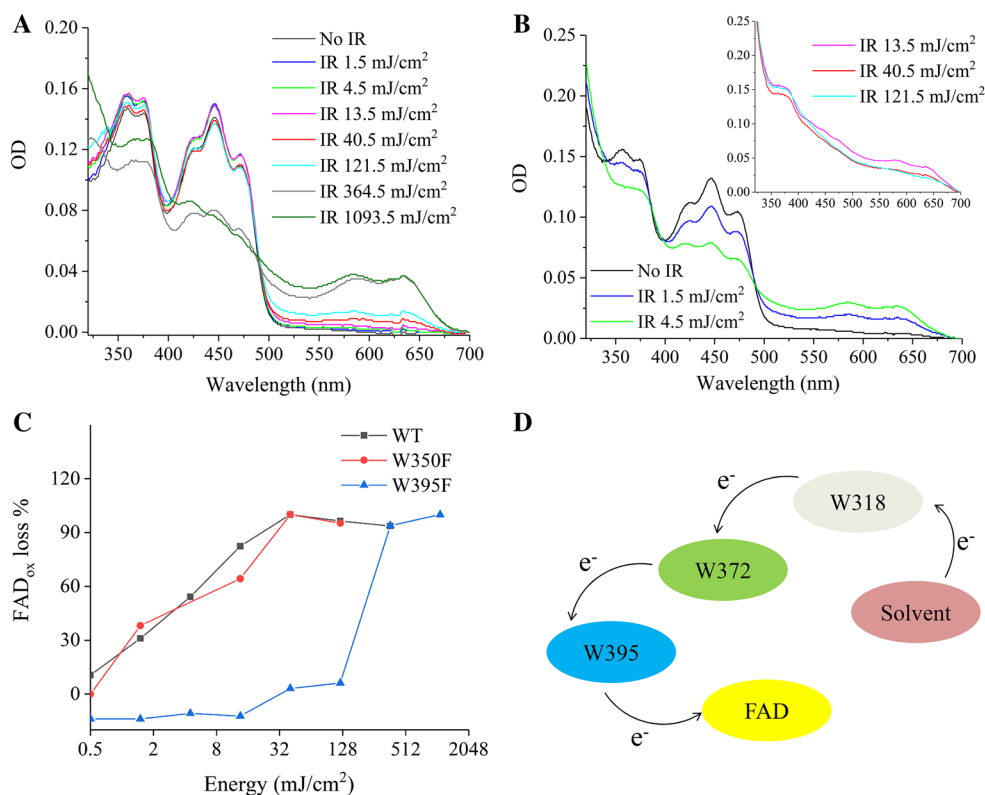
contained FAD in substoichiometric amounts if at all. Of the two mutants with detectable FAD, W395F was purified in greater abundance (300 µg from 1 L cells) than W372F (50 µg from per liter cells), so W395F was repurified from 2 L of cells and employed in subsequent experiments.

To assess photoreduction, the purified W395F (Fig. 4a) and W350F (Fig. 4b) mutant proteins were irradiated with different doses of light, and quantitative values for reduction as a function of dose are plotted in Fig. 4c. Clearly the control W350F protein exhibited WT reduction, and the W395F triad mutant was up to 100-fold less efficiently reduced. Based upon comparisons with other Type 1 Crys and PLs, W395F is the FAD-proximal trp of the triad (Fig. 4d). Our results suggest that the trp triad in pigeon Cry4 functions in photoreduction as it does in other Cry/PL family members, and the excited FAD in W395F is somehow able to inefficiently abstract an electron from some component of its immediate environment.

Light-dependent conformational change and photosensitivity of ClCry4

An interesting property of certain Crys is light-dependent conformational change, which was detected experimentally as light-dependent changes in sensitivity of the Crys towards trypsin digestion [20, 27, 51]. We applied this approach to detect whether light changes the conformation of ClCry4

Fig. 4 Photoreduction of W395F and W350F mutant ClCry4 proteins. **a** Photoreduction of trp triad mutant W395F with different IR energies. No IR indicates oxidized ClCry4. **b** Photoreduction of non-trp triad mutant W350F with different IR energies. **c** Oxidized FAD_{ox} loss versus light energy during photoreduction of WT (black line), W350F (red line) and W395F (blue line) ClCry4. The amount of FAD_{ox} was measured as percent of absorbance at 447 nm compared to unirradiated sample. **d** Electron transfer from the solvent to FAD via the trp triad



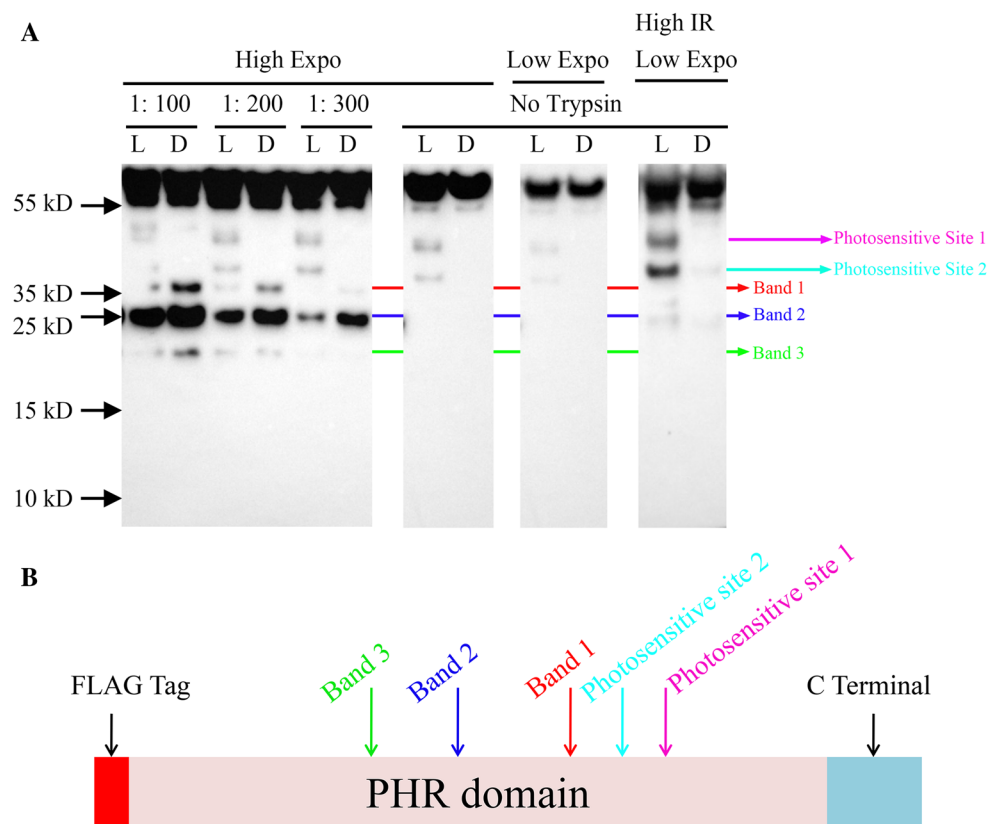


Fig. 5 Light-dependent conformational change and photosensitivity of ClCry4. **a** Effects of light and trypsin partial proteolysis on ClCry4. The ratios of protein: trypsin used for proteolysis are 1:100, 1:200, 1:300 and no trypsin, respectively. Protein was digested with trypsin for 30 min in either light (L) or dark (D) condition. Western blots were developed to show both high band exposures (High Expo) and low band exposures (Low Expo). The right panel was performed

with high IR (1.2 mW/cm^2), the others were low IR (0.3 mW/cm^2). Three partial proteolysis bands and two photosensitive sites are shown with different colors. **b** Illustration of approximate trypsin proteolysis sites and photosensitive sites of ClCry4. Recombinant ClCry4 contains a short N-terminal FLAG tag (red), a PHR domain (pink) and a C-terminal (cyan). The same colors are used to display the trypsin digestion and photosensitive sites for **a** and **b**

(Fig. 5). FLAG-tagged ClCry4 was digested for 30 min with varying, limited concentrations of trypsin either in the presence or absence of low IR (0.3 mW/cm^2). Afterwards the protein was resolved by SDS-PAGE and bands were visualized by western blot with anti-FLAG antibody. The results in Fig. 5a show two obvious effects of light. First, there are three trypsin digestion products (labeled as Bands 1, 2 and 3 with molecular weights of $\sim 36 \text{ kDa}$, $\sim 26 \text{ kDa}$ and $\sim 20 \text{ kDa}$, respectively) that are produced in the dark, but their appearance is inhibited by light exposure. Thus, light prevented proteolysis. In addition to these three proteolysis products, two bands labeled Photosensitive sites 1 and 2 (with a molecular weight of $\sim 50 \text{ kDa}$ and $\sim 42 \text{ kDa}$, respectively) are produced only with light exposure. These bands are produced in the absence of trypsin and are produced to a greater extent with higher IR (1.2 mW/cm^2). In the presence of light, FAD can generate reactive oxygen species capable of breaking bonds, which is the likely cause for these photolysis products of ClCry4. Figure 5b illustrates the relative positions of the proteolysis and photolysis sites.

Discussion

Sequences and functions of pigeon Cry

This study has characterized sequences, and biological and biophysical features of three Crys from pigeon. Our results and analyses have provided the first full-length sequences for two of the Crys, and our phylogenetic analysis has properly annotated ClCry1, ClCry2, and ClCry4. ClCry4 was formerly annotated as a ClCry1 isoform, and this misannotation appears to be common among birds.

ClCry1 and ClCry2 are phylogenetically grouped together as Type 2 Crys, both of which have highly similar sequences with mammalian Cry1s and Cry2s, respectively. Type 2 Crys have no FAD [8], and members of this group have been shown to function as the principal transcriptional repressors in the mammalian transcription–translation feedback loop that constitutes the core molecular circadian clock in a light-independent way [11, 52, 53]. This repressor function is essential and mice lacking their two Type 2 Crys (MmCry1

and MmCry2) have no functioning circadian clock [46, 54]. In addition to their role in mammals, clear circadian expression patterns of Type 2 Crys have been demonstrated in bird tissues recently [21, 45] and Type 2 Crys in both non-mammalian vertebrates and invertebrates have demonstrated properties of transcriptional repressors [9, 22, 24], supporting the idea that Type 2 Crys play a similar and integrated role in various species. Thus, it is presumed that ClCry1 and ClCry2 have essential roles as transcriptional repressors of the pigeon circadian clock. This role is consistent with our findings: purified ClCry1 had no detectable FAD, ClCry1 is a nuclear protein, modest rhythmicity of ClCry1 and ClCry2 was detected in some tissues, and both ClCry1 and ClCry2 were widely distributed among pigeon tissues.

Interestingly, some avian Cry1s are reported to be located in the cytosol of retinal cells [55] and therefore, seem to have a non-circadian function. However, since, avian Cry1s do not appear to bind FAD (8), they are not able to function as photoreceptors and/or magnetoreceptors. The reason why they are located in the cytosol remains unclear.

ClCry4 is a Type 4 Cry, to date a relatively recently discovered and investigated group of proteins. This group is characterized by the presence of FAD cofactor (this study and [19, 56]), some photoinduced activities *in vitro* (this study and [19, 20]), cytosolic localization in bird retina (see also [21]), absence of circadian/transcriptional repressor properties [21–23, 45] and a relatively short C-terminal extension following the PHR domain. Meanwhile, as shown in a recent study, the mRNA expression level in European robin retinae is significantly higher during the migratory season compared to the non-migratory seasons [21]. According to the integrated properties and hypothesis of FAD-containing Crys, ClCry4 is proposed as a photoreceptor and/or magnetoreceptor candidate. However, Type 4 Crys have no known function, and the discussion below compares and contrasts Type 4 Crys with Type 1 Crys which also possess FAD cofactor and function to entrain the biological clock to light/dark cycles.

Photoreduction and electron transfer of ClCry4

ClCry4 (this study) and the Type 1 *Drosophila* Cry [14, 57] were both purified with FAD in the fully oxidized state. An earlier study [19] photoreduced Type 1 and Type 4 Crys with a single high dose of near-UV light. The Type 1 Cry was reduced to anionic radical form, which is probably its ground state *in vivo* [13, 14, 57]. The Type 4 Crys were fully reduced. Our experiments employed graded light doses of photoreactivating light which permitted detection of a ClCry4 neutral radical as an intermediate in photoreduction. Reoxidation of the Type 1 DmCry in the dark was rapid and complete [19], while ClCry4 was similar to AtCry1, GgCry4 and PLs in that reoxidation was slow (overnight)

and proceeded through a neutral radical intermediate [20, 58, 59]. The relatively stable reduced form of ClCry4 is notable; however, stability is likely due in part to dialysis of the purified protein into 55% glycerol, which impedes equilibration to fully aerobic state. Therefore, the fact that the enzyme was found to be in oxidized state following a 4-h purification procedure does not necessarily mean that the protein was present in oxidized state *in vivo* at the beginning of the purification procedure.

This study identified the residues comprising the trp triad of Type 4 Crys. As expected, changing one of the triad residues to phe reduced the rate of photoreduction approximately 100-fold. Changing each of the other two triad residues individually to phe destabilized both of the mutant proteins and prevented FAD binding to varying degrees. Notably, the trp triad residues are highly homologous even among the animal Type 2 Crys, which do not possess FAD, suggesting a structural role for these residues in addition to their role in electron transfer *in vitro* and their postulated role in electron transfer *in vivo*.

Light-dependent conformational change of ClCry4

We find that ClCry4 undergoes light-dependent conformational change as probed by sensitivity towards trypsin digestion. Whether this property is associated with any function is not known. The light-dependent conformational change of DmCry increases both its proteolytic degradation rate and its binding to the core clock Tim protein, which accelerates the degradation of Tim [15, 16, 60]. These actions help keep the *Drosophila* clock entrained to the local light–dark cycle. However, a photoentrainment role for ClCry4 is doubtful. A side by side comparison showed that *in vivo*, light induced the degradation of DmCry, but not the zebrafish Cry4 (a Type 4 Cry), and it was concluded that if Type 4 Crys were found to entrain the biological clock, it would be by a mechanism different from Type 1 Crys. Thus, the light-induced conformational change of Type 4 Crys is not associated with light-dependent proteolysis. In addition, birds possess melanopsin in intrinsically photosensitive retinal ganglion cells, which likely mediate entrainment of the avian circadian clock [61]. Our finding of light-dependent conformational change in ClCry4 indicates that experimental efforts to search for interacting partners of ClCry4 should take illumination conditions under consideration.

All Crys have two major domains, the PHR domain and the C terminus. In Type 1 Crys, the PHR domain plays a role in FAD binding and photoreduction [62], while the C-terminus mediates functional conformational change and protein–protein interaction [27, 62, 63]. Our recombinant ClCry4 has a short N-terminal FLAG tag (~1 kDa) and the full-length recombinant protein is ~62 kDa. Sequence alignment reveals that the protein has a long enough PHR

domain but a rather short C-terminus (less than ~ 10 kDa). In contrast to Type 1 Crys whose conformational changes happen mainly at the C-terminus [27], our study (and that of Mitsui et al. [20] with GgCry4) shows that the changes in tryptic digestion of ClCry4 are detected within the PHR domain (Fig. 5b). Another study with GgCry4 found that light exposure affects binding of an anti-Cry4 antibody directed against a C-terminal epitope [56]. Unfortunately, these methods used to probe conformation are crude and it is not possible to systematically compare light-induced conformational changes in Type 1 and Type 4 Crys. Our related observation, direct (trypsin-independent) photolysis of ClCry4 likely reflects the combination of several factors including the wavelength distribution and dose of our light source and the presence of a susceptible protein structural element near the flavin in ClCry4.

Conclusion

Pigeons possess ClCry1, ClCry2 and ClCry4. ClCry1 and ClCry2 are properly annotated as Type 2 Crys, and they exhibit properties in vitro and characteristics in vivo of Type 2 Crys; therefore, they are presumed to function as Type 2 Crys, as transcriptional repressors in the core circadian clock mechanism. ClCry4 is a member of a newly discovered group with unknown function, the Type 4 Crys. Like other Type 4 Crys, ClCry4 possesses FAD cofactor. The cofactor is in oxidized state following purification, and is photoreduced via the trp triad to a relatively stable fully reduced FADH⁻ form in vitro, with the formation of a semireduced FADH[·] intermediate. ClCry4 undergoes a light-dependent conformational change upon irradiation. Located in the cytosol, ClCry4 also appears widely distributed among pigeon tissues. Our comparative properties between Type 2 and Type 4 Crys of pigeon reveal their sequence and functional distinctions. Further characterization of Cry4 photo-reactivity and positioning in tissues will be interesting partly in light of speculation that the geomagnetic field may influence the behavior of FAD-containing Crys in a light-dependent manner resulting in geomagnetic sensing.

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Compliance with ethical standards

Ethical approval All applicable guidelines for the care and use of animals in China and the National University of Defense Technology Ethics Committee were followed.

Conflict of interest The authors declare that they have no competing interests.

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