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De - crypting cryptochromes: electromagnetic field sensors and clockwork for quantum biology and medicine

Edited by
Margaret Ahmad and Brian Crane

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De - crypting cryptochromes: electromagnetic field sensors and clockwork for quantum biology and medicine

Topic editors

Margaret Ahmad — Université Paris-Sorbonne, France

Brian Crane — Cornell University, United States

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REVIEW
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The Magnetic Compass of Birds: The Role of Cryptochrome

Roswitha Wiltschko, Christine Nießner and Wolfgang Wiltschko**

FB Biowissenschaften, Goethe-Universität Frankfurt, Frankfurt am Main, Germany

The geomagnetic field provides directional information for birds. The avian magnetic compass is an inclination compass that uses not the polarity of the magnetic field but the axial course of the field lines and their inclination in space. It works in a flexible functional window, and it requires short-wavelength light. These characteristics result from the underlying sensory mechanism based on radical pair processes in the eyes, with cryptochrome suggested as the receptor molecule. The chromophore of cryptochrome, flavin adenine dinucleotide (FAD), undergoes a photocycle, where radical pairs are formed during photo-reduction as well as during re-oxidation; behavioral data indicate that the latter is crucial for detecting magnetic directions. Five types of cryptochromes are found in the retina of birds: cryptochrome 1a (Cry1a), cryptochrome 1b, cryptochrome 2, cryptochrome 4a, and cryptochrome 4b. Because of its location in the outer segments of the ultraviolet cones with their clear oil droplets, Cry1a appears to be the most likely receptor molecule for magnetic compass information.

Keywords: compass orientation, inclination compass, magnetoreception, radical pair model, cryptochrome, FAD, photocycle, UV/V cones

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Margaret Ahmad,
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Daniel Kattnig,
University of Exeter, United Kingdom

****Correspondence:***
Roswitha Wiltschko
wiltschko@bio.uni-frankfurt.de
Wolfgang Wiltschko
wiltschko@zoology.uni-frankfurt.de

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THE GEOMAGNETIC FIELD

The geomagnetic field surrounding the Earth is generated mainly by the Earth itself. Its two poles lie close to the rotational (geographic) poles but are not identical with them. The magnetic field lines emerge from the pole in the South (which is a physical North pole), where they go straight upward, then run around the Earth and reenter its surface at the pole in the North. Near the geographic equator, they form the "magnetic equator," being horizontal, parallel to the Earth's surface. The angle between the field lines and the horizontal, called "inclination," or "dip," is defined as positive when it is downward as in the northern hemisphere and negative when it is upward; it is 0° at the magnetic equator and -90°, respectively, +90°, at the poles. Magnetic intensity (field strength) is low compared to technically applied fields; it is largest at the magnetic poles, where it is about 60 μT (microTesla) and decreases toward the magnetic equator, with a local low of 23 μT at the South American east coast. At Frankfurt am Main, Germany (50°N, 9°E), for example, the inclination today is about 67° and the total intensity about 47 μT (for more information, see Winklhofer, 2009; Finlay et al., 2010).

We humans need instruments like a technical compass for indicating directions and a magnetometer for measuring intensity. Many animals from a wide variety of groups, however, are directly sensitive to the geomagnetic field and can use its information for navigation. Birds are by far the best-studied group, and the respective findings indicate that they have two sensors for different qualities of the geomagnetic field: one based on chemical processes to obtain directional information from the course of the field lines to use as a compass and another one

based on magnetite (a special form of Fe_3O_4) to measure magnetic intensity as a component of the navigational mechanisms indicating the location (for an overview, see, e.g., R. Wiltschko and Wiltschko, 2019). Here, we will focus on the avian magnetic compass, its functional properties and what is presently known about the detection of magnetic directional information and the putative role of the cryptochrome.

THE AVIAN MAGNETIC COMPASS

The ability of birds to use compass information from the magnetic field was first described in the 1960s in migratory birds. During the migratory seasons in autumn and in spring, these birds have a strong urge to move into their innate migratory direction. They show this directional preference also in suitable test cages, and when the North direction of the magnetic field around the cage is shifted by a coil system, the bird changes its heading accordingly, maintaining the same magnetic bearing. This was first demonstrated in European Robins, *Erithacus rubecula* (Turdidae), a small songbird (W. Wiltschko, 1968), but has meanwhile been shown in more than 20 other bird species from various avian lineages that are not closely related (for a list, see W. Wiltschko and Wiltschko, 2007). Among them are also a number of non-migrants, like, e.g., Homing Pigeons, *Columba livia domestica*, where it was demonstrated in homing experiments (Keeton, 1971; Walcott and Green, 1974) and by conditioning (Wilzeck et al., 2010). It was also demonstrated by directional training in Domestic Chickens, *Gallus gallus domesticus* (Phasanidae; Freire et al., 2005), and Zebra Finches, *Taeniopygia guttata*, (Estrildidae; Voss et al., 2007; Pinzon-Rodriguez and Muheim, 2017). Using the geomagnetic field as a compass thus seems to be a general feature of birds that employ this compass for orientation within their home range as well as during migration (for review, see, e.g., R. Wiltschko and Wiltschko, 2014, 2019).

The functional mode of the avian magnetic compass was also analyzed in behavioral experiments with migrating birds by exposing the test birds to different magnetic conditions and observing their responses. Three characteristics became evident:

(1) The magnetic compass of birds does not sense the polarity of the magnetic field but the axial course of the field lines. It is a so-called *inclination compass* that decides between the directions of this axis by the inclination of the field lines. For birds, the magnetic compass does not indicate magnetic North and South, as our technical compass does, but instead "poleward," the direction where the field lines are tilted downward and "equatorward," where they are pointing upward (W. Wiltschko and Wiltschko, 1972). This type of magnetic compass was found in all bird species tested for it (see R. Wiltschko and Wiltschko, 2014).

(2) The avian magnetic compass works only in a functional window around the local geomagnetic intensity, with fields more than about 20 to 25% weaker and stronger than the local field leading to disorientation. In particular, the disorientation in stronger fields was unexpected and seems unusual for stimuli. However, the functional window is not fixed but rather proved to be flexible: after staying in a field with higher or lower intensity, birds were able to orient at the respective intensity. European Robins caught and kept at 47 μT could adjust to fields as low as 4 μT and as high as 150 μT without losing their ability to oriented in the local field of 47 μT. (W. Wiltschko, 1978; Winklhofer et al., 2013) Yet they could not orient in an intermediate field that they had not experienced before – it appears that staying in a field outside the original functional window creates a new functional window at the respective intensity (W. Wiltschko, 1978; W. Wiltschko et al., 2006; Winklhofer et al., 2013). A similar response to magnetic intensity was found in Domestic Chickens (W. Wiltschko et al., 2007).

(3) The magnetic compass of birds is light-dependent (W. Wiltschko and Wiltschko, 1981). It works in bright sunshine under the full spectrum light (see, e.g., R. Wiltschko et al., 1981), but further studies showed that light from the short-wavelength end of the spectrum is required and that the light intensities necessary are very low. Birds were oriented under narrow-band light with a peak wavelength of 373 nm ultraviolet (UV), 424 nm blue, 501 nm turquoise, and 565 nm green light; under 568 nm and 585 nm yellow light and 617 nm, 635 nm, and 645 nm red light they were disoriented (W. Wiltschko et al., 1993; W. Wiltschko and Wiltschko, 1995, 1999; Rappl et al., 2000; Muheim et al., 2002; Pinzon-Rodriguez and Muheim, 2017). These studies were done under rather low intensities (8×10^{16} quanta s^{-1} m^{-2}, see, e.g., R. Wiltschko et al., 2010).

THE "RADICAL PAIR" MODEL

These characteristics of the avian magnetic compass imply an unusual sensory mechanism, and they caused Ritz et al. (2000) to propose the "Radical Pair"-model based on spin-chemical processes in photo-pigments that interact with the geomagnetic field. Absorption of photons leads to the formation of radical pairs, which can occur in two states, namely *singlet* with antiparallel spin and *triplet* with parallel spin, with the ratio singlet/triplet depending on the directional relation of the radical pair to the ambient magnetic field. Since the singlet and triplet products are different, this ratio could indicate magnetic directions. Ritz et al. (2000) proposed the eyes as a site of magnetoreception because light is available and because of their round shape, the receptor cells are arranged in all spatial directions. This would result in a specific activation pattern across the retina that is centrally symmetric to the direction of the magnetic vector and thus can indicate the axis of the field lines. As photo-pigment mediating magnetic directions, cryptochrome was suggested because it is the only photo-pigment known in animals that forms radical pairs.

This model can explain the specific characteristics of the avian magnetic compass: Since the relationship of the radical pair to the field lines and thus the singlet/triplet ratio ignores the polarity of the field, the consequence is an inclination compass

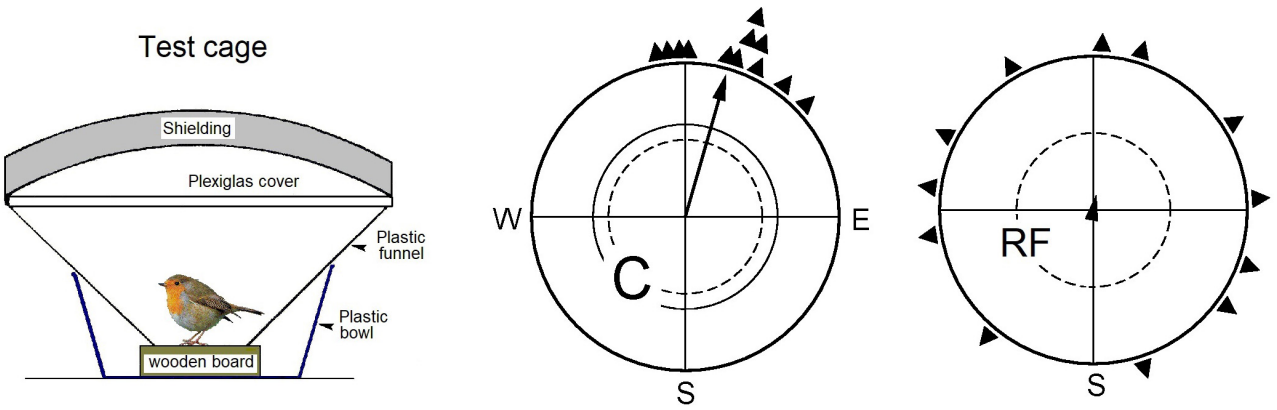

FIGURE 1 | Experiments with European Robins to demonstrate the disorienting effect of radiofrequency fields, indicating the involvement of radical pair processes (see Henbest et al., 2004). Left: Bird in a cage for testing migratory birds for orientation; the funnel is lined with coated paper where the bird leaves marks as it moves. – Center and right: Results of testing the same 12 birds: center, as control (C) in the local static field only; right, with a radiofrequency field of 7.0 MHz field added vertically, i.e., in an angle of 24° to the field lines (RF). The triangles at the peripheries of the circles mark the mean headings of the individual birds based on three recordings each; the arrows represent the grand mean vectors drawn proportional to the radius of the circle, and the two inner circles mark the 5% (dotted) and the 1% significance border of the Rayleigh test. (Data from Ritz et al., 2004).

as found in birds. The fact that the activation pattern would change with intensity explains the flexible functional window; birds experiencing a sudden intensity change are faced with a novel activation pattern. This may at first be confusing; however, because the pattern retains its central symmetry to the magnetic vector, birds can eventually learn to interpret it. The dependency of magnetic compass orientation on short-wavelength light is largely in agreement with the absorbance spectrum of cryptochrome (see, e.g., Müller and Ahmad, 2011), which was found in the avian eyes (Nießner et al., 2011, 2013).

Experimental evidence meanwhile supports the Radical Pair-Model. In an electro-retinographic study, differences in responses to varying magnetic directions were recorded in the retina under blue, but not under red light (Astakhova et al., 2020). Radio-frequency fields in the MHz (MegaHertz)-range are a diagnostic test for the involvement of radical pair processes (see, e.g., Ritz, 2001; Henbest et al., 2004); such radio-frequency fields indeed cause disorientation in birds (**Figure 1**; e.g., Ritz et al., 2004, 2009; Thalau et al., 2005; Kavokin et al., 2014; Bojarinova et al., 2020). In a recent study, oscillating magnetic fields were applied directly to the eyes by a small battery-operated coil placed on the head of the bird; here, the birds remained oriented (Bojarinova et al., 2020). However, it is not clear whether their orientation was still based on their inclination compass or whether it involved a so-called "fixed direction response," as they are frequently observed in situations where the normal inclination compass is disrupted (see R. Wiltschko et al., 2010 for details and discussion).

CRYPTOCHROME FORMING RADICAL PAIRS

Cryptochromes are proteins with flavin adenine dinucleotide (FAD) as the chromophore (see, e.g., Sancar, 2003; Chaves et al., 2011). FAD undergoes a redox cycle that is best studied in plants; the oxidized form, FADox, is the dark resting form. Photon absorption of wavelengths from UV to about 500 nm blue photo-reduces FADox to the semiquinone FADH•, forming a first radical pair with a tryptophan radical (Trp•). FADH• can be re-oxidized independently of light, or, in a next step, it can be further photo reduced by light of wavelength from UV to green to FADH-, the fully reduced form. FADH- is then re-oxidized independently of light to FADox. During this step, a second radical pair is formed (**Figure 2**), possibly with $O_2^{\bullet}$ (see, e.g., Ritz et al., 2009; Müller and Ahmad, 2011), but the true partner of FADH• and details of this process are still

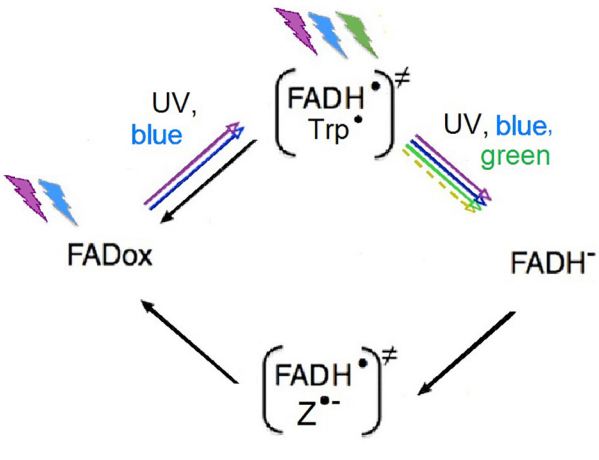

FIGURE 2 | Redox-cycle of FAD, the chromophore of cryptochrome. The radical pairs are given in parentheses; colored arrows indicate photoreduction by the respective wavelengths (see text); black arrows mark light-independent reactions of re-oxidation (after Müller and Ahmad, 2011, modified). "Z" in the radical pair generated during re-oxidation stands for a radical whose nature is not yet clear (see text).

under debate (see, e.g., Hogben et al., 2009; Lee et al., 2014; Worster et al., 2016; Kattnig, 2017; Nielsen et al., 2017; Player and Hore, 2019; Babcock and Kattnig, 2020; Procopio and Ritz, 2020).

In view of this, the question arises which of the radical pairs would mediate the direction of the magnetic field. In plants, when cryptochromes are indicating the presence and amount of light for controlling biological processes like, e.g., hypocotyl growth or flowering, the radical pair formed during photo-reduction, $FADH^{\bullet}/Trp^{\bullet}$ is considered to be the crucial one. Green light, which reduces the amount of $FADH^{\bullet}$ by further photo-reducing $FADH^{\bullet}$ to the fully reduced state FADH-, was found to act antagonistically to blue light *in vitro* as well as in living plants (e.g., Banerjee et al., 2007; Bouly et al., 2007). However, in the case of the avian magnetoreception, the situation is different. Many birds—European Robins, Australian Silvereyes, *Zosterops l. lateralis*, Garden Warblers, *Sylvia borin*, and Zebra Finches—are oriented under narrow-band lights with peak wavelengths at blue as well as green light (W. Wiltschko et al., 1993, W. Wiltschko and Wiltschko, 1995; Rappl et al., 2000; Pinzon-Rodriguez and Muheim, 2017). Since green light cannot photo-reduce FADox, this speaks against the involvement of the radical pair formed during photo-reduction being the one mediating magnetoreception but points to the other radical pair (see **Figure 2**).

Further behavioral experiments, however, showed that the orientation under green light is a transient phenomenon—it was possible only for less than 1 h. After this time, the birds were disoriented, whereas they continued to be oriented under the blue and turquoise light, that is, under wavelengths that can be absorbed by oxidized flavin. Also, orientation under green light was possible only when the birds had been before under "white" light, which included wavelengths that could photo-reduce oxidized cryptochrome—after staying in total darkness for 1 h, the birds were disoriented under green light, but they showed oriented headings under blue and turquoise light where the entire FAD-cycle can run (R. Wiltschko et al., 2014). This suggests that avian orientation under green light is possible only as long as there is a certain supply of $FADH^{\bullet}$ available for further photo-reduction to the fully reduced FADH- by green light and again implies that the radical pair formed during light-independent re-oxidation is the one that mediates magnetic directions in birds.

To test this hypothesis, birds were exposed to flickering light and a pulsed magnetic field in the following manner: With a frequency of 1 Hz, light was available for 300 ms with the magnetic field compensated; during the remaining time, there was total darkness, but, except for security intervals of 10 ms at the beginning and the end, the geomagnetic field was present. In this situation, the birds showed oriented behavior (**Figure 3**; R. Wiltschko et al., 2016). This clearly shows that the actual process of sensing magnetic directions can occur in darkness—it identifies the radical pair formed during re-oxidation as the crucial one for sensing directions. Light, however, is necessary for photo-reduction to provide the fully reduced form FADH- that is re-oxidized for the formation of this radical pair.

This is different from what is known about cryptochrome when it controls light-dependent processes in plants; here, the radical pair $FADH^{\bullet}/Trp^{\bullet}$ is important because $FADH^{\bullet}$ is formed, which was found to be the crucial form to indicate light. In the case of avian magnetoreception, however, radical pairs are to indicate the direction of the (geo)magnetic field. This is a different task, and it seems conceivable that evolution developed a modified mechanism that is better suited for this function.

In the future, it may help our understanding of cryptochromes and their functions if more attention was given to the re-oxidation process. A recent study (Pooam et al., 2019) suggests that magnetic effects in plants can also be mediated by processes during re-oxidation in the dark; yet here, not the direction, but the intensity of the magnetic field was effective.

CRYPTOCHROMES IN THE AVIAN EYES

In the eyes of birds, five types of cryptochromes – cryptochrome 1a (Cry1a), cryptochrome 1b (Cry1b), cryptochrome 2 (Cry2), Cryptochrome 4a (Cry4a), and Cryptochrome 4b (Cry4b) – have been found (see also R. Wiltschko and Wiltschko, 2019).

Cryptochrome 1 was first described in birds by Haque et al. (2002) based on mRNA expression in the photoreceptor layer and the ganglion cell layer of chickens (see also Fu et al., 2002; Mouritsen et al., 2004). In Robins, Möller et al. (2004) identified two splice products of the *Cry1* gene, Cry1a and Cry1b; they differ in their *C*-termini, which suggests different functions. In an immuno-histochemical study, Nießner et al. (2011) located Cry1a in the outer segments of the ultraviolet/violet (UVS/VS, SW1) cones in the retinae of Chickens and European Robins where immuno-marking for electron microscopy shows it positioned along the disks, where also the UV-opsin is located (**Figure 4**). Bolte et al. (2021) described Cry1a in the outer segments of the UV/V cones also in pigeons, Blackcaps, *Sylvia atricapilla,* and Zebra Finches, so Cry1a in these locations appears to be common in all birds. Cell fractionation revealed Cry1a in the membrane fraction, suggesting that Cry1a is anchored along membranes (Nießner et al., 2011). In birds, photoreceptor cells contain oil droplets that absorb different amounts of short wavelengths in order to shift the maximum absorbance of the color receptors further apart for better color vision. Only the UVS/VS cones have clear oil droplets that allow all wavelengths of light to pass (see Hart, 2001). This type of photoreceptor is present all across the retina, i.e., in different directional relationships to the magnetic vector; hence they can give rise to the activation pattern suggested by Ritz et al. (2000).

Based on studies with purified Cry1, Kutta et al. (2017) questioned whether vertebrate Cry1 indeed had FAD bound and hence could be photo-reduced (see also Hochstoeger et al., 2020), although Liedvogel et al. (2007) reported that avian Cry 1a contained FAD and could absorb blue light. An *in vivo* study also showed that at least avian Cry1a is directly activated by light (see **Figure 5**). It is activated by all wavelengths that are absorbed by flavin: An immuno-chemical study showed activated Cry1a under all light conditions where birds were oriented (Nießner et al., 2013). Yet Bolte et al. (2021) failed to reproduce this finding; the reasons are unknown.

In contrast to Cry1a, Cry1b was located by immuno-histochemistry in the cytosol of ganglion cells, displaced ganglion

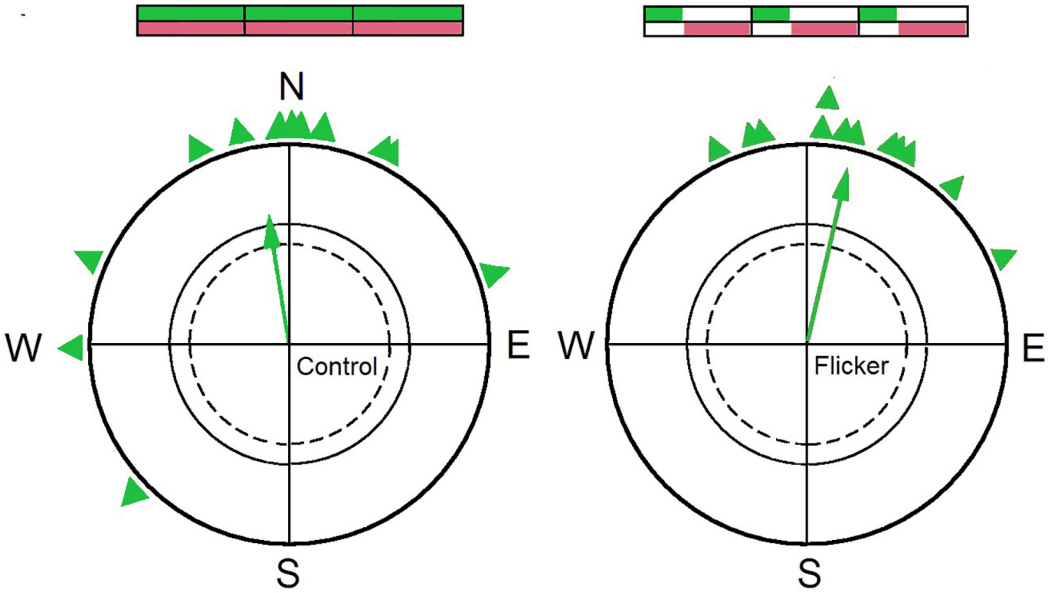

FIGURE 3 | Orientation of a group of 12 birds during spring migration. Left: under control conditions with continuous light and the magnetic field permanently present, the birds are significantly oriented in their northerly migratory direction. Right: under *flicker* conditions, when the magnetic field and light was present alternatingly, the same birds are likewise oriented in their migratory direction (for details, see text). The schemes above the circles symbolize the distribution of light (above, green) and magnetic field (below; brown). Symbols as in **Figure 1** (Data from R. Wiltschko et al., 2016).

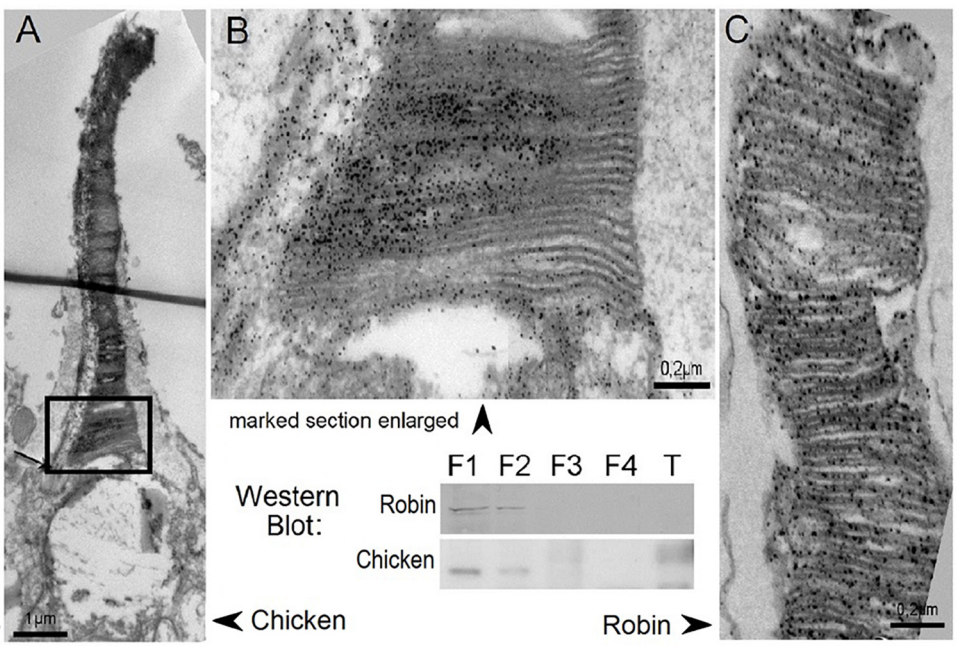

FIGURE 4 | Cry1a immuno-labeled in the outer segments of the UV/V cones of chickens and robins. Electron-microscopic images of the outer segments of the UV/V-cones, with labeled Cry1a visible as dark dots along the disk membranes. **(A)** Entire outer segment of a chicken V-cone. **(B)** Higher magnifycation of the lower part of this outer segment. **(C)** Part of the outer segment of the UV-cone of a robin. -Western blots of retinae showing Cry1a in the cytosol (F1) and membrane fraction (F2); F3, nuclear fraction; F4, cytoskeletal fraction; and T, tongue tissue as control (from Nießner et al., 2011).

cells, and also in the inner segments of the photoreceptors, free as well as bound to membranes (Mouritsen et al., 2004; Bolte et al., 2016; Nießner et al., 2016). In night-migrating birds, its expression varies with the season; it was much stronger during the migratory season when the birds were active during the night (Fusani et al., 2014; Nießner et al., 2016). It has been speculated

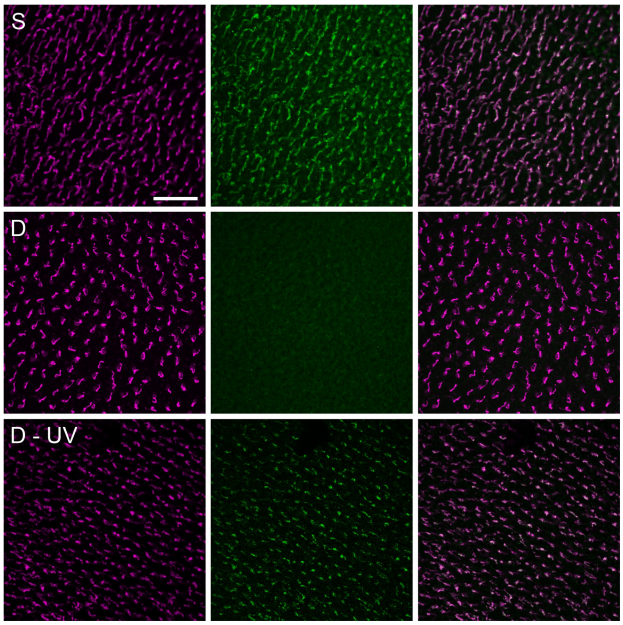

FIGURE 5 | Double immuno-fluorescence labeling of chicken retinae for violet (SW1) opsin marking the violet cones (left column, magenta fluorescence) and that of Cry1a with a specific antiserum against the *C*-terminus (center column, green fluorescence), and merging of the two labels (right column). The images in each row show the labels in the same patch of the retina; with the merge indicating that Cry1a occurs only together with the violet opsin in the SW1 (violet) cones. Treatment of the birds: Upper row, *S*: pre-treatment in daylight: 30 min in sunlight: Cry1a is labeled. Center row, *D*: pre-treatment in daylight, 30 min in total darkness: no Cry1a label is visible, indicating that the antiserum only recognizes activated Cry1a. Bottom row, D-UV: 30 min pre-treatment in darkness, 5 min in 373 nm UV light: Cry 1a is labeled, showing that Cry1a has been activated by light. The scale bar represents 50 μm applies to all panels (data from Nießner et al., 2013).

that Cry1b possibly plays a role in magnetoreception (Bolte et al., 2016), but, because the use of a magnetic compass appears to be a general sensory ability of birds, also shared by non-migrants (see above), the seasonal changes suggest another role—one that possibly involves the shift from diurnal activity to nocturnal activity (*Zugunruhe*) by night migrants.

Bailey et al. (2002) and Fu et al. (2002) described Cry2; identifying it by its mRNA in chickens and Japanese Quails, *Coturnix japonica* (Phasanidae). It was found in a number of organs, among them the pineal gland and, in the eyes, in the photoreceptors, and in ganglion cells. This cryptochrome includes a sequence that suggests a location in the cell nucleus (Möller et al., 2004; Mouritsen et al., 2004), indicating a possible role as clock protein (see, e.g., Sancar, 2003 for discussion of this role of cryptochromes).

Cryptochrome 4 was described in the retina of chickens first by Watari et al. (2012) using mRNA-expression and immunohistochemistry (see also Pinzon-Rodriguez et al., 2018). Günther et al. (2018) could identify Cry4 in the outer segments of the longwave-sensitive (LWS) single cones and of the double cones of European Robins. Recently, an isoform of Cry4, Cry4b, was identified by analyzing the DNA sequence. This splice product appears to be widespread; by genomic BLAST database search, it was also indicated in more than 25 avian species of various orders (Einwich et al., 2020). Another study localized Cry4 also in the outer plexiform layer, where it seemed associated with the synapses between photoreceptors and horizontal cells (Hochstoeger et al., 2020).

A possible role of Cry4 in magnetoreception has been frequently discussed (e.g., Günther et al., 2018; Pinzon-Rodriguez et al., 2018; Hochstoeger et al., 2020). Especially its location within the double cones prompted speculations on such a role of Cry4 because of the possibility that the input of two adjacent receptors with the magneto-receptive molecules oriented in different directions could be compared; it was proposed that this might help to overcome problems with different light intensities and polarization (Worster et al., 2017; Günther et al., 2018). However, the principal cone is associated with an oil droplet that acts as a cut-off filter absorbing short wavelengths (Hart, 2001) and thus most of the wavelengths required for cryptochrome photo-reduction. This, together with the gap junctions between the two cones, would interfere with a comparison, making such a role of Cry4 in the double cones rather unlikely. Hochstoeger et al. (2020) speculated about a possible role of Cry4 in the outer plexiform layer. The Cry4 in the LWS single cones seems even less suitable for magnetoreception because the red oil droplets in these cones transmit only long wavelengths that cannot photo-reduce cryptochrome. Moreover, a study by Mitsui et al. (2015) implies that Cry 4 requires a very long time for re-oxidation, which would not be favorable for receptive processes.

Altogether, if cryptochrome is indeed the receptor molecule in birds, the presently available evidence indicates Cry1a as the most likely receptor for sensing directions. The observation that its gene expression shows a diurnal rhythm (Pinzon-Rodriguez et al., 2018) does not speak against such a role, because it only indicates a rhythmic production in the inner segment of the cones, but it does not say anything about its general availability in the sensory active outer segment and its use. It appears to be a parallel case to vision, where the production of the visual pigment opsin also shows a diurnal pattern (e.g., Pierce et al., 1993; von Schantz et al., 1999), but animals can see the entire day.

OUTLOOK

More than 50 years after the discovery of the avian magnetic compass, we finally have a concrete idea about the primary mechanism leading to the detection of directions by the magnetic field in birds: a radical pair process, with cryptochrome playing a crucial role in this process. Yet there are still several conflicting findings that have to be resolved, and a number of open questions that have to be answered.

One of the most important questions concerns the mechanisms by which the information obtained by the radical pair is transformed into a biological signal. Cryptochrome is found in the outer segments of cones, i.e., in a cell type that is also important for color vision. Is the visual information from the opsin and information on magnetic directions from cryptochrome transmitted separately or together? One might

expect the latter since the cone has only one known way of transmitting information. This would mean, however, the two types of information have somehow to be separated, either already in the eyes or later in higher centers in the brain. There are speculations about possibilities (see, e.g., Bischof et al., 2011), but it will require many more considerations and experiments until we hopefully reach a complete understanding of how birds perceive the direction of the geomagnetic field.

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RW and WW wrote this review together, CN checked the text and contributed the histological data. All authors discussed the article and approved the submitted version.

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frontiers in Plant Science

REVIEW
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The World of Algae Reveals a Broad Variety of Cryptochrome Properties and Functions

Jan Petersen[1†], Anxhela Rredhi[1†], Julie Szyttenholm[1†], Sabine Oldemeyer[2†], Tilman Kottke[3,4†] and Maria Mittag[1†]*

[1]*Matthias Schleiden Institute of Genetics, Bioinformatics and Molecular Botany, Friedrich Schiller University, Jena, Germany,* [2]*Experimental Molecular Biophysics, Department of Physics, Freie Universität Berlin, Berlin, Germany,* [3]*Department of Chemistry, Bielefeld University, Bielefeld, Germany,* [4]*Biophysical Chemistry and Diagnostics, Medical School OWL, Bielefeld University, Bielefeld, Germany*

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Margaret Ahmad,
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Alfred Batschauer,
University of Marburg, Germany

***Correspondence:**
Maria Mittag
m.mittag@uni-jena.de

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†ORCID:
Jan Petersen, orcid.org/0000-0002-8469-6679
Anxhela Rredhi, orcid.org/0000-0001-9787-7711
Julie Szyttenholm, orcid.org/0000-0001-9373-0608
Sabine Oldemeyer, orcid.org/0000-0001-7139-7218
Tilman Kottke, orcid.org/0000-0001-8080-9579
Maria Mittag, orcid.org/0000-0003-3414-9850

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Algae are photosynthetic eukaryotic (micro-)organisms, lacking roots, leaves, and other organs that are typical for land plants. They live in freshwater, marine, or terrestrial habitats. Together with the cyanobacteria they contribute to about half of global carbon fixation. As primary producers, they are at the basis of many food webs and they are involved in biogeochemical processes. Algae are evolutionarily distinct and are derived either by primary (e.g., green and red algae) or secondary endosymbiosis (e.g., diatoms, dinoflagellates, and brown algae). Light is a key abiotic factor needed to maintain the fitness of algae as it delivers energy for photosynthesis, regulates algal cell- and life cycles, and entrains their biological clocks. However, excess light can also be harmful, especially in the ultraviolet range. Among the variety of receptors perceiving light information, the cryptochromes originally evolved as UV-A and blue-light receptors and have been found in all studied algal genomes so far. Yet, the classification, biophysical properties, wavelength range of absorbance, and biological functions of cryptochromes are remarkably diverse among algal species, especially when compared to cryptochromes from land plants or animals.

Keywords: blue-light receptor, *Chlamydomonas*, flavin, *Ostreococcus*, *Phaeodactylum*, photoreceptor, photosynthetic microorganisms

INTRODUCTION

What Are Algae?

Algae are photosynthetic eukaryotes defined primarily by their lack of roots, leaves, and the stem that are typical for higher plants (Parker et al., 2008). They are divided into microscopically small microalgae that are a major part of phytoplankton and macroalgae, including seaweeds. Algae are found anywhere from soil to lakes, rivers and oceans and they are crucial to food webs (Steele, 1974). Algal activities can even influence biogeochemical processes as observed with the Greenland ice sheet (McCutcheon et al., 2021). Together with cyanobacteria, aquatic algae are responsible for about 50% of global carbon fixation (Field et al., 1998) and thus are of high ecological relevance. Evolutionarily, algae can be divided into two major groups, with one group derived from primary endosymbiosis and the other one from secondary endosymbiosis

(Keeling et al., 2005). For primary endosymbionts, a unicellular eukaryotic cell engulfed a cyanobacterium to become a chloroplast. In the case of secondary endosymbionts, a unicellular eukaryotic cell engulfed either a green alga (secondary green) or a red alga (secondary red). Recently, it was shown that tertiary and possibly even higher-order endosymbiotic events occurred (Sibbald and Archibald, 2020), but these will not be part of this review. Primary endosymbionts (named Archaeplastida) include glaucophytes (algae with cyanelle plastids), rhodophytes (red algae), chlorophytes (green algae), and all land plants together with ferns and mosses (Bowman et al., 2007; Adl et al., 2012). Green algae and land plants are also grouped as Chloroplastida. All these groups possess plastids surrounded by two envelope membranes. In the lineages with secondary endosymbionts, plastids are encircled by either four (e.g., diatoms, brown algae, haptophytes, and cryptophytes) or three membranes (euglenophytes, dinoflagellates; Gentil et al., 2017). Some of these lineages still contain the nucleus of the engulfed green or red alga, but in a strongly reduced form, called nucleomorph (e.g., in cryptophytes). Others such as euglenophytes, diatoms, dinoflagellates, or brown algae have completely lost this nucleus (Gentil et al., 2017). In this review, we focus solely on algal groups with cryptochromes that have been so far phylogenetically described based on existing genomes or have been characterized by their photoreceptors.

The Influence of Light on Algal Life and the Diversity of Algal Photoreceptors

Light is an important source of energy and information for algal life on our planet (**Figure 1**). As for all photosynthetic organisms, algae transform the radiation energy of the sunlight into chemical energy (Eberhard et al., 2008). They perceive light *via* pigments or specialized photoreceptors. Light regulates their photosynthesis and balances their photosynthetic apparatus, controls their behavior (photoorientation), entrains their circadian clocks, and influences their cell and sexual cycles as well as developmental processes (reviewed in Hegemann, 2008; Kianianmomeni and Hallmann, 2014; Kottke et al., 2017; Rredhi et al., 2021).

Cyanobacteria, also known as prokaryotic blue-green algae, and eukaryotic algae have an amazing repertoire of different photoreceptors compared to land plants or animals. For many members, we still do not know their detailed functions and/or mechanisms (Kianianmomeni and Hallmann, 2014; Greiner et al., 2017; Jaubert et al., 2017; Kottke et al., 2017; Kroth et al., 2017; König et al., 2017b; Rockwell and Lagarias, 2020). In addition to the classically known photoreceptors from land plants which absorb in the red/far red (phytochrome) and blue region of the visible spectrum (cryptochrome, phototropin, Zeitlupe) as well as in the UV-B (UVR8; Kim et al., 2007; Fankhauser and Christie, 2015; Ahmad, 2016; Wang and Lin, 2020; Podolec et al., 2021), algae bear several novel types of light sensors. Moreover, cyanobacteria and algae also possess a larger variety of the above-mentioned "classical" photoreceptors with new properties and biological functions. For example, cyanobacteriochromes, which are phytochromes in cyanobacteria, absorb strongly shifted from the well known red/far-red range to shorter wavelengths of visible light (Rockwell et al., 2012). Moreover, new types of cryptochromes are present in algae with novel biophysical properties such as red-light absorption and will be detailed in the chapters below.

Numerous photoreceptors are unique to algae. For example, aureochrome photoreceptors are only present in a single group of algae, the photosynthetic stramenopiles, including Xanthophyceae (yellow-green algae), diatoms, and brown algae (Kroth et al., 2017). These aureochrome photoreceptors contain a light-oxygen-voltage (LOV) domain for light reception and a basic region leucine zipper (bZIP) domain for DNA binding and act as light-driven transcription factors. In the green unicellular model alga *Chlamydomonas reinhardtii* (Cr), even 18 different types of photoreceptors have been reported, including two channelrhodopsins that are fundamental to its photoorientation and several histidine kinase rhodopsins (HKRs) whose functions are largely unknown (Greiner et al., 2017; Luck and Hegemann, 2017). HKRs contain a His-kinase-, response regulator-, and a rhodopsin domain. Some of the HKRs even have guanylyl cyclase activity that has been recently used for application as an optogenetic tool (Tian et al., 2021). The field of optogenetics is self-evolving since knowledge on the exceptional properties of Cr channelrhodopsins became available (Hegemann and Nagel, 2013).

Further types of (possibly) novel light sensors were recently discovered in phytoplankton in the open ocean (Coesel et al., 2021). The genomes of these marine microalgae encode light-sensing proteins with new combinations of known domain structures or even fusions of different types of photoreceptors. For example, LOV domains were found within one protein together with different DNA-binding domains such as a homeobox or a heat shock factor domain or with signal-transduction motifs (e.g., an EF-hand; Coesel et al., 2021). A new type of dual cryptochrome that is fused to another photoreceptor was also found in these genomes (Makita et al., 2021) and is included in this review.

It should also be stated that the functions of photoreceptors in algae may differ from those in higher plants. For example, Cr phototropin influences the sexual cycle (Huang and Beck, 2003), the development of the eyespot, a primitive visual system of the alga (Trippens et al., 2012), and mediates the feedback regulation of photosynthesis in the green alga (Petroutsos et al., 2016).

Blue-light-activated adenyl cyclases represent another type of light sensors in algae. They mediate photoavoidance in the secondary green alga *Euglena gracilis* (Iseki et al., 2002) and contain blue-light sensor using flavin adenine dinucleotide (BLUF) domains (Ito et al., 2010). In the green alga Cr as well as in animal spermatozoa, the BLUF domain is directly associated with dynein and involved in ciliary motility (Kutomi et al., 2021).

VARIETY OF ALGAL CRYPTOCHROMES

In this review, we focus on the variety of cryptochromes found in algae. Cryptochromes are flavoproteins that were first described

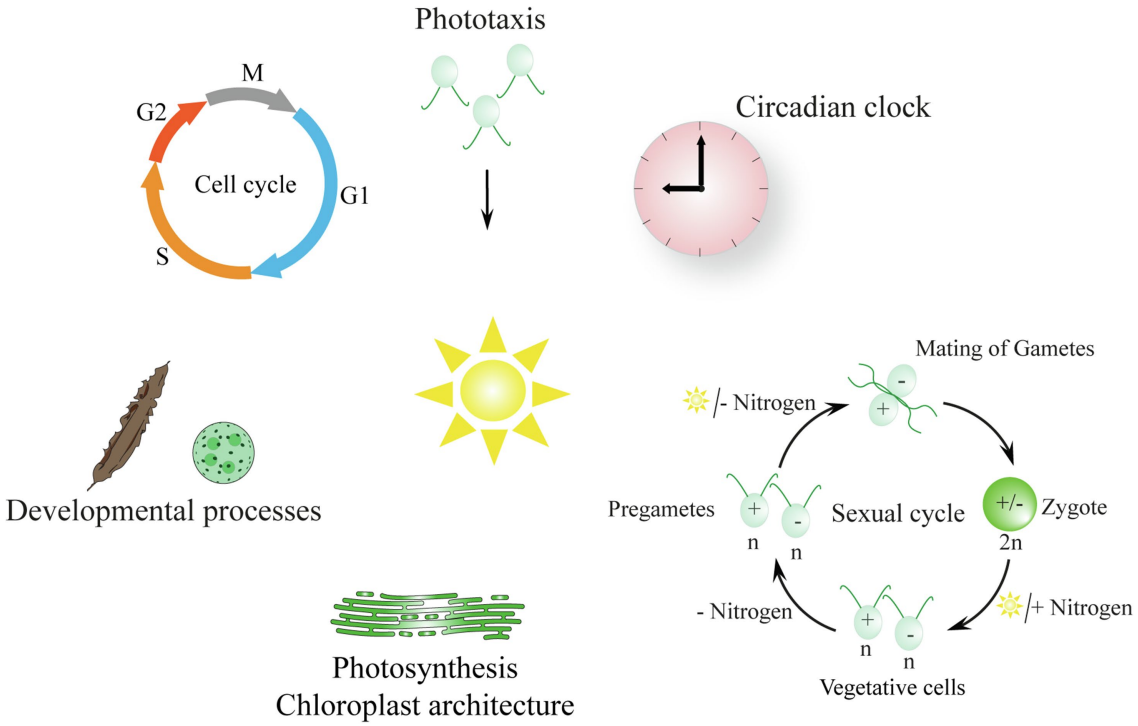


FIGURE 1 | Biological processes in algae that are regulated by light (reviewed in Hegemann, 2008; Kianianmomeni and Hallmann, 2014, Kottke et al., 2017; Sasso et al., 2018). The description of the symbolized processes begins at the top of the scheme and moves in a clockwise manner: Photoorientation of flagellate algae: tactic movement to the light is shown; phobic movement occurs with strong light (not shown). | The algal circadian clock is entrained by light–dark cycles. | The sexual cycle of *Chlamydomonas*: vegetative cells turn into pregametes in the dark without a nitrogen source and then become gametes in the light in the absence of a nitrogen source; gametes fuse and a resilient zygote is formed that needs a nitrogen source and light to undergo meiosis. | Light is needed for photosynthesis and influences the chloroplast architecture; thylakoid membranes are shown exemplarily (Rredhi et al., 2021) | Light influences developmental processes in the multicellular algae *Saccharina* and *Volvox* (Kianianmomeni and Hallmann, 2015; Yang et al., 2020). | The cell cycle is symbolized; algal cells are synchronized by light–dark cycles; M, mitosis; G1, gap1; S, synthesis; G2, gap2 (Coesel et al., 2009; Cross and Umen, 2015).

as blue-light receptors in plants and animals (Chaves et al., 2011). They are derived from the blue-light-dependent DNA repair enzymes called photolyases that can be found in pro- and eukaryotes (Ahmad and Cashmore, 1993; Sancar, 2003; Chaves et al., 2011). As primary light sensors, cryptochromes and photolyases carry a non-covalently bound flavin adenine dinucleotide (FAD) molecule within their photolyase homology domain (PHR). Additionally, they may bind an antenna chromophore like 5,10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazaflavin (8-HDF). The antenna chromophore harvests additional light and transfers the energy to the FAD (Hoang et al., 2008). Photoexcitation of the FAD may lead to a change of its redox state that can be either oxidized (FAD_{ox}), semireduced as an anion radical ($FAD\bullet^{-}$), semireduced as a neutral radical ($FADH\bullet$) or fully reduced ($FADH^{-}$; Chaves et al., 2011). Conformational changes are promoted within the cryptochrome protein structure depending on the FAD redox state, resulting in different signaling properties. Most cryptochromes possess a C-terminal extension (CCT) whose structure is rather undefined and only shows poor conservation. The CCT varies in length depending on the cryptochrome. Nevertheless, the CCT is of great importance for cryptochrome function and downstream signaling (Chaves et al., 2011). The first cryptochromes were analyzed as blue-light receptors in the land plant *Arabidopsis thaliana* (At CRY1 and At CRY2; Ahmad and Cashmore, 1993, Chaves et al., 2011) and in the fly *Drosophila melanogaster* (dCRY) as an animal-type I CRY (Emery et al., 1998). Later, it was found that the cryptochromes from mice or humans group to animal type II CRYs. They do not act as light sensors but instead have a function in the central oscillator of the circadian clock (Okamura et al., 1999; Todo, 1999; Chaves et al., 2011).

Cryptochrome Categories in Algae

To date, four different classes of cryptochromes are known in algae based on phylogenetic analyses and further characterizations. Moreover, a fifth class was recently described consisting of a fusion of a cryptochrome with another photoreceptor. The following section will present details on the five different classes:

1. The classical plant cryptochromes (pCRYs) present in land plants (At CRY1 and At CRY2) are found in some but not in all algae. Plant CRYs, like all cryptochromes, share the conserved PHR at the N-terminus binding FAD (Chaves et al., 2011), and contain a comparatively long CCT

(Reisdorph and Small, 2004; Müller et al., 2017). Cr pCRY has the longest known extension with about 500 amino acids (**Figure 2**). Plant CRYs may also bind MTHF as antenna chromophore as derived from their homology to CPD (cyclobutane pyrimidine dimer) photolyases (Chaves et al., 2011; Beel et al., 2012). There is no indication that pCRYs still have photolyase activity (Lin and Shalitin, 2003). Plant CRYs are found in many investigated Chloroplastida (**Figure 3**) but seem to be absent in the green picoalga *Ostreococcus tauri* (Ot), in the red macroalga *Porphyra umbilicalis* (Pum) or in the diatom *Phaeodactylum tricornutum* (Pt; **Figure 3**).

2. The cryptochrome–photolyase family (CPF1) of animal-like cryptochromes. For a long time, it was thought that all cryptochromes have lost photolyase activity. However, it was recently discovered that some algal animal-like CRYs from Pt, Ot, and Cr have maintained photolyase activity (Coesel et al., 2009; Heijde et al., 2010; Franz et al., 2018). These animal-like cryptochromes group by their phylogeny closely to the cryptochrome (6-4)–photolyase family (CPF1) and are thus phylogenetically located in between the animal-type I and -type II cryptochromes from animals (Beel et al., 2012). They exert further biological functions including light perception and circadian clock control as well as the control of the algal Cr life cycle (Coesel et al., 2009; Heijde et al., 2010; Beel et al., 2012; Zou et al., 2017). Cr aCRY binds 8-HDF as antenna chromophore (Franz et al., 2018; **Figure 2**). The CCTs of the animal-like CRYs are usually shorter as compared to the pCRYs. Notably, CPF animal-like CRYs are widely distributed in algae but are missing in land plants (**Figure 3**).
3. The CRY-DASH (*Drosophila*, *Arabidopsis*, *Synechocystis*, *Homo*) cryptochromes. CRY-DASH (-like) proteins are found in many organisms from bacteria to vertebrates (Brudler et al., 2003; Kiontke et al., 2020). They group closely to CPD photolyases (Beel et al., 2012; Fortunato et al., 2015). Bacterial, plant, and fungal CRY-DASHs can repair CPD in single-stranded and even in double-stranded DNA, as reported for some members of the mucoromycotina fungi (Selby and Sancar, 2006; Pokorny et al., 2008; Tagua et al., 2015; Navarro et al., 2020). Studies on repair activities by members of the cryptochrome/photolyase family in algal systems are rare. It was found that two out of three putative CRY-DASHs of the red picoalga Cm repair CPD lesions, but only in single-stranded and not in double-stranded DNA (Asimgil and Kavakli, 2012). Interestingly, CRY-DASH proteins can be located in organelles. The land plant At CRY3 was found in the chloroplast and in mitochondria (Kleine et al., 2003), while the algal Cr CRY-DASH1 protein was only found in the chloroplast (Rredhi et al., 2021; **Figure 2**). Recently, algal CRY-DASHs were shown to be involved in further biological functions beside DNA repair, supporting their additional or alternative role as photoreceptors (Yang et al., 2020; Rredhi et al., 2021). CRY-DASH proteins were found in all selected algal systems (**Figure 3**), including an Antarctic *Chlamydomonas* sp. ICE-L in which its highest transcript expression level is at 5°C and a salinity of 32% (Zhang et al., 2020).
4. The plant-like CryP, found in algae and metazoans. Instead of a classical pCRY, the genome of Pt encodes a plant-like CRY, named CryP (Juhas et al., 2014; König et al., 2017b) that was later identified in metazoan genomes (Oliveri et al., 2014). CryP binds FAD and MTHF as chromophores and has a CCT of about 70 residues (**Figure 2**). As in CRY-DASH proteins, the FAD in CryP is present in the neutral radical state after isolation as opposed to the oxidized state found in pCRYs (see below; Juhas et al., 2014). A plant-like CRY is also present in the picoalga Ot that lacks a classical pCRY. Interestingly, the red picoalga *Cyanidioschyzon merolae* (Cm) and the green picoalga *Pycnococcus provasolii* (Pp) have both plant-like CRYs and plant CRYs (**Figure 3**).
5. Dualchrome, a new dual-photoreceptor chimera. Recently, a new type of photoreceptor from the marine picoalga Pp was discovered in metagenome data of ocean picoplankton, named dualchrome (DUC1, **Figures 2, 3**). DUC1 bears a fusion of a phytochrome and a plant CRY (Makita et al., 2021) and was not found in any other alga or other organisms so far.

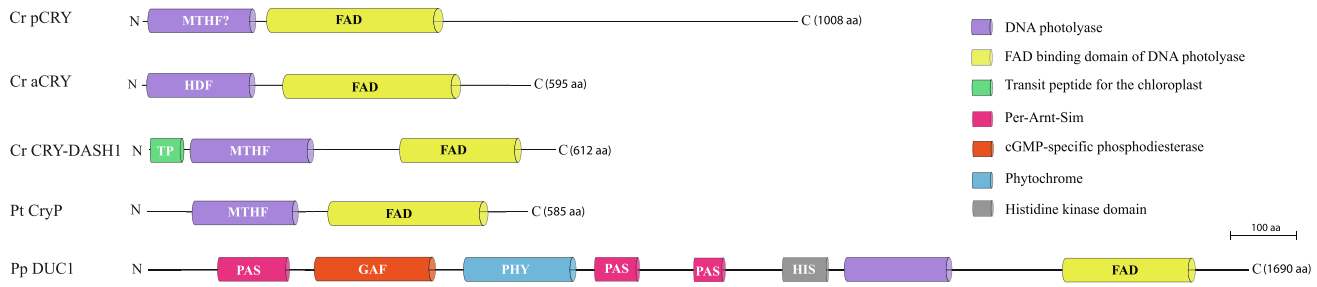


FIGURE 2 | Cryptochrome categories in algae (Juhas et al., 2014; Kottke et al., 2017; Makita et al., 2021; Rredhi et al., 2021). A representative member of each of the five known categories is shown, starting with the classical plant cryptochrome of the green flagellate alga *Chlamydomonas reinhardtii* (Cr pCRY) and its long C-terminal extension. It is followed by the Cr animal-like CRY of the cryptochrome/photolyase family (Cr aCRY), the Cr CRY-DASH1 with its chloroplast transit peptide, the plant-like CryP of the diatom *Phaeodactylum tricornutum* and the dual cryptochrome DUC1 from the green picoalga *Pycnococcus provasolii*, a fusion of a phytochrome and a cryptochrome. Domains are indicated, including the names of the chromophores (FAD) and antenna chromophores (MTHF, 8-HDF); FAD, flavin adenine dinucleotide; MTHF, 5,10-methenyltetrahydrofolate; 8-HDF, 8-hydroxy-7,8-didemethyl-5-deazaflavin.

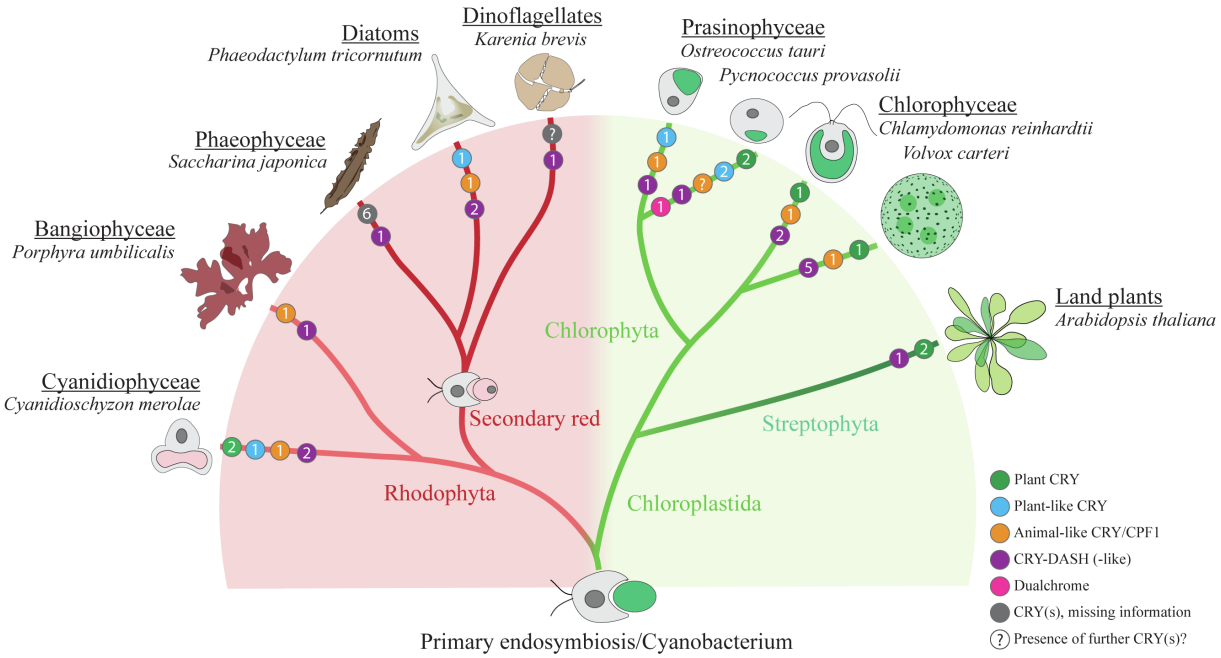


FIGURE 3 | Cryptochrome distribution within selected algal species. Simplified schematic cladogram of cryptochrome (CRY) distribution within selected algal species in comparison with the land plant *Arabidopsis thaliana*. Colored circles indicate the numbers (presented in each circle) and representatives of different types of CRYs within the presented species (green, plant CRY; blue, plant-like CRY; orange, animal-like CRY/CPF1; purple, CRY-DASH and CRY-DASH-like; magenta, dualchrome; gray, CRY(s) that are currently not assigned to the former groups; "?," indicating that there may be further potential CRY(s) in this organism that have not been identified so far). Information for the presented data was taken from the following references: *Cyanidioschyzon merolae* (Cm; Brawley et al., 2017), *Porphyra umbilicalis* (Pum; Brawley et al., 2017), *Saccharina japonica* (Sj; Deng et al., 2012; Yang et al., 2020), *Phaeodactylum tricornutum* (Pt; Juhas et al., 2014; Oliveri et al., 2014; Fortunato et al., 2015), *Karenia brevis* (Kb; Brunelle et al., 2007), *Ostreococcus tauri* (Ot; Heijde et al., 2010; Kottke et al., 2017), *Pycnococcus provasolii* (Pp; Makita et al., 2021), *Chlamydomonas reinhardtii* (Cr; Beel et al., 2012; Kottke et al., 2017), *Volvox carteri* (Vc; Kottke et al., 2017) and *Arabidopsis thaliana* (At; Chaves et al., 2011). Please note that three putative CRY-DASHs are predicted for Cm in the analysis of Asimgil and Kavakli (2012). For the genus *Karenia*, three putative CRY/photolyase members are predicted (Shikata et al., 2019). The number of cryptochrome/photolyase members from an additional dinoflagellate and from three stramenopile radiophytes, which all form noxious red tides (Shikata et al., 2019), has been omitted from the figure for clarity. As there is no data available of CRYs from the secondary green line, this line was omitted. The cladogram was modified after Handrich et al. (2017).

Spatiotemporal Expression of Algal Cryptochromes

Expression Profiles in Light–Dark Cycles

The expression of algal cryptochromes was so far mainly studied under light–dark cycles (diurnal conditions). In most cases, it is still unknown if their expression is also controlled by the circadian clock. Algal cryptochromes are routinely expressed in a diurnal way whereby the amplitude of the rhythm and the phase of the peak can vary. The green algal Cr pCRY protein (formerly known as *Chlamydomonas* photolyase homolog 1, CPH1) is nearly absent during the day, but accumulates strongly (amplitude increase up to tenfold) during the night (Reisdorph and Small, 2004; Müller et al., 2017). Cr pCRY protein abundance seems to be independent of the circadian clock (Müller et al., 2017). The protein is known to be rapidly degraded by light *via* the proteasome pathway. Transcript levels of Cr pCRY also increase during the night (Kottke et al., 2017), which is also the case for a plant CRY from the chlorophyte *Haematococcus pluvialis* under the tested high light conditions (Hang et al., 2020). These findings along with the negative regulation of Cr pCRY in gametogenesis (see below) suggests a "dark" function of green algal pCRYs (Müller et al., 2017). In contrast to Cr pCRY, the Cr aCRY protein is rather consistently expressed over the light–dark (LD) cycle (chosen cycle of 12 h light:12 h darkness), being highest from the beginning until the middle of the day and lowest at the beginning of the night (change in amplitude about 2-fold; Beel et al., 2012; Kottke et al., 2017). The third CRY from Cr, Cr CRY-DASH1 reaches its maximal protein abundance at midday and is lowest at the beginning of the night (change in amplitude about 4-fold; Rredhi et al., 2021). Taken together, the acrophases of peak expression vary with all three investigated Cr CRYs, ranging from early day until late night. For some other algal cryptochromes, only transcript abundances have been determined so far. In the kelp *Saccharina japonica* (Sj), the maximum transcript level of a *CRY-DASH* is reached at midday in a LD 12 h:12 h cycle (Yang et al., 2020). Transcript levels of the marine picoalga Ot *CPF1*, as determined by microarrays, peak during the day, while levels of Ot *CPF2*, belonging to the CRY-DASH family, peak at the beginning of the day (Heijde et al., 2010). Also in Pt, all studied transcript levels of different *CRYs* (studied by qPCR) had rhythmic expression in an LD cycle of 16 h:8 h with peaks in different

acrophases, indicating a possible functional diversification within the diurnal cycle (Oliveri et al., 2014).

Localization of Cryptochromes

Depending on the expression and function, the subcellular localization of CRYs may vary. The green algal Cr aCRY plays a role in vegetative cells as well as in pregametes and gametes (Zou et al., 2017). During daytime (LD4 being 4 h after light was switched on in a 12 h:12 h LD cycle), Cr aCRY is localized to a significant extent in the nucleus of vegetative cells, but is mainly distributed over the cell body (most likely in the cytosol) at late night (LD22 being 10 h after light was switched off). In sexual cells (pregametes, gametes, and dark inactivated gametes) Cr aCRY was never found in the nucleus, but always distributed over the cell body. The presence of Cr aCRY in the nucleus of vegetative cells at daytime is in congruence with its activity as (6-4) photolyase, repairing UV-B induced damages (Franz et al., 2018). Thus, Cr aCRY localization varies depending on its function. In contrast, the subcellular localization of Pt CPF1 in the nucleus was not differentially regulated by darkness and light (blue or ultraviolet) treatment (Coesel et al., 2009).

For Cr pCRY, experimental data is lacking, but it is predicted to be localized in the nucleus (Kottke et al., 2017).

In the case of the colony-forming alga *Volvox carteri* (Vc) that consists of somatic cells and sexually active gonidia, transcript levels were determined for different photoreceptors in the different cell types. Vc aCRY and Vc pCRY were found to accumulate during final cellular differentiation (Kianianmomeni and Hallmann, 2015). Some Vc photoreceptor genes, including Vc pCRY, are highly expressed in the somatic cells. These data give first insights into the possibility that the different CRYs may also be involved in developmental cycles.

As mentioned above, CRY-DASH proteins like CRY3 from *A. thaliana* have been shown to be localized in organelles; CRY3 is found in chloroplasts and mitochondria (Kleine et al., 2003). Localization studies revealed the presence of CRY-DASH from the dinoflagellate *Karenia brevis* (Kb) only in chloroplasts (Brunelle et al., 2007). The green algal Cr CRY-DASH1 bearing a chloroplast transit peptide was also found solely in the chloroplast but not in mitochondria, using biochemical fractionation (Rredhi et al., 2021). Its localization is thus in close relation with its biological function, as detailed below.

Localization of the novel type dualchrome was analyzed heterologously in tobacco cells in fusion with GFP. It was primarily found in the nucleus and its localization was not changed by light treatment (Makita et al., 2021).

Biological Functions of Algal Cryptochromes

Although a large repertoire of algal cryptochromes is known (**Figure 3**), only a few members have been studied in depth regarding their biological function(s). These studies were mostly done in model algal species that can be transformed and where overexpressing lines and knockdown or knockout mutants can be generated. **Table 1** summarizes the main functions of these cryptochromes. Like land plant CRYs, algal CRYs are involved in the regulation of several cellular functions.

Dual Function Cryptochromes: DNA Repair and More

The first evidence for an algal animal-like cryptochrome with repair activity came from a marine diatom. Pt CPF1 was found to be a member of the cryptochrome (6-4) photolyase family. In contrast to other animal and land plant cryptochromes, Pt CPF1 shows (6-4) photoproduct repair activity (Coesel et al., 2009). Using a Pt *CPF1*-overexpressing line, blue-light regulated transcript levels of wild type were compared to that line. The analyses suggest that Pt CPF1 has a regulatory role in controlling the photosynthetic light harvesting complex and photoprotection as well as cell-cycle progression in addition to its photolyase activity (Coesel et al., 2009). Moreover, a clock function was postulated as detailed in the section below. Similarly, Ot CPF1 from the marine picoalga Ot exerts (6-4) photolyase activity and is involved in circadian rhythms (Heijde et al., 2010). Also the freshwater green alga Cr that lives mainly in wet soil (Sasso et al., 2018) has a dual function cryptochrome, Cr aCRY. It repairs (6-4) photoproducts; a knockdown mutant is less resistant to UV-B treatment than wild type (Franz et al., 2018). In addition, aCRY is involved in regulating transcript levels that are controlled by blue, yellow or red light, including those that encode a light harvesting protein (LHCBM6), glutamine synthetase 1, an enzyme of nitrogen metabolism or the circadian clock component C3, a subunit of the RNA-binding protein CHLAMY1 (Beel et al., 2012).

Algal CRYs and the Biological Clock Machinery

As indicated above, dual function animal-like cryptochromes are also involved in the circadian clock machinery. For Pt and Ot CPF1, the dual function was shown by positively testing their repressor activities in a heterologous system. The transcription factors CLOCK and BMAL were used for this purpose. They form a dimer and are part of the mammalian clock-controlled feedback loop. The inhibition of the CLOCK-BMAL-mediated transcription by Pt and Ot CPF1 was positively verified in COS cells using an E-box bearing luciferase reporter system (Coesel et al., 2009; Heijde et al., 2010). Moreover, Ot *CPF1* knockdown lines were generated along with a luciferase reporter that was put under the control of a circadian promoter. In several of these lines, period lengthening (of 1 up to 5 h) was observed compared to wild type, and the amplitude was dampened (Heijde et al., 2010). In a Cr *aCRY* knockdown line, light-induced induction of the C3 subunit of the circadian RNA-binding protein CHLAMY1 that influences the acrophase was altered (Beel et al., 2012). These experiments suggest that dual function CPF1/aCRYs play an important role within the circadian system.

For the green algal Cr pCRY, an involvement in the circadian clock was also found, using knockdown lines and a representative circadian clock-controlled process named photoaccumulation or phototaxis. Detection of this rhythm is automated (Forbes-Stovall et al., 2014; Müller et al., 2017). Phase response curves (PRCs) with blue-light pulses taken from wild type and a Cr *pCRY* knockdown line revealed the influence of Cr pCRY on PRC

TABLE 1 | Biological functions of algal CRYs, including comparative data from transgenic lines.

Type	(6-4) repair	(CPD) repair	Circadian clock	Sexual cycle	Photosynthesis: Components, apparatus	Other	References
Cr pCRY			X	X			Forbes-Stovall et al., 2014; Müller et al., 2017, Kamrani et al., 2018
Pt CryP					X	X[a]	Juhas et al., 2014; König et al., 2017b
Cr aCRY	X		X	X	X	X	Beel et al., 2012; Zou et al., 2017; Franz et al., 2018
Pt CPF1	X		X		X	X	Coesel et al., 2009
Ot CPF1	X		X				Heijde et al., 2010
Ot CPF2 (CRY-DASH)		X[b]	X				Heijde et al., 2010
Cr CRY-DASH1					X	X[c]	Rredhi et al., 2021

[a]*Photoreceptor network;*
[b]*weak CPD activity;*
[c]*growth curve;*
Cr, Chlamydomonas reinhardtii; Ot, Ostreococcus tauri, Pt, Phaeodactylum tricornutum.

behavior. The largest differences in phase resetting of about 10 h between wild type and the mutant were observed in early subjective night (Müller et al., 2017). These data suggest a role of pCRY in the entrainment of the circadian clock. Moreover, the Cr *pCRY* knockdown line exerts a significantly lengthened period of about 27.9 h compared to wild type (24.5 h in average); it becomes arrhythmic after a few days under free-running conditions that are used to analyze clock properties. It was also suggested that pCRY positively regulates the clock component, ROC75, a putative transcription factor (Kamrani et al., 2018). These data support the model that Cr pCRY is not only involved in circadian input but is also linked to the central oscillator.

CRYs and Life Cycle Regulation

The sexual cycle of the unicellular alga Cr, which is regulated by light and nitrogen availability [reviewed in Kottke et al. (2017); Sasso et al. (2018)], is well studied. Cr vegetative cells of both mating types (mt^+ and mt^-) turn into pregametes in the dark upon the removal of the nitrogen source. In the presence of light and without nitrogen source, pregametes become gametes. This process is controlled by three photoreceptors. It is promoted by phototropin and inhibited by two algal cryptochromes, aCRY and pCRY (Huang and Beck, 2003; Müller et al., 2017; Zou et al., 2017). It is assumed that the inhibition process allows gametes to reconvert to pregametes and vegetative cells if a nitrogen source becomes available short term. Sexually active gametes finally fuse to form a quadriflagellated cell that is converted to a resilient zygote (**Figure 1**). Zygotes can survive in the dark and without a nitrogen source for months (Sasso et al., 2018). Upon light and nitrogen availability, germination is induced, and a tetrad of haploid vegetative cells is formed which results in individual vegetative cells. Activation of germination is again mediated by the above-mentioned three photoreceptors (phototropin, aCRY, and pCRY), but in this case all three act in concert as positive regulators (Huang and Beck, 2003; Müller et al., 2017; Zou et al., 2017). Thus, both aCRY and pCRY play central roles in the sexual cycle of Cr either as negative regulators (gamete formation) or as positive regulators (germination). In this context, it is also of interest that blue light is the main trigger for gamete formation and germination. However, red light also has a significant albeit smaller influence on germination (Zou et al., 2017). aCRY with its property to absorb also in the red spectral region in addition to blue (Beel et al., 2012, see also chapter on properties below) seems to be the relevant receptor for red light because pCRY and phototropin do not absorb in this range.

CRYs and the Photosynthetic Apparatus Regulation

CRYs have a broad influence on the photosynthetic machinery by regulating transcript levels and/or protein abundance of photosynthetic components, pigments or the entire photosynthetic machinery. Here, we will present exemplarily the effects of two representative CRYs, the plant-like CryP from Pt and CRY-DASH1 from Cr, but it should be mentioned that also Pt CPF1 and Cr aCRY were shown to be involved in the regulation of transcript levels of photosynthetic compounds (Coesel et al., 2009; Beel et al., 2012).

Plastids and the photosynthetic apparatus of diatoms are different from that of Chloroplastida in several aspects. Diatoms belong to the secondary red endosymbionts (**Figure 3**). Their plastids are surrounded by four membranes, and they possess additional fucoxanthin pigments that give them an orange, brownish color. Based on a Pt *CryP* knockdown line, effects of this plant-like CRY on proteins of the photosynthetic apparatus were studied. Here, the most prominent members of the diatom light harvesting chlorophyll-fucoxanthin family (Lhcf) as well as proteins involved in their photoprotection (Lhcx) were analyzed. Lhcf1 -Lhcf11 protein abundance was enhanced in the *CryP* knockdown strains in comparison to wild type cells (Juhas et al., 2014). Contrarily, the protein level of Lhcx was decreased in the knockdown lines (Juhas et al., 2014). Changes were also observed in transcript levels under slightly different conditions, but were not always in agreement with the protein levels suggesting that posttranscriptional regulation plays a role

(König et al., 2017a). Taken together, these data show that CryP contributes significantly to changes in photosynthetically relevant compounds.

Characterization of the green algal Cr CRY-DASH1 protein represents a first functional in-depth study of an algal CRY-DASH protein. The Cr *CRY-DASH1* knockout line showed a significantly reduced growth rate (Rredhi et al., 2021) in contrast to Pt *CryP* knockdown lines that were influenced in photosynthesis components, but had a similar growth rate to wild type (Juhas et al., 2014). Intriguingly, the content of chlorophyll a and b as well as of the carotenoids was increased in the *CRY-DASH1* knockout line. This was even visible by the naked eye; cultures of the knockout line were of a darker green than wild type (Rredhi et al., 2021). The increase in pigments went hand in hand with hyper-stacking of thylakoid membranes and an increase in two of the central proteins of photosystem II, D1 and the antenna protein CP43 in the mutant line (Rredhi et al., 2021). CRY-DASH1 thus acts as a repressor that prevents the synthesis of excessive pigments and membranes and thus balances the photosynthetic machinery. Its regulatory role seems to be exerted at the posttranscriptional/translational level as the transcript levels of the genes encoding D1 and CP43 are not altered in the mutant compared to wild type (Rredhi et al., 2021). It is postulated that the observed reduction in growth is due to the higher pigment amount resulting in a shading effect. Indeed, light intensity within a culture flask of the knockout line was reduced compared to that of a wild-type-containing-flask (Rredhi et al., 2021). Notably, Cr CRY-DASH1 absorbs primarily in the UV-A range (see chapter below), where the photosynthetic pigments absorb only to a smaller extent.

Others

The recently described dualchrome (DUC1) represents a chimera consisting of a phytochrome and a cryptochrome in the rather unknown green picoalga Pp (**Figure 2**). It was found that the Pp genome encodes also further homologue proteins of the model plant *A. thaliana* involved in light signaling such as phototropin, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), or ELONGATED HYPOCOTYL 5 (HY5) in addition to the mentioned CRY variants (**Figure 3**). Thus, it possesses a gene set necessary for adapting to various light conditions (Makita et al., 2021).

The presence of several CRYs and other photoreceptors in algae elicits the question of if they form a photoreceptor network influencing each other. Indeed, it was found in Pt *CryP* knockdown lines that the transcript levels of other photoreceptors (Pt phytochrome and Pt CPF1) are influenced by the reduction of Pt CryP. These data suggest exactly such a scenario (König et al., 2017a).

Biophysical Properties of Algal Cryptochromes

The physiological responses to light are the result of a chain of events on the molecular level. First, light is absorbed by a chromophore bound to the receptor, which then leads to a photochemical reaction. This reaction causes a change in structure and/or conformation of the protein, which then may change the interaction of the receptor with a signaling partner such as a specific binding protein or DNA. Finally, a signal transduction cascade leads to a change in the physiology of cells and organisms.

Light-Induced Oligomerization of Cryptochromes

An interesting aspect of the cryptochrome response to light is that the photochemical reaction of the FAD may cause a change in the oligomerization state, i.e., in the number of identical receptors that associate within a dynamic complex. A formation of large complexes called photobodies of At CRY2 has been observed in plant cells (Mas et al., 2000), which is in agreement with a homooligomerization to clusters of receptors upon blue-light illumination (Bugaj et al., 2013). These clusters are reversible in the dark and dissociate within minutes. Accordingly, the reversible clustering has drawn much attention to the development of optogenetic tools to localize fusion proteins to one spot within mammalian cells by illumination (Bugaj et al., 2013; Taslimi et al., 2014). Recent structural characterization by cryo-electron tomography and X-ray crystallography has revealed that At CRY2 forms tetramers of the PHR upon illumination (Ma et al., 2020; Shao et al., 2020; Palayam et al., 2021).

Oligomerization of algal cryptochromes has been studied on some selected members and light-induced changes in oligomerization have been found. A similar clustering behavior to At CRY2 has been observed for the light-sensitive domain of algal Cr pCRY as a fusion protein labeled with a fluorescence protein. The light-induced degradation of Cr pCRY in *C. reinhardtii* prevents a study of a potential functional role of this clustering *in vivo* (Reisdorph and Small, 2004; Müller et al., 2017). However, it has been shown that a complex containing Cr pCRY is formed at the end of the night in *C. reinhardtii*, possibly in context with its dark-related function. It remains open whether this complex includes homooligomers of pCRY or if it is a heteromeric complex with yet unknown partner(s) (Müller et al., 2017).

The oligomerization state has been characterized for isolated Cr aCRY in great detail. Here, red light induces the transition from a dimer in the dark to a tetramer in the light (Oldemeyer et al., 2016). Interestingly, the presence of the CCT is required for the formation of the dimer in the dark. Truncation of the CCT produces a monomer, which forms a dimer upon illumination only to a small extent (Oldemeyer et al., 2016). Some deviating results on oligomerization states of Cr aCRY can be explained by the different exclusion volumes of the columns used for gel filtration (Franz-Badur et al., 2019).

In summary, light-induced changes in oligomerization state have been observed in algal cryptochromes but differ between pCRY and aCRY. In general, little is known about complex formation of algal cryptochromes with signaling partners or other photoreceptors. These signaling partners need to be identified to link the changes in oligomerization state to the signal transduction cascade.

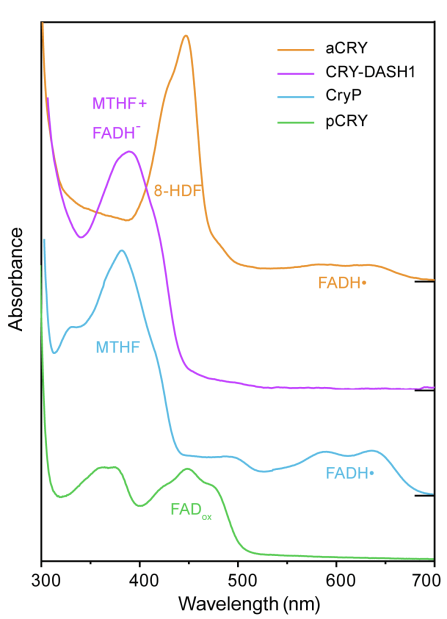
Strong Variations in the Absorption Spectra Define the Different Roles of Algal Cryptochromes

The absorption spectrum of a photoreceptor in the dark is decisive for the region of the sun's spectrum, by which this receptor is activated. Ideally, the absorption spectrum matches the action spectrum that was recorded for the function *in vivo*. The absorption spectrum of cryptochromes reflects the redox state of the FAD cofactor, i.e., whether it binds oxidized FAD (FAD_{ox}), the anion radical ($FAD^{\bullet -}$), the neutral radical ($FADH^{\bullet}$), or fully reduced FAD ($FADH^{-}$). Different redox states of FAD can be stabilized in the dark by the specific protein environment of a cryptochrome. Accordingly, a cryptochrome acts as a blue and UV-A light receptor, if (FAD_{ox}) is the stable redox state in the dark.

The interpretation of the absorption spectra of cryptochromes is complicated by the fact that usually the recording is performed after isolation from a heterologous expression system, which might lead to an oxidation of the FAD. In addition, an antenna chromophore is bound to some cryptochromes, which might be lost upon purification or might not even be produced by the heterologous expression system. The antenna molecule, which has a higher extinction coefficient as compared to flavin, serves the purpose to absorb the light and to transfer energy to the FAD cofactor, thereby increasing the probability of light absorption.

The plant cryptochrome Cr pCRY shows a typical spectrum for a blue-light receptor carrying oxidized FAD (FAD_{ox}) with maxima in the blue and UV-A region (**Figure 4A**; Immeln et al., 2007). The presence of the CCT does not influence the absorption spectrum (Goett-Zink et al., 2021). Some residual MTHF has been identified after purification from bacterial cells, but a functional role as an antenna chromophore and binding of MTHF in the alga is not likely. FAD_{ox} in pCRY is even present in the strongly reducing cytosol of living *Escherichia coli* cells (Goett-Zink et al., 2021), which is a strong argument for FAD_{ox} being the native chromophore of pCRY in *Chlamydomonas* in the dark similar to findings for pCRYs from land plants (Bouly et al., 2007).

Members of the animal-like CRY/CPF1 family such as Ot CPF1, Pt CPF1 and Cr aCRY bind FAD_{ox} after purification (Coesel et al., 2009; Heijde et al., 2010; Beel et al., 2012). The more unusual was the finding that Cr aCRY uses the flavin neutral radical ($FADH^{\bullet}$) as the dark state for a sensory function thereby accessing almost the complete visible spectral region including yellow and red light (Beel et al., 2012; **Figure 4A**). This fact suggests that $FADH^{\bullet}$ is the predominant form of FAD in the alga in the dark, which is supported by a very efficient photoactivation reaction from FAD_{ox} to $FADH^{\bullet}$ *in vitro* (Lacombat et al., 2019). The tight binding of an antenna molecule is another difference of aCRY/CPF1 to plant cryptochromes. The binding of 8-HDF was suggested for Ot CPF1 from structural considerations (Brazard et al., 2012) and then identified for Cr aCRY after implementing the synthesis of 8-HDF in *E. coli* (Franz et al., 2018). The antenna 8-HDF strongly absorbs light in the blue region of the visible spectrum (**Figure 4A**) and additionally stabilizes the $FADH^{\bullet}$ in the dark, preventing oxidation (Oldemeyer et al., 2020).



B

$FAD_{ox} \xrightarrow[+e^- +H^+]{h\nu} FADH^{\bullet} \xrightarrow[+e^-]{h\nu} FADH^{-} \xrightarrow{h\nu} (FADH^{-})^*$

Cr pCRY	dark form	signaling state	deactivated	
Pt CryP	?	dark form	signaling state ?	
Cr aCRY	?	dark form	signaling state	
Cr CRY-DASH1	?	?	dark form	?

FIGURE 4 | Absorption spectra of different cryptochromes from algae recorded *in vitro* directly after isolation and the postulated roles of the redox states of FAD in these cryptochromes. **(A)** The absorption spectra of cryptochromes are characterized by the different oxidation states of the FAD chromophore and the binding of antenna molecules. The plant cryptochrome Cr pCRY binds FAD_{ox} and acts as blue-light receptor. Plant-like cryptochrome Pt CryP and animal-like cryptochrome Cr aCRY both absorb almost in the full visible spectral region because of the absorption of $FADH^{\bullet}$. However, Pt CryP binds MTHF as antenna, whereas Cr aCRY binds 8-HDF leading to different absorption maxima in the UV-A and blue region, respectively. The UV-A-receptor Cr CRY-DASH1 absorbs mainly at around 380 nm dominated by the contribution of MTHF, whereas its chromophore $FADH^{-}$ absorbs only weakly. For simplicity, the absorption of FAD_{ox}, $FADH^{\bullet}$ and $FADH^{-}$ is indicated at the maximum with highest wavelength, but all FAD species also contribute to the absorption at lower wavelengths. Spectra were displaced vertically for better visibility. **(B)** The redox states of FAD play different roles in the algal cryptochromes, as postulated on the basis of the absorption spectra and functional studies of representative members. The dark form indicates the redox state present *in vivo* in the dark. Light absorption induces a reduction of the FAD. The signaling state is associated with conformational changes and/or changes in oligomerization state of the receptor that drive signal transduction.

Much less is known about the plant-like subfamily and its member Pt CryP. The binding of stable $FADH^{\bullet}$ and MTHF in the dark after purification might indicate a light response similar to the aCRY family, with the distinct difference that the antenna MTHF absorbs shifted to the UV-A region compared

to 8-HDF (**Figure 4A**; Juhas et al., 2014). Functional studies have focused so far only on the blue-light effects of CryP on Pt physiology (König et al., 2017a). It would be of high interest to study also potential responses of Pt CryP to green and red light.

The large subfamily of CRY-DASH proteins (Brudler et al., 2003) is also well represented with several members in algal genomes (**Figure 3**). Few members have been isolated and characterized by absorption spectroscopy. A typical behavior for members of CRY-DASH has been found for Ot CPF2 *in vitro*, which showed a dominant absorbance by the antenna MTHF and some contribution from FAD_{ox} (Heijde et al., 2010). Similar findings have been presented for Cm PHR5 (Asimgil and Kavakli, 2012). Recent investigations on Cr CRY-DASH1 revealed that FAD is present in the fully reduced state ($FADH^-$) directly after purification, which together with the photochemical results indicates a presence of $FADH^-$ in Cr *in vivo* (Rredhi et al., 2021). As a result, Cr CRY-DASH1 mainly absorbs light in the UV-A-region (**Figure 4A**), supported by functional studies as a UV-A receptor in Cr. Of note, the UV-A absorption of CRY-DASH1 is maximal in a spectral region where the typical blue-light receptors pCRY and phototropin as well as photosystems I and II show only weak absorption. Accordingly, CRY-DASH1 may complement the other receptors in the alga by the spectral range in which it is activated.

In summary, the strong differences in the absorption spectra of cryptochromes in the dark reflect the variety of functions for which these receptor subfamilies evolved (Kottke et al., 2017; **Figure 4B**). Cryptochromes act in algae as UV-A receptor (Cr CRY-DASH1), as UV-A/blue-light receptor (Cr pCRY) and as "white light" receptor covering almost the full spectral region (Cr aCRY), defined by the redox state of FAD cofactor in the dark. The FAD is reduced after the absorption of light and forms the signaling state, accompanied by conformational changes and/or changes in oligomerization state of the receptor. The identification of the signaling state and the mechanism of signal propagation within the receptor are important biophysical questions to be answered for each member. The details of the different pathways identified to date go beyond the scope of this review. Even more differences in the light responses will be revealed as more and more candidates of algal cryptochromes are being characterized biophysically.

OUTLOOK

It is expected that metagenome analysis from phytoplankton will lead to the discovery of even more novel types of photoreceptors, including new cryptochrome variants such as the recently identified DUC1. The high number of different light sensors in aquatic organisms is correlated with the fact that light quality and light quantity for aquatic algae change dynamically. The light conditions depend on the depth within the water column where the algae occur at a given time in the ocean, on the presence of solved particles and on other light-absorbing organisms at this location. The challenge will be to analyze the role of all these photoreceptors and their mutual interaction partners in the *in vivo* systems.

Algae represent a rich source for novel cryptochrome photoreceptors. These might be used as optogenetic tools to generate light switches in algal cells for controlling gene expression or in combination with signaling molecules such as cAMP or Ca^{2+} (Kianianmomeni and Hallmann, 2014). They might trigger light-dependent adaptations of organismal physiology, development, and behavior in nature (Losi et al., 2018) and thus create potent tools for biotechnological approaches. They may also be used in heterologous systems for neurobiology and in medicine, as it was recently done with an algal rhodopsin to partly recover vision in blind people (Sahel et al., 2021). The land plant At CRY2 was already used for optogenetical protein clustering (Park et al., 2017) and for regulating gene expression in mammalian cells (Hernández-Candia et al., 2019). In general, the high variety of algal CRYs offers a high potential for engineering new sensor tools.

Currently, we are only beginning to understand the biophysical properties and the involved signal transduction pathways as well as the biological functions of the different algal cryptochromes. Their interplay within one algal system and along with the other photoreceptors of this system remains largely enigmatic, even in the today selected model algal organisms. Double-, triple-, or even higher-order mutants need to be produced to gain further insights in their complex photobiology. Yet, the basis of these light signaling events is the key to understand (i) algal fitness and thus (ii) their central contribution as photosynthetic organisms to life on Earth. As our knowledge on the role and function of algal cryptochromes improves, it will be possible to manipulate growth, reproduction and the development of algae as well as to optimize photosynthesis, for example by application of targeted illumination protocols.

AUTHOR CONTRIBUTIONS

JP, AR, JS, and MM designed and drew **Figures 1–3**. SO and TK created **Figure 4**. JP and MM arranged **Table 1**. JP, AR, TK, and MM wrote the paper, with input from all authors. All authors contributed to the article and approved the submitted version.

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REVIEW
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Plant Cryptochromes Illuminated: A Spectroscopic Perspective on the Mechanism

Lukas Goett-Zink[1†] *and Tilman Kottke*[1,2*†]

[1]*Department of Chemistry, Bielefeld University, Bielefeld, Germany,* [2]*Biophysical Chemistry and Diagnostics, Medical School OWL, Bielefeld University, Bielefeld, Germany*

Plant cryptochromes are central blue light receptors for the control of land plant and algal development including the circadian clock and the cell cycle. Cryptochromes share a photolyase homology region with about 500 amino acids and bind the chromophore flavin adenine dinucleotide. Characteristic for plant cryptochromes is a conserved aspartic acid close to flavin and an exceptionally long C-terminal extension. The mechanism of activation by excitation and reduction of the chromophore flavin adenine dinucleotide has been controversially discussed for many years. Various spectroscopic techniques have contributed to our understanding of plant cryptochromes by providing high time resolution, ambient conditions and even in-cell approaches. As a result, unifying and differing aspects of photoreaction and signal propagation have been revealed in comparison to members from other cryptochrome subfamilies. Here, we review the insight from spectroscopy on the flavin photoreaction in plant cryptochromes and present the current models on the signal propagation from flavin reduction to dissociation of the C-terminal extension.

Keywords: photoreceptor, photolyase, in-cell spectroscopy, flavin, UV-vis spectroscopy, EPR (electron paramagnetic resonance), FTIR (Fourier transform infrared spectroscopy), blue light receptor

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***Correspondence:**
Tilman Kottke
tilman.kottke@uni-bielefeld.de

***ORCID:**
Lukas Goett-Zink
0000-0002-0076-5308
Tilman Kottke
0000-0001-8080-9579

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INTRODUCTION

Plant cryptochromes (pCRY) are a subfamily of the large cryptochrome/photolyase superfamily (CPF) of photoreceptors, DNA repair enzymes and clock proteins (Chaves et al., 2011). pCRY share a 500 amino acid photolyase homology region (PHR) and differ in the length of the unconserved C-terminal extension (CCT) with little structural elements (**Figure 1**). Flavin adenine dinucleotide (FAD) is bound noncovalently as a chromophore to the FAD binding pocket of the PHR. pCRY regulate many photomorphogenetic responses such as the flowering time as well as the determination of the day length in land plants and the cell cycle in green algae (Wang et al., 2014; Kottke et al., 2017).

The homology in sequence and structure of pCRY to cyclobutane pyrimidine dimer (CPD) photolyases might suggest that they share a common mechanism. However, extensive spectroscopic studies have shown that the mechanisms of these two protein subfamilies are distinct. A key difference between CPD photolyases and pCRY is the initial state of the cofactor. CPD photolyases bind fully reduced FAD ($FADH^-$) as the dark form prior to catalysis, which is formed by a so-called photoactivation reaction from the precursors oxidized FAD (FAD_{ox}) and FAD neutral radical (FADH•) (Sancar, 2003). In contrast, the dark form of pCRY is FAD_{ox}, which has been confirmed by a series of studies *in vitro* and in cells (Banerjee et al., 2007; Bouly et al., 2007; Engelhard et al., 2014;

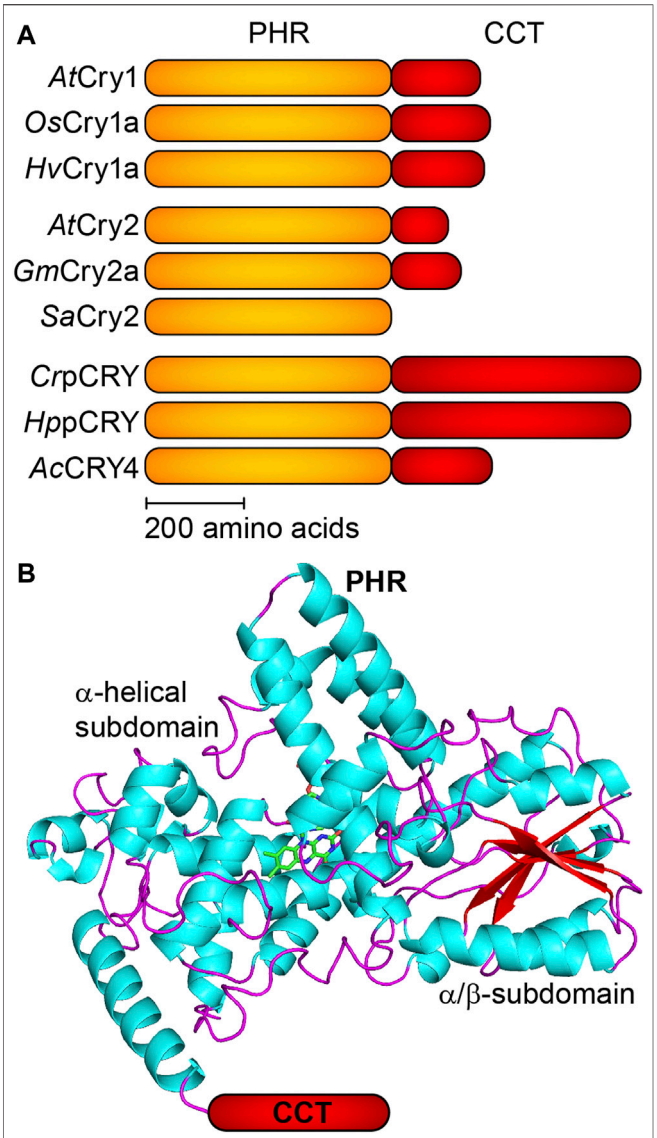

FIGURE 1 | Schematic domain topology and structure of plant cryptochromes (pCRY). **(A)** Plant cryptochromes share a conserved PHR domain and a CCT of varying length. *Crp*CRY exhibits the longest CCT with ~500 amino acids. The selected pCRY originate from *Arabidopsis thaliana* (*At*CRY1 and *At*CRY2), rice *Oryza sativa* (*Os*CRY1a), barley *Hordeum vulgare* (*Hv*CRY1a), soybean *Glycine max* (*Gm*CRY2a), white mustard *Sinapis alba* (*SaCRY2*), green alga *Chlamydomonas reinhardtii* (*Crp*CRY), green alga *Haematococcus pluvialis* (*Hpp*CRY) and fern *Adiantum capillus-veneris* (*Ac*CRY4). **(B)** The PHR domain of pCRY comprises an α-helical subdomain binding FAD and ATP as well as an α/β-subdomain with a parallel β-sheet (PDB:1U3D). The CCT is connected to the C-terminal region of the PHR but is not resolved in the crystal structure.

Goett-Zink et al., 2021). Still, some unifying aspects have been found between the photoactivation reaction of CPD photolyase and the photoreaction of FAD_{ox} in pCRY.

It should be noted that spectroscopic studies have almost exclusively been performed on only two members of the pCRY family, cryptochrome 1 from *Arabidopsis thaliana* (*At*CRY1) (Lin et al., 1995) and plant cryptochrome from *Chlamydomonas reinhardtii* (*Crp*CRY or CPH1) (Reisdorph and Small, 2004). Issues with stability and solubility of the full-length proteins limited these investigations mostly to the PHR, neglecting the influence of the CCT. In the following, pCRY will be used synonymously to pCRY-PHR and the few studies on the full-length proteins will be highlighted.

Here, we will discuss the initial steps in the photoreaction of pCRY along with similarities and differences to CPD photolyases. Moreover, we will reveal the current status of insight into the subsequent signal propagation from the chromophore through the PHR to the CCT. Last, we will address key differences in mechanism to other members of the cryptochrome superfamily and give an outlook on open questions with respect to the light-induced clustering of the PHR.

FORMATION OF THE FLAVIN NEUTRAL RADICAL STUDIED BY TIME-RESOLVED APPROACHES

Light absorption in pCRY is dominated by the chromophore FAD_{ox}. An antenna molecule is not bound to pCRY, at least not stoichiometrically (Immeln et al., 2007; Hoang et al., 2008), in contrast to the binding of the antenna 5,10-methenyltetrahydrofolic acid (MTHF) to CPD photolyase (Jorns et al., 1984). This difference might be rationalized by the much higher extinction coefficient of FAD_{ox} in pCRY than of $FADH^-$ in CPD photolyase. The crystal structure of pCRY shows side chains filling the binding pocket that might lower the affinity to MTHF (Brautigam et al., 2004). Instead, pCRY bind adenosine triphosphate (ATP) (Bouly et al., 2003) and other nucleotides (Engelhard et al., 2014), most likely in the access cavity close to the chromophore, which is in DNA photolyase responsible for binding of damaged DNA (**Figure 2A**) (Brautigam et al., 2004; Ma et al., 2020).

The FAD_{ox} in pCRY absorbs in the UVA and blue spectral region up to $\lambda \sim 500$ nm resulting in a loss of FAD_{ox} and the formation of FADH• (Lin et al., 1995; Giovani et al., 2003). This photoreaction in pCRY was studied at high time resolution by femtosecond broadband transient UV-vis spectroscopy. With a time constant of 400 fs, an electron transfer takes place from the nearby tryptophan ($TrpH_{400}$) to the excited FAD_{ox}* forming the flavin anion radical ($FAD•^-$) and the corresponding tryptophan cation radical ($TrpH_{400}•^+$) (**Figure 2B**) (Immeln et al., 2012). The redox potentials for reduction of FAD_{ox} to FADH• in pCRY and oxidation of TrpH to ($Trp•,H^+$) in solution are −153 mV (Balland et al., 2009) and 1.00 V (Mahmoudi et al., 2016), respectively, precluding reduction of FAD_{ox} in the dark. Excitation results in configurations with similar energy of FAD_{ox}* and the charge transfer state $TrpH_{400}$ to FAD_{ox}* (Cailliez et al., 2014). The $FAD•^-$ is stabilized against recombination by a tryptophan triad, which increases the distance between the cation and anion radicals by electron hopping between $TrpH_{400}$, $TrpH_{377}$ and $TrpH_{324}$ (or alternatively $TrpH_{379}$) within 30 ps (Immeln et al., 2012). This triad is conserved in the CPF family and the respective electron transfer processes have been studied in detail for photoactivation of CPD photolyase, albeit starting with

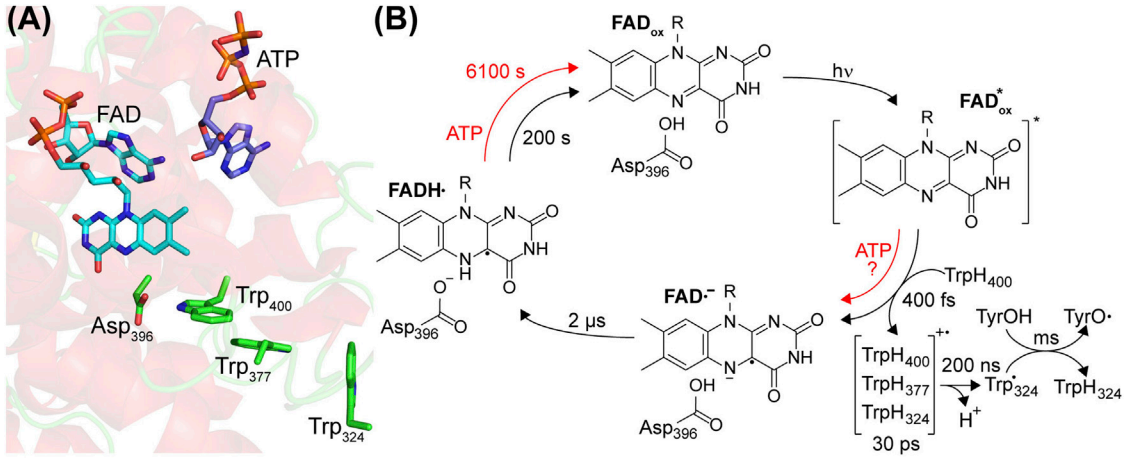

FIGURE 2 | Center of the photoreaction of plant cryptochromes and photocycle of FAD. **(A)** The α-helical domain of the PHR binds oxidized FAD and ATP. Close to the FAD, a tryptophan-triad Trp_{400}, Trp_{377} and Trp_{324} acts as electron donor and aspartic acid Asp_{396} acts as proton donor (according to *At*CRY1 numbering). **(B)** FAD_{ox} is excited by UV-A/blue light initiating an ultrafast electron transfer from Trp_{400}. In the presence of ATP, an alternative electron pathway has been proposed. The resulting FAD anion radical ($FAD^{\bullet-}$) is stabilized by electron hopping events in the tryptophan triad and deprotonation of $TrpH_{324}^{\bullet+}$ to the bulk. Subsequently, Asp_{396} protonates $FAD^{\bullet-}$ to the FAD neutral radical ($FADH^{\bullet}$), which represents the signaling state. The formation of a tyrosyl radical ($TyrO^{\bullet}$) takes place in the millisecond time region. Binding of ATP enhances the yield of the photoreaction and decelerates the reoxidation to FAD_{ox} as indicated by the time constants determined for *Crp*CRY.

excitation of FADH• (Aubert et al., 2000). In the next step, $TrpH^{\bullet+}$ in pCRY deprotonates to Trp• with τ = 200 ns, most likely releasing the proton to the bulk (Müller et al., 2014).

Time-resolved UV-vis spectroscopy revealed that $FAD^{\bullet-}$ is protonated to give FADH• with a time constant of 2 μs (**Figure 2B**) (Langenbacher et al., 2009; Maeda et al., 2012). The FADH• is considered to be the signaling state in pCRY. Infrared difference spectroscopy, in particular the time-resolved step-scan technique, was used to identify the proton donor as the conserved Asp_{396} (Kottke et al., 2006; Hense et al., 2015; Thöing et al., 2015), which is fully protonated in the dark at physiological pH (Müller et al., 2014; Schroeder et al., 2018). Accordingly, proton transfer is completely decoupled from the electron transfer, which was confirmed by a quantum mechanical molecular dynamics approach (Lüdemann et al., 2015). The presence of the intrinsic proton donor Asp_{396} close to FAD is one of the major differences to CPD photolyase, which contains Asn at this position (**Figure 2**).

As final electron transfer step, Trp• reacts with a surface-exposed tyrosine to a tyrosine radical ($TyrO^{\bullet}$) with τ = 1 ms in full-length pCRY (Giovani et al., 2003), which is then reduced in the millisecond time range by the bulk, strongly depending on the concentration of external reductant and on the pCRY member (Giovani et al., 2003; Thöing et al., 2015). Interestingly, the identification of this tyrosine in pCRY is lacking, whereas in other cryptochromes specific tyrosines have been identified (Oldemeyer et al., 2016; Zoltowski et al., 2019).

The quantum yield of FADH• formation is low with only 2% (Giovani et al., 2003), but can be significantly increased by the addition of ATP and reductant to ~14% for *At*CRY1 (Müller et al., 2014). The increased FADH• formation has been attributed to structural changes in pCRY caused by the binding of ATP (Iwata et al., 2020), which leads to a closer contact of FAD and Trp_{400} enhancing the electron transfer (Cailliez et al., 2014). This quantum yield is still comparatively low for a photoreceptor pointing to several loss processes, considering that other cryptochromes have shown quantum yields of up to 43% (Zoltowski et al., 2019). Moreover, the effect of ATP is less pronounced in *Crp*CRY in the absence of reductant, for which only 2% of the absorbed photons lead to a stable product on the minute time range (Schroeder et al., 2018). Interestingly, experiments on pCRY demonstrated a sensitivity in yield of FADH• on the external magnetic field acting on the singlet/triplet interconversion of the radical pair $FAD^{\bullet-}/Trp^{\bullet+}$ (Maeda et al., 2012), which inspired further investigations on the role of cryptochromes in magnetoreception.

FADH• is strongly stabilized by protonation (Hense et al., 2015), by reduction of Trp• (Müller et al., 2014) and by the presence of ATP (Immeln et al., 2007; Hense et al., 2015). Accordingly, dependent on the specific pCRY member and on the buffer conditions, the reoxidation to FAD_{ox} by oxygen takes a few minutes to hours at room temperature (**Figure 2B**). Important insight was provided by the finding in a screen that two point mutations distant from the FAD binding pocket at helix α13 strongly modulate the recovery time of FAD_{ox} (Taslimi et al., 2016), which might be related to the signaling mechanism. A competing pathway for reoxidation requires the presence of high light intensity and strong reductant to produce $FADH^{-}$, which then readily reacts with oxygen (Müller and Ahmad, 2011).

IN-CELL SPECTROSCOPIC APPROACHES CONTRIBUTE TO OUR UNDERSTANDING OF THE MECHANISM

The formation of FADH• from FAD_{ox} in the photoreaction of pCRY was confirmed by in-cell fluorescence and in particular by

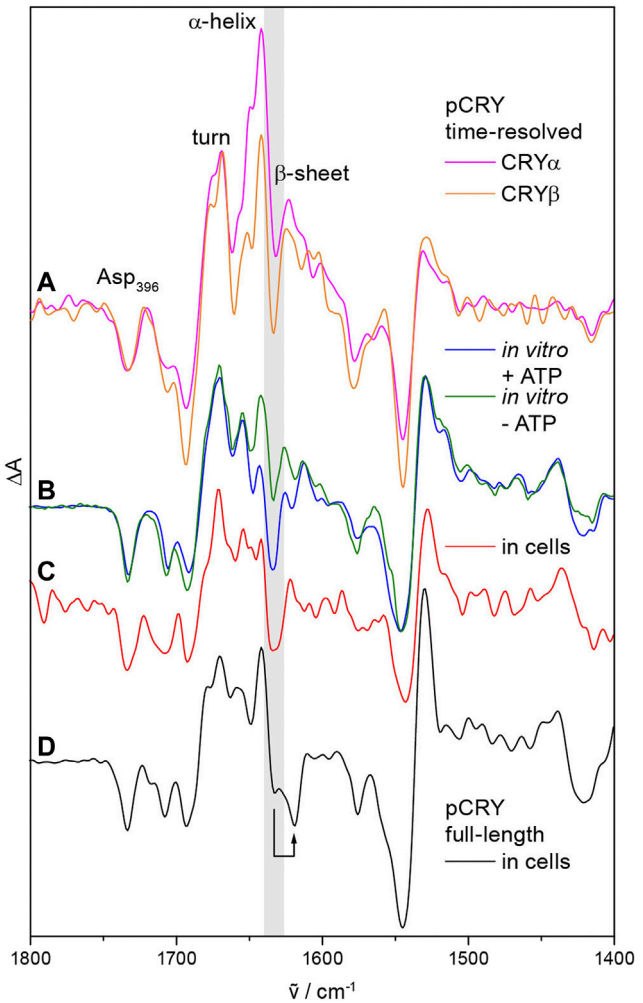

FIGURE 3 | Conformational changes in pCRY observed by time-resolved, static and in-cell infrared difference spectroscopy. **(A)** Time resolved step-scan experiments on the PHR domain detected an increase in α-helical and turn elements, which is characteristic for the intermediate CRYα with a lifetime of 500 µs. Subsequently, a loss of β-sheet content is detected representing the intermediate CRYβ (highlighted in gray) with a lifetime of 29 ms. **(B)** The presence of ATP stabilizes the CRYβ intermediate *in vitro* into the time range of minutes as observed by static experiments. **(C)** Similarly, cellular nucleotides stabilize CRYβ in bacterial cells as determined by in-cell spectroscopy. **(D)** Full-length pCRY shows a shift of the β-sheet signal attributed to the association of the CCT close to the β-sheet of the PHR as shown by in-cell spectroscopy. Spectra were taken from (Thöing et al., 2015; Schroeder et al., 2018; Goett-Zink et al., 2021).

in-cell electron paramagnetic resonance (EPR) spectroscopy on frozen insect cells (Banerjee et al., 2007; Bouly et al., 2007). The decay of FADH• after illumination was slowed down in living *E. coli* cells to a similar extent as in the presence of ATP *in vitro*, as shown by in-cell UV-vis spectroscopy (Goett-Zink et al., 2021). Importantly, the decay of EPR signals in insect cells agreed with the time range of the physiological response, further supporting the role of FADH• as signaling state (Herbel et al., 2013). However, the role of the conserved tryptophan triad as an essential part of the photoreaction has been controversial (Ahmad, 2016), because single point mutations in the tryptophan triad of pCRY *in planta* did not abolish the physiological response (Li et al., 2011; Gao et al., 2015). Instead, the tryptophan triad might play a role in the structural integrity because some of these mutants show constitutively active phenotypes (Li et al., 2011; Gao et al., 2015). Photoreduction of FAD_{ox} in pCRY in insect cells similarly proceeded despite such point mutations (Engelhard et al., 2014). Moreover, small metabolites, in particular ATP, enhanced the photoreaction also with mutations in the tryptophan triad (Engelhard et al., 2014). It should be noted that a role of ATP as a reducing agent can be excluded. Therefore, an alternative electron pathway in the presence of cellular nucleotides was proposed, which is independent of the tryptophan triad. All these observations in cells have changed the view on the activation mechanism of pCRY *in vivo*.

SIGNAL PROPAGATION IN THE RECEPTOR FROM FLAVIN ADENINE DINUCLEOTIDE TO C-TERMINAL EXTENSION—INSIGHT FROM SPECTROSCOPY

pCRY responds with conformational changes to the photoreduction of FAD_{ox}. Time-resolved step-scan and rapid-scan infrared difference spectroscopy identified two major intermediates after light-induced activation of the PHR, CRYα and CRYβ (**Figure 3A**) (Thöing et al., 2015). Changes in α-helical elements and turn structures are detected already few microseconds after excitation representing the CRYα intermediate. Subsequently, a loss of β-sheet content takes place with a time constant of 500 µs, which is characteristic for CRYβ and occurs concomitant with the formation of TyrO•. It should be highlighted that the only β-sheet of pCRY is present in the α/β-subdomain at a distance of ~25 Å to FAD (**Figure 1B**) (Brautigam et al., 2004). This assignment indicates that the signal propagates from FADH• to this parallel β-sheet leading to a reorganization of the β-sheet rather than an unfolding. The presence of ATP stabilizes the conformational changes of CRYβ *in vitro* (**Figure 3B**) and in bacterial cells (**Figure 3C**) from a transient species with τ = 29 ms into the minute time range (Schroeder et al., 2018; Goett-Zink et al., 2021). Both, *At*CRY1 and *Cr*pCRY showed such stabilization of conformational changes in the presence of ATP (Schroeder et al., 2018; Iwata et al., 2020) suggesting that CRYβ is a key component in the signal progression of pCRY.

The isolated CCT of pCRY is largely unstructured as found by circular dichroism and nuclear magnetic resonance (NMR) spectroscopy (Partch et al., 2005). Recent studies on full-length pCRY in bacterial cells by in-cell infrared difference spectroscopy showed an association of the CCT close to the β-sheet of the PHR in the dark, thereby downshifting the signal of the β-sheet (**Figure 3D**) (Goett-Zink et al., 2021). Upon illumination, the CCT dissociates from the PHR and increases the diffusion coefficient with τ = 400 ms as demonstrated by transient grating spectroscopy on full-length pCRY (Kondoh et al.,

2011). The dissociation increases the accessibility of the CCT to proteolytic digestion *in vitro* (Partch et al., 2005). Together, spectroscopic studies on pCRY indicate that the light-induced signal propagates from FAD to the β-sheet with bound CCT resulting in a β-sheet reorganization, which then induces the dissociation of the CCT from the PHR (Goett-Zink et al., 2021).

The initiating step of the signal propagation from the chromophore to the protein is not fully understood. A model has been proposed in which the negative charge of deprotonated Asp_{396} repels bound negatively charged ATP and leads to a dissociation of the CCT covering the ATP binding site (Müller and Bouly, 2014). Direct experimental evidence for a light-induced release of ATP or a coverage of the ATP binding site by the CCT is lacking. It is challenging to design studies on pCRY mutants without a negative charge in the FAD binding pocket, because the exchange of Asp_{396} to Asn and Cys leads to the light-induced formation of charged $FADH^-$ and $FAD•^-$, respectively (Burney et al., 2012; Hense et al., 2015). Infrared spectroscopic studies on pCRY lacking Asp_{396} (in the D396C mutant) showed that $FAD•^-$ is already sufficient to induce β-sheet reorganization in pCRY, albeit the formation of FADH• and/or deprotonated Asp_{396} stabilizes the conformational changes into a physiological relevant time region (Hense et al., 2015; Schroeder et al., 2018). Furthermore, time resolution has not been sufficient yet to link the formation of the intermediate CRYα to either formation of $FAD•^-/Asp_{396}OH$ or $FADH•/Asp_{396}O^-$. Hence, electrostatic interactions/repulsion exerted by $FAD•^-$ and deprotonated Asp_{396}, respectively, are likely key components of signal propagation in pCRY. Similar mechanisms have been proposed to be active in *Drosophila* cryptochrome by the negative charge of $FAD•^-$ (Ganguly et al., 2016) as well as in other photoreceptor families such as photoactive yellow proteins (Kottke et al., 2018).

SIMILARITIES AND KEY DIFFERENCES IN MECHANISM COMPARED TO OTHER CRYPTOCHROME SUBFAMILIES

A unifying aspect of the mechanism of pCRY valid also for other cryptochrome subfamilies is the decoupled electron and proton transfer to FAD_{ox}. As a result, $FAD•^-$ is formed and stabilized to a different extent. pCRY is differentiated from other cryptochrome subfamilies by the conserved Asp_{396} as an intrinsic proton donor to $FAD•^-$ within few microseconds. In other cryptochromes such as CRY-DASH, animal and animal-like cryptochromes the Asp_{396} is exchanged by an asparagine (Brudler et al., 2003). These cryptochromes show a millisecond protonation of $FAD•^-$, most likely from the bulk (Lacombat et al., 2019). Different extents of stabilization have been reported within the DASH subfamily (Iwata et al., 2010). For insect cryptochromes, a cysteine is conserved at this position and $FAD•^-$ is stable for minutes (Berndt et al., 2007; Zoltowski et al., 2011). It should be noted that a single exchange of cysteine for aspartate in insect cryptochromes led to formation of a neutral radical (Öztürk et al., 2008), albeit not with the characteristic blue shift of the FADH• absorbance bands in pCRY attributed to the charge of deprotonated Asp_{396} close to FADH• (Immeln et al., 2010). These observations show that a specific, hydrophobic environment in the FAD binding pocket of pCRY generates a protonated Asp_{396} in the dark (Kottke et al., 2006). The full protonation of Asp_{396} is aided by the binding of ATP, which results in an upshift of the pK_a value (Müller et al., 2014; Iwata et al., 2020).

Asp_{396} in pCRY not only acts as proton donor, but influences the redox potential of the FAD. The potential for reduction of FADH• to $FADH^-$ is lowered as compared to CPD photolyase with an Asn conserved at this position (Balland et al., 2009), supported by the finding that the D396N mutant of pCRY forms $FADH^-$ instead of FADH• after illumination (Burney et al., 2012). Moreover, replacement of Asn by Asp in CPD photolyase leads to stabilization of FAD_{ox} instead of FADH• in the dark *in vitro*, whereas hydrogen bonding and the protein environment for FAD are quite similar (Damiani et al., 2011). Accordingly, pCRY is primed to bind FAD_{ox} as the dark form for blue light reception. In contrast, animal-like cryptochromes have been claimed to bind FADH• as the dark form for white light reception (Beel et al., 2012), aided by a very efficient photoreduction from FAD_{ox} (Lacombat et al., 2019). Recently, CRY-DASH have been postulated to bind $FADH^-$ in the dark for UVA reception (Rredhi et al., 2021).

In other members of the CPF than pCRY, an antenna chromophore is additionally associated in a stoichiometric ratio such as 5,10-methenyltetrahydrofolic acid (MTHF) to CRY-DASH or 8-hydroxy-deazaflavin (8-HDF) to animal-like cryptochromes (Song et al., 2006; Franz et al., 2018). These antenna molecules aid in increasing the extinction coefficient for excitation, which might be rationalized by the lower extinction coefficient of FADH• or $FADH^-$ as compared to FAD_{ox}.

Another property distinguishing pCRY from other cryptochromes is the exceptional length of the CCT for most of the pCRY members (**Figure 1A**). This difference needs to be considered in the comparison of mechanisms to *Drosophila* CRY with a CCT of only 23 amino acids or to CRY-DASH, for which some fungal members have CCT with up to 200 amino acids (Froehlich et al., 2010). Nevertheless, as for pCRY, undocking of the CCT from the PHR after photoactivation was demonstrated in *Drosophila* CRY (Chandrasekaran et al., 2021). Interestingly, *Sinapis alba* pCRY does not contain a CCT, which poses some questions on its signal transduction mechanism (Malhotra et al., 1995).

OUTLOOK ON THE MOLECULAR BASIS OF CLUSTERING

Several open questions in the mechanism of pCRY have been highlighted in the previous sections. A further fascinating aspect of pCRY is the light-induced homooligomerization and formation of photobodies as observed with phytochrome B in *A. thaliana* (Mas et al., 2000; Bugaj et al., 2013). Blue-light illumination of pCRY induces clustering via PHR, which is essential for the function in *A. thaliana* (Wang and Lin, 2020). The photooligomerization of PHR has been successfully established on *At*CRY2 and applied in optogenetic tools for light-induced activation of effector proteins (Bugaj et al., 2013; Taslimi et al., 2014). Illuminated *At*CRY2 forms tetrameric units of the PHR in a "doughnut" shaped structure with interaction

sites at the α/β-subdomain and the C-terminal region of the α-helical subdomain as observed by cryogenic electron microscopy (Ma et al., 2020). The introduction of several single point mutations in the α-domain of *At*CRY2 abolished homooligomerization, whereas a E490G mutation shows increased oligomerization properties (Taslimi et al., 2014; Ma et al., 2020). Albeit these observations indicate the involvement of these amino acids in oligomerization, the molecular basis of clustering in pCRY is not yet understood. Time-resolved spectroscopic methods for studying structural changes on pCRY with single point mutations might address these unresolved questions.

AUTHOR CONTRIBUTIONS

LG-Z created the figures with input from TK; LG-Z and TK wrote the manuscript.

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REVIEW
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Structural and Chemical Biology Approaches Reveal Isoform-Selective Mechanisms of Ligand Interactions in Mammalian Cryptochromes

*Simon Miller and Tsuyoshi Hirota**

Institute of Transformative Bio-Molecules, Nagoya University, Nagoya, Japan

Cryptochromes (CRYs) are core components of the circadian feedback loop in mammals, which regulates circadian rhythmicity in a variety of physiological processes including sleep–wake cycles and metabolism. Dysfunction of CRY1 and CRY2 isoforms has been associated with a host of diseases, such as sleep phase disorder and metabolic diseases. Accumulating evidence for distinct roles of CRY1 and CRY2 has highlighted the need for CRY isoform-selective regulation; however, highly conserved sequences in CRY ligand-binding sites have hindered the design of isoform-selective compounds. Chemical biology approaches have been identifying small-molecule modulators of CRY proteins, which act in isoform-non-selective and also isoform-selective manners. In this review, we describe advances in our understanding of CRY isoform selectivity by comparing X-ray crystal structures of mammalian CRY isoforms in apo form and in complexes with compounds. We discuss how intrinsic conformational differences in identical residues of CRY1 and CRY2 contribute to unique interactions with different compound moieties for isoform selectivity.

Keywords: cryptochromes, circadian clock, X-ray crystallography, small-molecule modulators, isoform selectivity

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Andrew C. Liu,
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Jihwan Myung,
Taipei Medical University, Taiwan

***Correspondence:**
Tsuyoshi Hirota
thirota@itbm.nagoya-u.ac.jp

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INTRODUCTION

The circadian clock is a biological timekeeper that regulates daily physiological rhythms in almost all organisms. In humans, sleep–wake behavior, hormone secretion, body temperature, and metabolism exhibit ~24-h circadian rhythms (Bass and Lazar, 2016). The suprachiasmatic nucleus (SCN) in the hypothalamus forms the central clock that orchestrates physiological rhythms in tissues and cells throughout the body *via* neuronal, hormonal, and behavioral signaling (Ralph et al., 1990; Reppert and Weaver, 2002). External stimuli, such as light-mediated activation of retinal ganglion cells, prompt the SCN to synchronize with environmental day–night cycles (Hatori and Panda, 2010). In contrast, feeding is the major timing cue for the peripheral clocks that locally regulate circadian rhythms in different tissues (Schibler et al., 2003).

At the core of the circadian clock, CLOCK and BMAL1 transcription factors form a heterodimer that binds to E-box elements and activates the transcription of thousands of genes

regulating metabolism and other outputs (Rey et al., 2011; Koike et al., 2012). CLOCK-BMAL1 also activates *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes, whose protein products repress CLOCK-BMAL1 to form a transcription–translation negative feedback loop (Takahashi, 2017). Alleviation of CLOCK-BMAL1 repression is regulated by proteasomal degradation of PER and CRY proteins, which is enhanced by post-translational modifications. Casein kinase I (CKI) phosphorylates PERs for degradation (Narasimamurthy and Virshup, 2021), and CKI inhibitors cause period lengthening of behavioral rhythms (Meng et al., 2010; Lee et al., 2019). In contrast, AMP-activated protein kinase (AMPK)- and glycogen synthase kinase-3β (GSK-3β)-dependent phosphorylation of CRYs regulate their degradation (Lamia et al., 2009; Kurabayashi et al., 2010). Proteasomal degradation of CRYs is mediated by ubiquitination *via* the E3 ligase FBXL3 (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007), whereas FBXL21 induces CRY stabilization (Hirano et al., 2013; Yoo et al., 2013). The balance of CRY degradation and stabilization is also important for the regulation of circadian period length, such that degradation facilitates a new cycle to begin, and stabilization prolongs the current cycle. Both CRY1 and CRY2 isoforms can repress CLOCK-BMAL1 as part of a large complex with PER proteins, but only CRY1 is able to form a late repressive complex with CLOCK-BMAL1 in the absence of PERs (Koike et al., 2012; Michael et al., 2017; Rosensweig et al., 2018; Fribourgh et al., 2020). Furthermore, CRY2, but not CRY1, promotes c-MYC degradation *via* the recruitment of FBXL3 (Huber et al., 2016). This and other accumulating evidence have shown distinct regulatory roles of CRY1 and CRY2 isoforms.

CRY dysfunction has been implicated in diseases, such as circadian sleep disorders (Hirano et al., 2016; Patke et al., 2017) and diabetes (Zhang et al., 2010; Lamia et al., 2011). Furthermore, CRY1 and CRY2 isoforms have been differentially associated with cancer and may function as pro- or anti-tumorigenic factors depending on the type of cancer (Chan and Lamia, 2020; Chan et al., 2021; Shafi et al., 2021). CRYs therefore represent attractive drug targets for the treatment of CRY-mediated and circadian clock-related diseases. Because dysfunctional CRY1 and CRY2 appear to have overlapping and distinct associations with diseases, isoform-selective CRY-targeting compounds, in addition to isoform-non-selective compounds, would facilitate the understanding of molecular mechanisms underlying CRY functions to form the basis of therapeutics. In this review, we will introduce the development of small-molecule modulators of CRY proteins by chemical biology approaches, and how structural biology (**Box 1**) has enhanced our understanding of isoform-selective mechanisms that regulate compound interactions and activity.

BOX 1 | Macromolecular structure determination.
X-ray crystallography is a technique that enables the determination of tertiary protein structures at an atomic level. To determine CRY structures, purified CRY proteins are mixed with precipitant solutions and positioned (either sitting or hanging drops) over a reservoir containing a buffer, salt, and precipitant. Vapor diffusion removes water from the protein-containing drop increasing protein concentration and reducing solubility and thus driving it into a supersaturated state where crystal nucleation can occur. Protein crystals are cryoprotected in solutions containing high concentrations of a precipitant, such as glycerol or polyethylene glycols (PEGs), mounted in cryo-loops, and flash-cooled in liquid nitrogen to produce a vitreous, frozen state that is free of crystalline ice. The cryo-loops containing protein crystals are mounted on a goniometer at a synchrotron facility and rotated in 0.1–1° increments in a monochromatic beam of hard X-rays at a specific wavelength (typically ~0.9–1.0 Å). Coherently scattered X-rays produce diffraction patterns that are recorded on a detector. The intensities of reflections are computationally processed and Fourier transforms are used to calculate structure factors that contain information regarding the amplitudes and phases of reflections (NB. Phase information is missing in the diffraction data and needs to be calculated from a protein model, isomorphous replacement with a heavy atom, or anomalous scattering from selenium incorporation or native sulfur-containing residues). Data processing produces a 3-D electron density map into which the protein and bound ligands are modeled.

IDENTIFICATION OF CRY-TARGETING COMPOUNDS

The clock gene reporters *Bmal1-dLuc* and *Per2-dLuc* enable cellular circadian rhythms to be measured (Hirota and Kay, 2015). By evaluating the effects of compound libraries on the period, phase, and amplitude of circadian rhythms *via* phenotypic screening, modulators of clock function have been identified (Miller and Hirota, 2020). Among synthetic compounds with diverse structures, a carbazole derivative, KL001 (**Figure 1**), was found as a period-lengthening compound. Development of an affinity probe based on KL001, followed by affinity purification of interacting proteins and mass spectrometry analysis, revealed that KL001 was the first-in-class CRY modulator (Hirota et al., 2012). KL001 binds to the FAD pocket of CRY (Nangle et al., 2013; Miller et al., 2021) and competes with the C-terminal tail of FBXL3 (Xing et al., 2013), resulting in stabilization and activation of CRY for period lengthening (Hirota et al., 2012; **Box 2**). Development of KL001 derivatives resulted in the identification of KL044 as a more potent compound (Lee et al., 2015), and GO044 and GO200 as period-shortening compounds (**Figure 1**; Oshima et al., 2015). In addition to clock regulation, CRYs have been shown to inhibit glucagon and glucocorticoid-dependent induction of gluconeogenesis in the liver (Zhang et al., 2010; Lamia et al., 2011). Consistent with these findings, KL001 treatment of primary hepatocytes inhibits glucagon-dependent induction of gluconeogenesis (Hirota et al., 2012), and the orally available KL001 derivatives, compound 41 and compound 50 (**Figure 1**), have been shown to improve glucose tolerance and fasting blood glucose levels in diet-induced obese and diabetic mice (Humphries

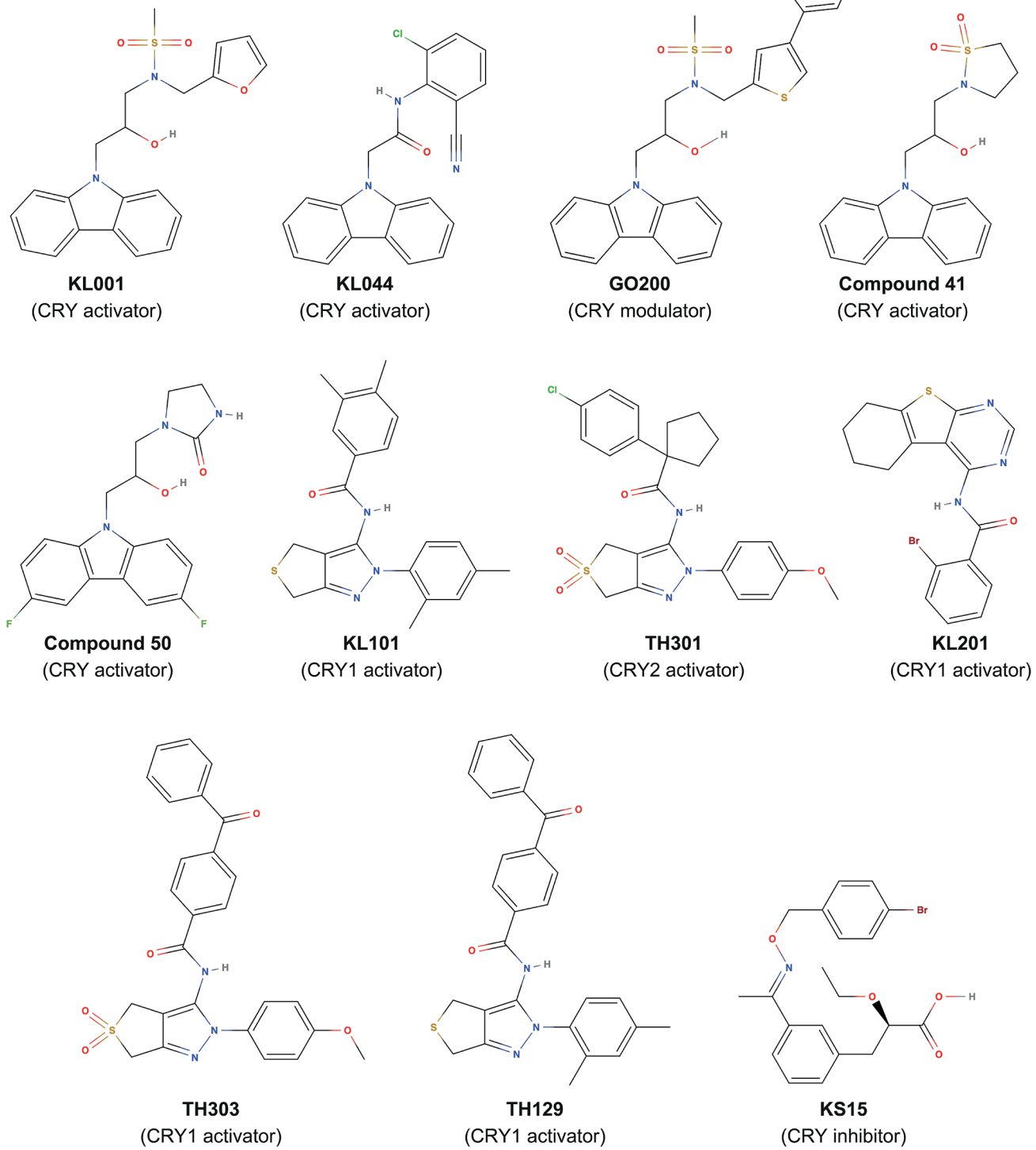

FIGURE 1 | Small-molecule compounds that target CRY proteins.

et al., 2016, 2018). CRY-targeting compounds therefore have therapeutic potential in the prevention of hyperglycemia. KL001 and its derivative are also efficacious in the treatment of glioblastoma, a malignant brain cancer, by reducing proliferation of patient-derived glioblastoma stem cells (Dong et al., 2019).

Despite KL001 being an effective tool for CRY activation, it binds to CRY1 and CRY2 without preference, in other words, isoform non-selective, making it difficult to obtain insights into distinct functions of CRY isoforms. Very high sequence identity in the FAD pockets (see section "Structure of Mammalian CRYs") in CRY1 and CRY2 has hindered the design and development of isoform-selective compounds. Analyses of period-lengthening compounds identified from phenotypic screens led to a breakthrough with the first discovery of CRY isoform-selective activators KL101 and TH301 (phenylpyrazole derivatives; **Figure 1**), which stabilize CRY1 and CRY2, respectively (Miller et al., 2020b; **Box 2**). Used as tools to activate CRY1 and CRY2, KL101 and TH301 helped to identify CRY-mediated activation of brown adipocyte differentiation (Miller et al., 2020b). Furthermore, a similar approach identified other CRY1 isoform-selective activators, the thienopyrimidine derivative KL201 (Miller et al., 2020a), and benzophenone derivatives TH303 and TH129 (Kolarski et al., 2021a), which together provide molecular tools to investigate CRY1 functions (**Figure 1**).

In addition to the activators, a CRY inhibitor was identified. The 2-ethoxypropanoic acid derivative KS15 (**Figure 1**) has been reported to target both CRY1 and CRY2 and block CRY-dependent inhibition of CLOCK-BMAL1 (Chun et al., 2014; Jang et al., 2018). KS15 has anti-proliferative effects on breast cancer MCF-7 cells by increasing *p53* and *Bax* gene expression (Chun et al., 2015). Considering that CRY1 and CRY2 have been associated with p53 suppression or degradation in other cancers (Chan et al., 2021; Jia et al., 2021) and that *Cry1* and *Cry2* knockout in *p53* mutant mice reduces cancer risk (Ozturk et al., 2009), KS15, or its derivatives (Jeong et al., 2021) may be efficacious in the treatment of several cancers, in addition to MCF-7 breast cancer. Together, these results identified CRYs as promising targets of small-molecule compounds.

BOX 2 | Period regulation by CRY-targeting compounds. Deletion of *Cry1* and *Cry2* genes results in period shortening and lengthening, respectively (van der Horst et al., 1999; Liu et al., 2007), suggesting opposite roles of these genes in period regulation. In contrast, CRY-activating compounds KL001, KL044, KL101, TH301, KL201, TH303, and TH129 cause period lengthening irrespective of their isoform selectivity (Hirota et al., 2012; Lee et al., 2015; Miller et al., 2020a,b; Kolarski et al., 2021a). Compound treatment in combination with mathematical modeling provided insights into the roles of CRY1 and CRY2 in period regulation (Hirota et al., 2012). FBXL3 is localized in the nucleus and degrades nuclear CRY1 and CRY2. Therefore, inhibition of FBXL3-dependent degradation by compounds results in nuclear accumulation of CRYs. Mathematical modeling predicted that stabilization of both nuclear CRY1 and CRY2 causes period lengthening. Consistent with this prediction, KL001 causes period lengthening in both *Cry1* and *Cry2* knockout cells (i.e., in the presence of only CRY2 or CRY1), and CRY1 or CRY2 isoform-selective compounds lengthen the period. Then, why do *Cry1* and *Cry2* knockouts result in opposite period change? Mathematical modeling suggested that the stronger repressor CRY1 and the weaker repressor CRY2 compete for a rate-limiting PER interaction in the cytosol to form a complex for nuclear localization. This competition determines the balance of CRY1 and CRY2 nuclear translocation, resulting in apparently opposite phenotypes of *Cry1* and *Cry2* knockouts: the weaker repressor CRY2 causes shorter period, and the stronger repressor CRY1 causes longer period. Compound treatment bypasses this rate-limiting step and directly stabilizes nuclear CRY1 and CRY2, resulting in period lengthening. Therefore, CRY1 and CRY2 have qualitatively similar roles in period regulation, while their potency is different. The molecular mechanisms of period shortening by GO200 (Oshima et al., 2015) and amplitude reduction by KS15 (Chun et al., 2014) are not clear and need further investigation.

STRUCTURE OF MAMMALIAN CRYs

Cryptochromes belong to a photolyase/cryptochrome family of FAD-binding proteins, and the photolyase homology region (PHR) in mammalian CRYs (**Figure 2A**) is structurally related to photolyases. The PHR comprises several subdomains: An N-terminal α/β domain and a C-terminal α-helical domain, connected by an extended linker region (**Figure 2B**; Czarna et al., 2013; Xing et al., 2013; Miller et al., 2020b). Important structural and regulatory features in the PHR include: (1) The FAD pocket in mouse CRYs shares very high sequence identity between CRY isoforms. It has an open form that is accessible to molecules, rather than the closed form in photolyases that traps FAD in the pocket, and therefore can bind FAD, ligands, or the C-terminal tail of FBXL3 even after protein folding (Czarna et al., 2013; Xing et al., 2013; Miller et al., 2021); (2) the lid loop is juxtaposed to the FAD pocket and frequently interacts with residues in the FAD pocket in CRY crystal structures (Miller et al., 2021). Complex formation of CRY with PER proteins results in the restructuring of the loop and pocket residues (Nangle et al., 2014; Schmalen et al., 2014); (3) the P-loop can regulate circadian rhythms *via* phosphorylation (Ode et al., 2017), although the structural mechanisms are unknown due to flexibility and disorder of this loop; and (4) the secondary pocket binds to the PAS-B domain of CLOCK (Michael et al., 2017; Rosensweig et al., 2018). Several variant residues between CRY1 and CRY2 are located in the vicinity of the secondary pocket loop, resulting in a non-structured loop in CRY1, which facilitates the tighter binding of CRY1 to CLOCK PAS-B (Fribourgh et al., 2020). This enables the formation of a late repressive complex with CLOCK in the absence of PER (Koike et al., 2012).

In addition to the PHR domain, CRYs also contain a CRY C-terminal tail (CCT), which displays considerable sequence divergence between CRY1 and CRY2. A study utilizing cell-based

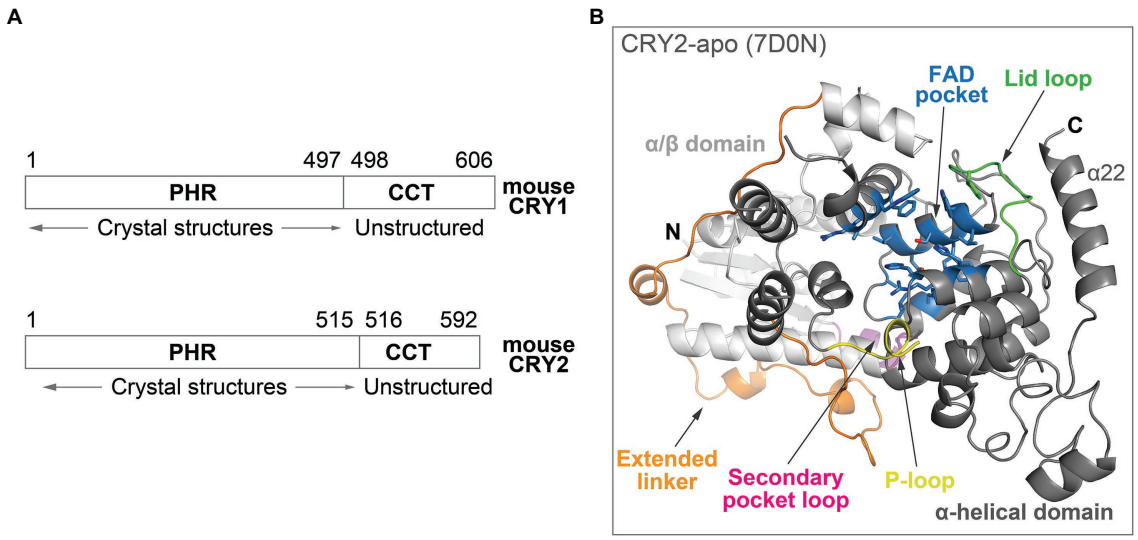

FIGURE 2 | Overall structure of mouse CRY. **(A)** Domain organization of mouse CRY1 and CRY2. The PHRs are highly ordered with ~80% sequence identity between CRY1 and CRY2, whereas the CCTs have divergent sequences, variable lengths and are predicted to be structurally disordered. **(B)** The crystal structure of the PHR domain of CRY2-apo (PDB ID: 7D0N). The N-terminal α/β domain (white) and C-terminal α-helical domain (gray) are connected by an extended linker (orange). Important structural and regulatory features include: The FAD pocket (blue), lid loop (green), P-loop (yellow), and secondary pocket loop (magenta).

CRY genetic rescue assays showed that the CCT is dispensable for rhythm generation, but regulates period length and rhythm amplitude (Khan et al., 2012). CCTs are mostly unstructured in secondary structure predictions, making their structural characterization challenging (**Figure 2A**; Parico and Partch, 2020). In contrast, the CCT in *Drosophila* CRY (dCRY) is much shorter than in mammalian CRYs, and its crystal structure has been determined (Czarna et al., 2013; Levy et al., 2013). dCRY CCT interacts with the lid loop and also directly with two key FAD pocket residues: H378 (equivalent to mouse CRY1 H355 and CRY2 H373) and W422 (equivalent to CRY1 W399 and CRY2 W417). H378 forms a bridge between the CCT and the FAD molecule, and both the protonation state and conformation of this His residue regulate CCT docking and undocking (Ganguly et al., 2016; Chandrasekaran et al., 2021). However, no mammalian apo form structures containing the CCT are available, so structural mechanisms of interactions among the FAD pocket, lid loop, and CCT are currently unknown. The region encoded by exon 11 of CRY1 in the CCT interacts with the PHR and regulates its affinity for CLOCK-BMAL1 (Parico et al., 2020). The CCT appears to be highly dynamic in nature and may regulate CRY activity by binding to different regions of the PHR to control circadian timing.

MOLECULAR INTERACTIONS OF CRYs WITH COMPOUNDS

The design of isoform-selective compounds has been hindered by almost identical sequences in the FAD pockets of CRY1 and CRY2 and their highly similar overall PHR structures. Recent crystal structures of mouse CRY1-PG4 [PDB: 7D0M; apo-like structure with a weakly bound cryoprotectant tetraethylene glycol (PG4) in the FAD pocket] and CRY2-apo (PDB: 7D0N), however, have provided insights into intrinsic structural differences in their FAD pockets and lid loops (Miller et al., 2021). The FAD pocket is composed of three subregions: (1) Hydrophobic region 1 consisting of mouse CRY1 residues W292, F296, W399, and L400 (W310, F314, W417, and L418 in CRY2); (2) Affinity region: CRY1 residues Q289, H355, H359, and S396 (Q307, H373, H377, and S414 in CRY2); and (3) Hydrophobic region 2: CRY1 residues R358, A362, F381, L385, A388, I392, and W397 (R376, A380, F399, L403, A406, V410, and W415 in CRY2; **Figures 3, 4**). These 15 residues represent residues that most often interact with compounds, and CRY1 S396/CRY2 S414 in the affinity region form a canonical hydrogen bond in all compound structures. Superposition of CRY1-apo (PDB: 6KX4), CRY1-PG4, and CRY2-apo shows how fully conserved residues adopt different conformations (Miller et al., 2021; **Figure 4**). Most notable are CRY1 W399 "out" and corresponding CRY2 W417 "in" conformations (these Trp residues are referred to as the gatekeeper due to interactions or steric clashes with compound functional groups depending on their "out" or "in" orientations; see below); CRY1 H355 corresponding to CRY2 H373; and lid loop residues CRY1 F406-Q407 and CRY2 F424-Q425.

X-ray crystal structures represent the gold standard for elucidating molecular conformations and interactions, but their structures are predominantly static and can have conformational restraints imposed by crystal packing (interactions between protein molecules that form the crystal lattice). It is therefore important to evaluate the integrity of protein structures with supporting methods and analyses. Molecular dynamics (MD) simulations computationally analyze energetically favorable conformations in dynamic structures. Comparisons of X-ray

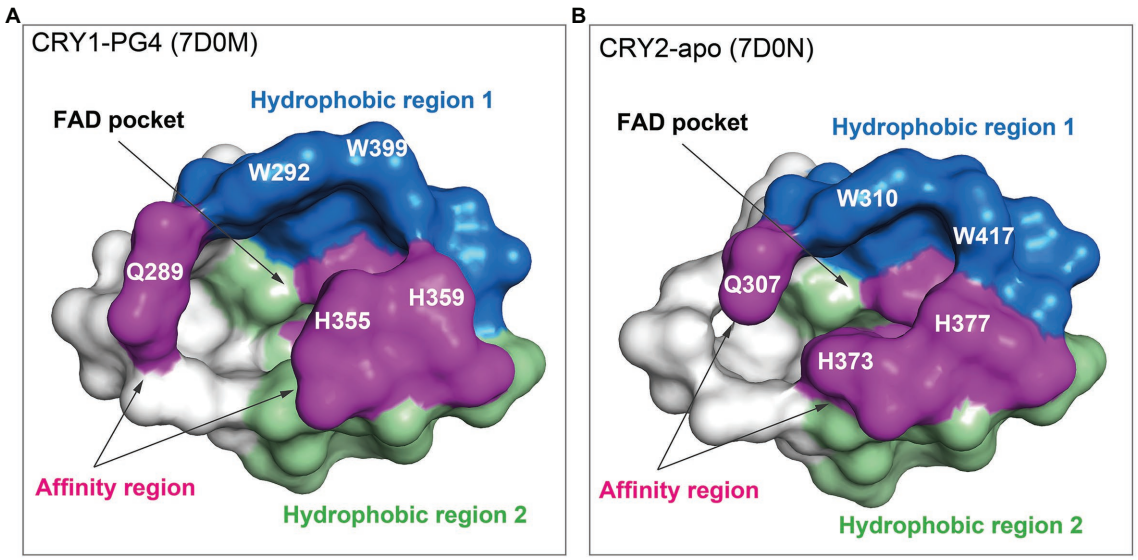

FIGURE 3 | FAD pocket surfaces in CRY1 and CRY2. The FAD pocket can be divided into three subregions: Hydrophobic region 1 (blue), the affinity region (magenta), and hydrophobic region 2 (green). Pockets are shown for CRY1-PG4 [the PG4 molecule (not shown) was present in the crystallization buffer and bound non-specifically in the FAD pocket; PDB ID: 7D0M] **(A)** and CRY2-apo (7D0N) **(B)**.

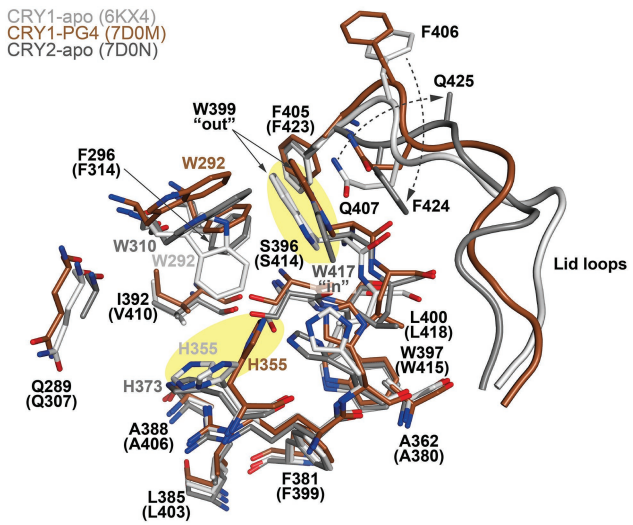

FIGURE 4 | Conserved residues in the FAD pockets and lid loops of CRY1 and CRY2 intrinsically adopt distinct conformations. Superposition of CRY1-apo (PDB ID: 6KX4; white), CRY1-PG4 (7D0M; brown; PG4 molecule not shown) and CRY2-apo (7D0N, gray) shows heterogeneity in the conformations of the FAD pocket residues CRY1 H355 and W399 (yellow shading), and the lid loop residues F406 and Q407 (CRY2 H373, W417, F424, and Q425, respectively). The opposite conformations of the CRY1 W399 and CRY2 W417 gatekeepers form differential interactions with reversed lid loop conformations (indicated by dotted arrows). CRY1 W292 displays some flexibility in CRY1 structures and occupied a "down" conformation in CRY1-apo (6KX4), but is repositioned to accommodate compounds (**Figures 5**–**7**).

crystal structures and MD simulations have shown agreement in the differential conformations of the gatekeepers and lid loops in CRY1 and CRY2, supporting that intrinsic conformational differences occur despite the high sequence similarity (Miller et al., 2021). In contrast, CRY1 H355/CRY2 H373 is more dynamic in CRY apo structures and did not show isoform-dependent differences. In the intrinsic "out" conformation in CRY1, the gatekeeper W399 interacts with Q407 in the lid loop, which helps to rigidify the N-terminal portion of the lid loop in combination with the canonical insertion of F405 into the auxiliary pocket located just behind hydrophobic region 1. In CRY2, F423 inserts into the auxiliary pocket in an equivalent manner to CRY1 F405, but the gatekeeper W417 adopts an "in" conformation and interacts with F424 in the lid loop. This stabilizes the N-terminal region of the loop in a different conformation to CRY1 and results in the lid loop residues CRY1 F406-Q407 and CRY2 F424-Q425 rotating ~180° relative to each other (Miller et al., 2021; **Figure 4**).

The intrinsic difference of the gatekeeper orientation explains well the isoform selectivity of KL101 and TH301. The phenylpyrazole groups of KL101 and TH301 occupied hydrophobic region 2 of the FAD pocket, although their binding positions were slightly offset due to substituent functional groups: a dimethylphenyl and a methoxyphenyl in KL101 and TH301, respectively, which formed distinct interactions with residue W397 in CRY1 and corresponding W415 in CRY2 (**Figures 5A**,**B**; Miller et al., 2020b). KL101 contains an upper dimethylphenyl that inserted into hydrophobic region 1 of CRY1 (PDB: 6KX6), and the steric bulk of the dimethylphenyl induced a slight outward push of the gatekeeper W399. Importantly, W399 retained the intrinsic "out" conformation observed in CRY1-PG4 and CRY1-apo structures (**Figures 4, 5A**). H355 also adopted a similar conformation to CRY1-apo and CRY1-PG4. In contrast, a notable conformational change occurred upon the binding

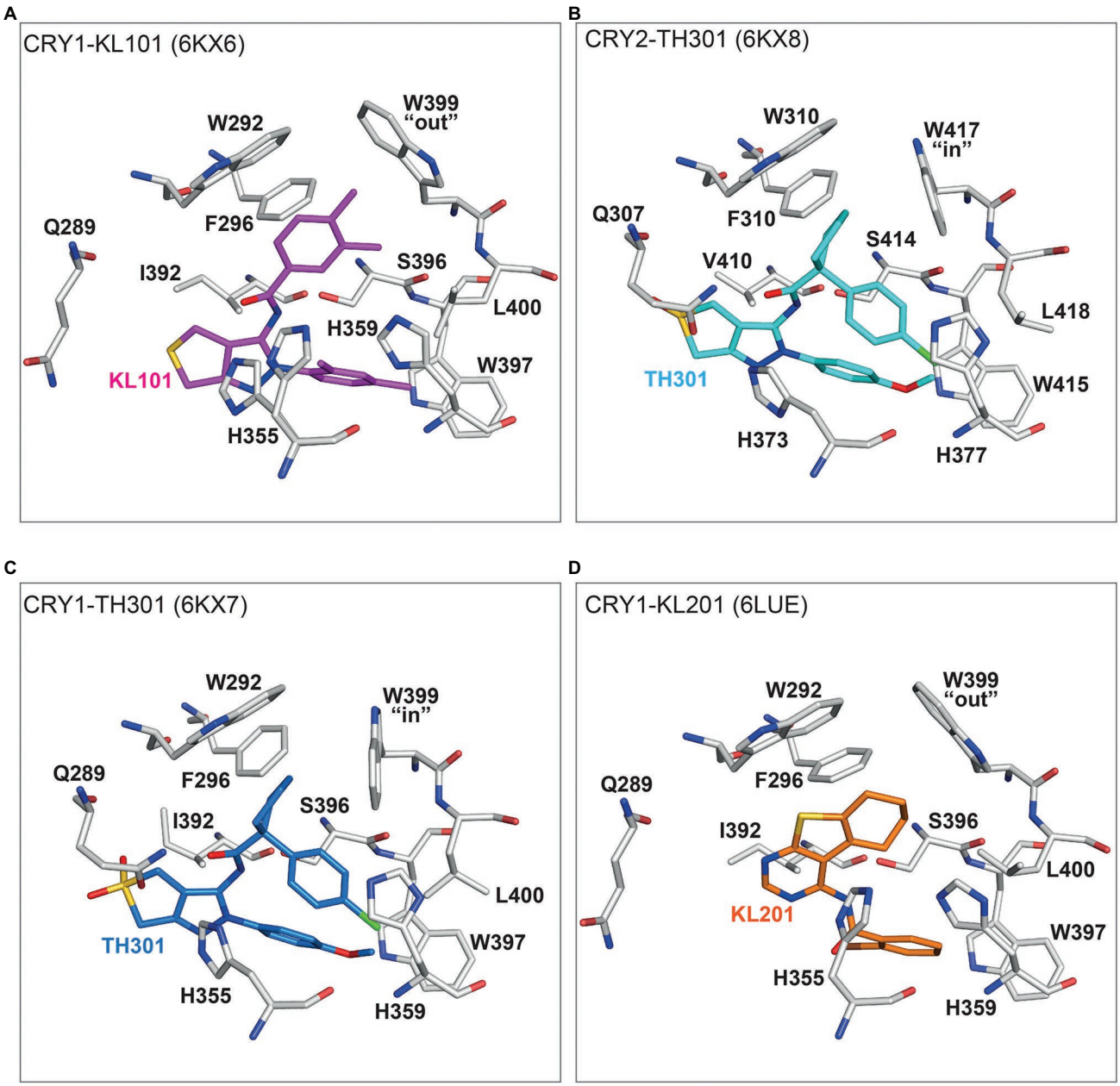

FIGURE 5 | Binding modes of isoform-selective compounds in CRY1 and CRY2. **(A)** Crystal structure of CRY1-KL101 (PDB ID: 6KX6). Key interacting residues (white) and the KL101 compound (magenta) are shown. The upper dimethylphenyl located in hydrophobic region 1 and fitted to an intrinsically "out" gatekeeper W399 conformation. The lower dimethylphenyl located in hydrophobic region 2 and formed hydrophobic interactions with W397. H355 was not restrained by KL101 binding and transiently adopted the same conformation observed in CRY1-PG4 (**Figure 4**) and CRY1-KL201 **(D)**. **(B)** Crystal structure of CRY2-TH301 (6KX8). The cyclopentyl group of TH301 (cyan) binds in hydrophobic region 1 and forms a stacking interaction with the intrinsically "in" conformation of gatekeeper W417 (white). The methoxyphenyl group (in hydrophobic region 2) forms a hydrogen bond with W415. H373 adopted the same conformation as in CRY2-apo (**Figure 4**). **(C)** The binding of TH301 (blue) to CRY1 (white) induces a conformational change in CRY1-TH301 (6KX7). The small steric bulk of the cyclopentyl of TH301 induced a stacking interaction with the gatekeeper W399, which rotated from an intrinsic "out" conformation to an "in" conformation. The conformational freedom of H355 is restricted by the methoxyphenyl group, preventing H355 adopting a conformation observed in CRY1-KL101 **(A)** and CRY1-PG4 (**Figure 4**). **(D)** A unique binding mode of KL201 (orange) to CRY1 (white) in CRY1-KL201 (6LUE). The fused tricyclic thienopyrimidine and cyclohexyl moiety of KL201 binds to hydrophobic region 1 of the FAD pocket. In this configuration, the compound fits to an intrinsically "out" gatekeeper W399 conformation. H355 adopted a stable conformation similar to CRY1-PG4 (**Figure 4**) and CRY1-KL101 **(A)**, but distinct from CRY1-TH301 **(C)**. The bromophenyl group (essential for compound effect) located in hydrophobic region 2.

of TH301 to CRY1 (PDB: 6KX7; Miller et al., 2020b). The gatekeeper rotated to an "in" position to form a stacking interaction with the cyclopentyl group (**Figure 5C**). The binding mode of TH301 in CRY2 (PDB: 6KX8) was almost identical

to that in the CRY1-TH301 complex, but critically the CRY2 gatekeeper W417 required no conformational change, compared to CRY2-apo, to form a favorable stacking interaction with the cyclopentyl (**Figure 5B**). Furthermore, H355 in CRY1-TH301 adopted a different conformation to CRY1-PG4 and CRY1-KL101, whereas corresponding H373 in CRY2-TH301 retained the same conformation as in CRY2-apo (**Figures 4, 5A,B**). Overall, the results showed that KL101 and TH301 can bind to the intrinsic conformations of the FAD pockets in CRY1 and CRY2, respectively, accounting for their isoform-selective properties. In contrast, TH301 (selective to CRY2) induced large conformational changes in the FAD pocket of CRY1, indicative of less favorable interactions. Structure-guided point mutations in the lid loop residues CRY1 Q407 and CRY2 F424, which differentially interact with the CRY1 W399 and CRY2 W417 gatekeepers, resulted in inversed responses to KL101 and TH301 (Miller et al., 2021), supporting that differential intrinsic gatekeeper-lid loop conformations in CRY isoforms contribute to compound selectivity.

Structure activity relationships (SARs) of KL101 and TH301 derivatives provided insights into the functional groups required for optimal compound effects (Miller et al., 2020b). The *meta*-methyl group (interacted with the gatekeeper in hydrophobic region 1) and the *para*-methyl group of KL101 (interacted with hydrophobic residues in hydrophobic region 2) were important for compound effect, and their removal or substitution resulted in severely reduced activity. In TH301, the removal and substitution of the methoxy group (interacted with W397 in hydrophobic region 2) caused inactivity and reduced activity, respectively, and both the cyclopentyl and chlorophenyl groups were essential for activity.

The structure of CRY1 in complex with KL201 (PDB: 6LUE) revealed a unique interaction with a thienopyrimidine scaffold (Miller et al., 2020a). Compared to KL001 and KL044 (see below), KL201 appears to bind upside down in the FAD pocket with the fused tricyclic thienopyrimidine and cyclohexyl moiety occupying hydrophobic region 1, instead of hydrophobic region 2 (**Figure 5D**). In this orientation, KL201 fits to a gatekeeper "out" conformation. The exact mechanisms of CRY1 selectivity are not clear, but the thienopyrimidine scaffold has greater steric bulk than the functional groups of KL101 and TH301 that occupy hydrophobic region 1, and might cause a steric clash with the CRY2 gatekeeper "in" conformation or W310 (equivalent to CRY1 W292), which is closer to the center of the FAD pocket in CRY2. Another mechanism potentially regulating selectivity is the conformation of H355 in CRY1-KL201, which is very similar to CRY1-PG4 and CRY1-KL101, but different to CRY2-apo (**Figures 4, 5D**). SAR analyses of KL201 derivatives identified an essential role of the bromophenyl group (interacted with residues in hydrophobic region 2) in compound activity and a size-dependent effect of the cyclohexyl (interacted with W292 and W399; Miller et al., 2020a). Together, the crystal structures of CRY–compound complexes identified a mechanism whereby compounds compatible with intrinsic FAD pocket conformations of CRY1 and CRY2 show preferential binding. However, it is important to note that these compounds are only marginally selective in the context of PHR-only CRY constructs and require the CCT for full selectivity (Miller et al., 2020b, 2021; see below).

In contrast, TH303 and TH129 are selective to CRY1 PHR constructs missing the CCT (Kolarski et al., 2021a). TH303 and TH129 contained the same phenylpyrazole scaffold as KL101, but attached to a benzophenone substituent that inserted into hydrophobic region 1 in CRY1-TH303 (PDB: 7D1C) and CRY1-TH129 (PDB: 7D19) structures. In order to avoid a clash with the large steric bulk of the benzophenone moiety, the gatekeeper W399 underwent a considerable and unique conformational change where it inserted into the auxiliary pocket causing the ejection of F405 (**Figures 6A**,**B**). The removal of F405 from this pocket induced rearrangement of the lid loop and facilitated an interaction of F409 with the benzophenone. The benzoyl group in the benzophenone moiety of TH129 was critical for activity, and its substitution resulted in dramatically reduced period-lengthening activity. Furthermore, TH303 and TH129, but not KL101, showed reduced effects on a CRY1 F409A lid loop mutant (Kolarski et al., 2021a), supporting an interaction of the lid loop with the benzophenone. Superposition of TH303 and TH129 with CRY2-apo shows how a more severe steric clash would occur between the benzophenone and the gatekeeper W417 (**Figure 6C**). Moreover, the intrinsic "in" conformation of the gatekeeper in CRY2 would require a much larger conformational change to insert into the auxiliary pocket, which is likely to be energetically unfavorable. Taken together, TH303 and TH129 may impart isoform selectivity *via* large conformational changes to the gatekeeper and lid loop in CRY1.

Crystal structures of the non-isoform-selective compound KL001 in complexes with CRY1 (PDB: 7DLI; Miller et al., 2021) and CRY2 (PDB: 4MLP; Nangle et al., 2013) revealed how this compound can bind to the FAD pocket of both CRY1 and CRY2 with minimal changes to intrinsic FAD pocket residues. The carbazole moiety and hydroxypropyl linker interacted with hydrophobic region 2 and the affinity region, respectively, and formed very similar binding modes in CRY1 and CRY2. In contrast, the more flexible methanesulfonamide and furan groups showed different binding modes in hydrophobic region 1 (**Figures 7A**,**B**). Importantly, the distinct "out" and "in" gatekeeper conformations of CRY1 and CRY2, respectively, can accommodate KL001, suggesting a mechanism for its isoform non-selective properties. A more potent derivative KL044 (Lee et al., 2015) formed strong interactions with FAD pocket residues in complex with CRY1 (Miller et al., 2020b; **Figure 7C**). Similar to KL001, the carbazole group in KL044 stably interacted with hydrophobic region 2. A chlorobenzonitrile moiety inserted into a hydrophobic annulus in hydrophobic region 1 interacting with W292, F296, and the gatekeeper W399 and also formed a CH–Cl interaction with L400. An amide linker connecting the bottom carbazole and top chlorobenzonitrile moieties formed a hydrogen bond with H359, in addition to a canonical interaction with S396. These interactions resulted in an optimized binding orientation of KL044 in the FAD pocket, accounting for its higher potency. The binding mode of KL044 in CRY2 has not been determined, but the small steric bulk of the chlorobenzonitrile may be able

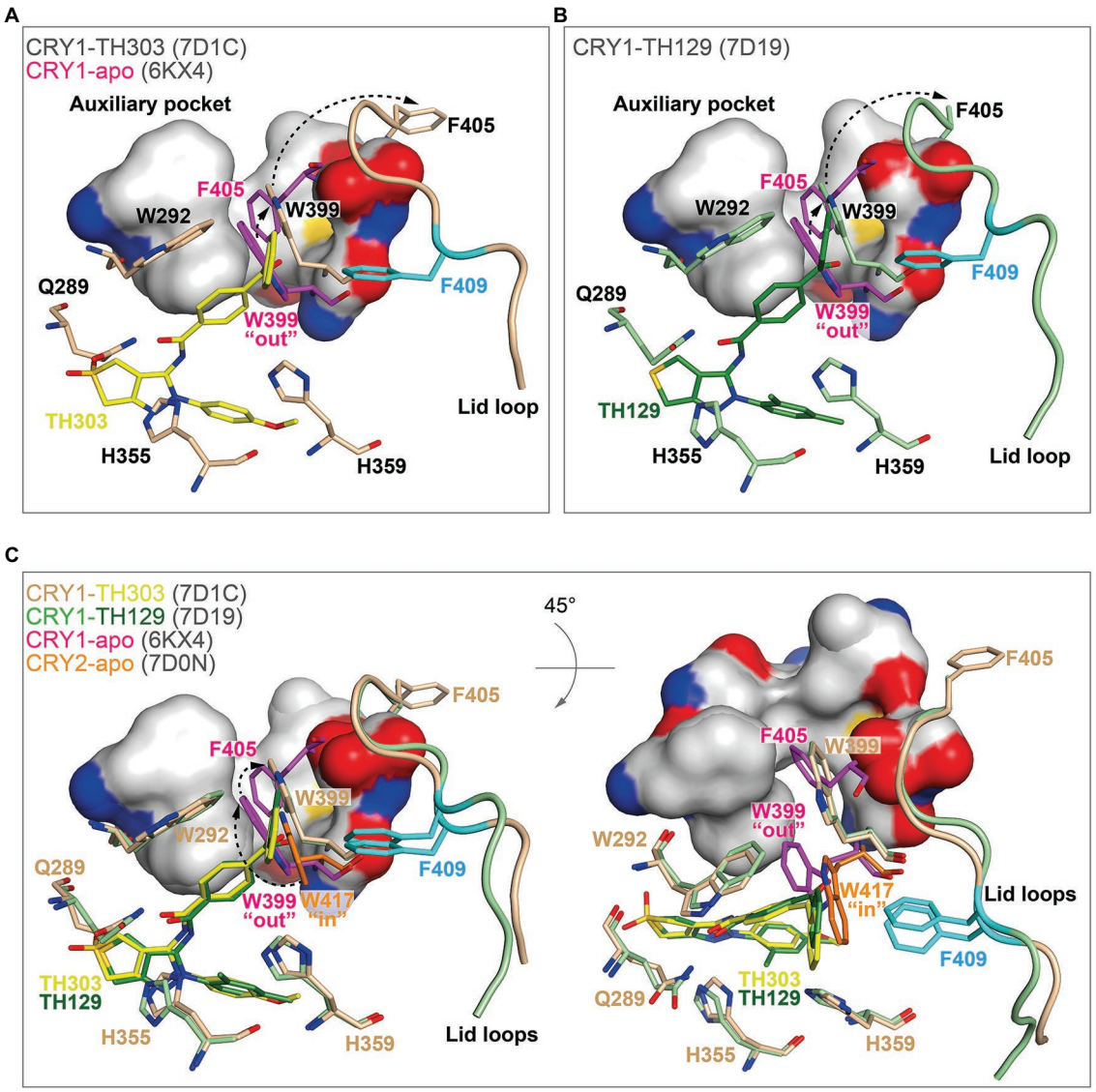

FIGURE 6 | The binding of benzophenone derivatives to CRY1 induced gatekeeper and lid loop reordering. **(A,B)** The benzophenone moieties of TH303 (**A**, yellow, 7D1C) and TH129 (**B**, green, 7D19) form a severe steric clash with the intrinsic gatekeeper W399 "out" conformation of CRY1-apo (6KX4; magenta). Repositioning of W399 (beige and light green) into the auxiliary pocket (small arrow) induced steric overlap with F405 (intrinsically inserts into the auxiliary pocket), which relocated to a distal position (large arrow) causing rearrangement of the lid loop. The lid loop residue F409 (cyan) was repositioned to form a stacking interaction with the benzophenone. The surface is shown for the auxiliary pocket residues F295, F296, A299, F306, I314, M398, and S404. **(C)** Conformational isomerism of CRY1 and CRY2 gatekeepers may regulate TH303 and TH129 selectivity. Superposition of CRY2-apo (7D0N) gatekeeper W417 onto CRY1 crystal structures. The intrinsic CRY2 gatekeeper W417 "in" conformation would likely require two conformational changes to enter the auxiliary pocket (dotted arrows, left panel). A rotated view (45° around the X-axis) shows the large conformational change required for the gatekeeper to enter the auxiliary pocket (right panel), making it less energetically favorable in CRY2.

to avoid a steric clash with the intrinsic "in" conformation of the gatekeeper W417.

Overall, crystal structures of CRYs in complexes with small-molecule activators have provided structural insights into their mechanisms of action. In contrast, there is currently no structural information available for the mechanisms by which CRY inhibitors, for example, KS15, exhibit their effects. Such structures could facilitate the development of new inhibitor derivatives, providing new tools for CRY regulation.

ROLE OF CCTs IN ISOFORM SELECTIVITY

Although structural information of the CCT is not available because of its disordered nature, functional assays have provided insights into the role of the CCT, in combination with the lid loop and gatekeeper, in compound selectivity. Chimeric CRY1 and CRY2 proteins containing their respective PHRs but with segment-swapped CCTs from their opposite isoforms

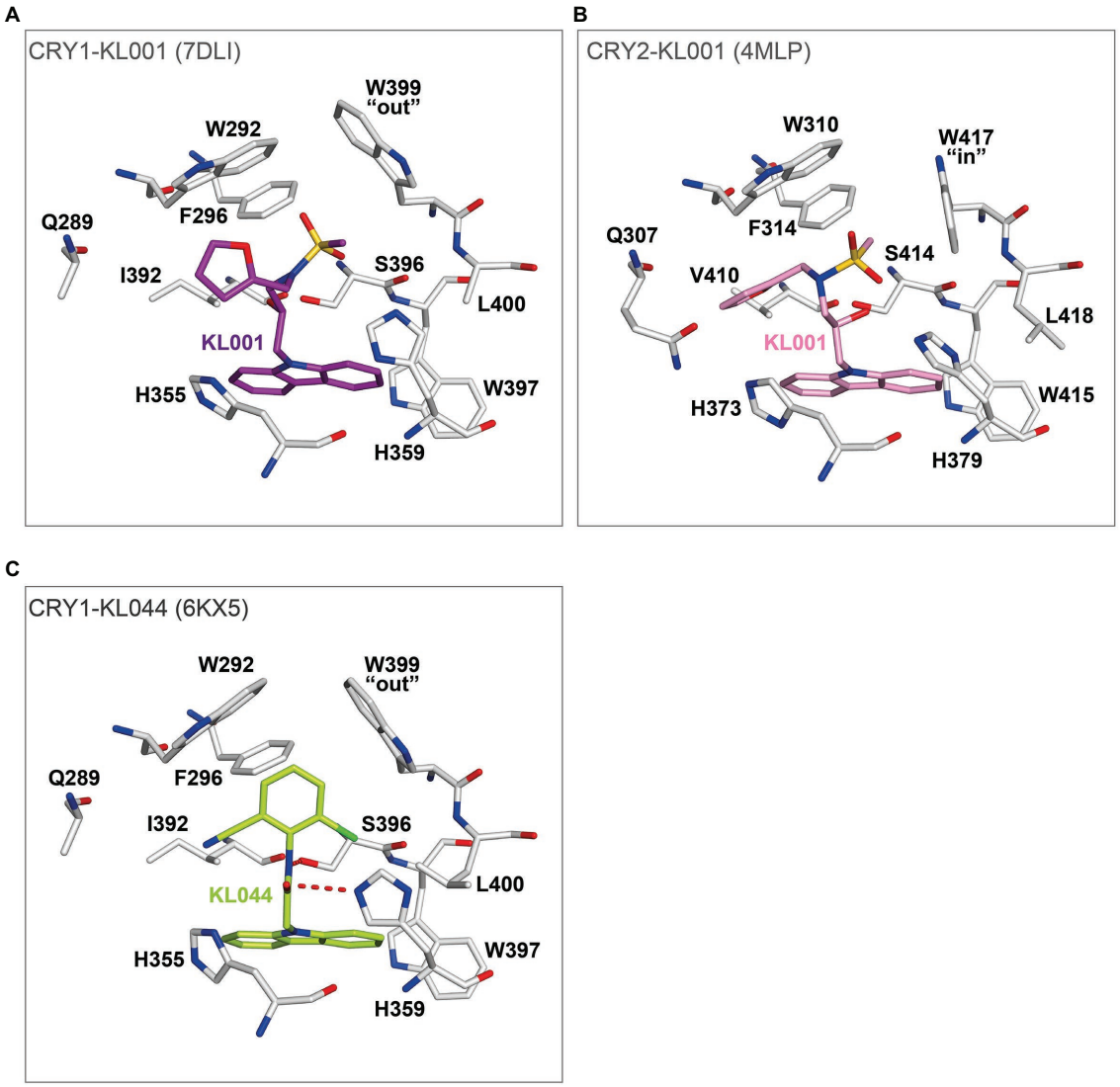

FIGURE 7 | The binding modes of isoform non-selective CRY compounds. **(A)** Crystal structure of CRY1-KL001 (7DLI). The carbazole and hydroxypropyl linker of KL001 (purple) stably interacted with hydrophobic region 2 and the affinity region of CRY1 (white), respectively. In contrast, the methanesulfonamide and furan groups have greater flexibility and located in hydrophobic region 1 with an intrinsic gatekeeper W399 "out" conformation. **(B)** Crystal structure of CRY2-KL001 (4MLP). KL001 (pink) can bind to intrinsic FAD pocket conformations, including gatekeeper W417 "in" of CRY2 (white). **(C)** The potent CRY activator KL044 formed stable hydrogen bonds with the FAD pocket. The amide linker in KL044 (lime green) formed a hydrogen bond with H359 in addition to a canonical hydrogen bond with S396. The chlorobenzonitrile group formed hydrophobic interactions with W292, F296, and W399, and a CH–Cl interaction with L400 in hydrophobic region 1. Furthermore, the intrinsic gatekeeper W399 "out" conformation could accommodate the chlorobenzonitrile. The carbazole interacted with multiple residues in hydrophobic region 2. Together, KL044 had an optimized binding mode.

are non-responsive to KL101 and TH301 (Miller et al., 2020b), demonstrating that CCT interactions with their respective PHRs differ between CRY isoforms. In contrast, CCT-swapping in combination with lid loop mutations (CRY1 Q407A and CRY2 F424A) can influence compound selectivity (Miller et al., 2021), suggesting their interplay. Segment swapping and truncations of CRY CCTs have determined that the region encoded by exon 10 is required for the effects of KL101 and TH301 (Miller et al., 2020b). Interestingly, unlike in mammalian cells, KL101 and TH301 did not change the circadian period in zebrafish, possibly due to markedly different sequences in the exon 10 region of zebrafish CRY1 and CRY2, compared to mammalian CRYs (Iida et al., 2021). This observation indicates a species-dependent difference in the effects of KL101 and TH301 and supports an important role of the exon 10 region in their effects. Furthermore, a hydrophobic patch (Phe-Met-Gly-Tyr) in human CRY1 exon 10 is reported to interact with the PHR by nuclear magnetic resonance spectroscopy (Parico et al., 2020). Together, these findings suggest a mechanism where the CCT might impart compound selectivity by interacting with residues in the FAD pocket and lid loop, and/or directly with compounds. It is possible that divergent CCTs in CRY1

and CRY2 bind to and stabilize intrinsically different FAD pocket and lid loop conformations observed in CRY PHR structures or that CCT binding induces conformational rearrangement of key pocket residues and the lid loop. However, specific mechanisms remain elusive, and crystal structures of full-length CRY proteins with and without bound compounds will be pivotal in the future elucidation of these mechanisms.

CONCLUDING REMARKS

Intrinsic differences in CRY1 and CRY2 structures have shed light on the isoform-selective binding of compounds. The differential conformations of FAD pocket residues are analogous to a lock, and compounds that show compatible binding to these conformations are analogous to a key. Further interactions among the FAD pocket, lid loop, and CCT appear to stabilize distinct conformations and impart isoform selectivity. Compounds with opposite selectivity that require conformational changes to intrinsic pocket residues, most likely incur steric hindrance to pocket entry or less energetically favorable interactions. Despite these advances in our understanding of CRY regulation by small-molecule compounds, there are many mechanisms yet to be structurally determined. In particular, molecular interactions between the PHR and CCT governing isoform selectivity, and intrinsic differences in highly conserved PHR residues that regulate the binding of PHR isoform-selective compounds, such as TH303 and TH129, still require elucidation. Furthermore, the regulatory mechanisms of period-shortening compounds, such as GO200, are poorly understood. Future research should determine these critical regulatory mechanisms. The understanding of compound-binding modes will facilitate the derivatization and development of new compounds with optimal binding properties. The development of improved isoform-specific CRY modulators could provide new insights into distinct CRY1 and CRY2 functions, as well as more efficacious treatment of clock-related diseases due to the activation or inhibition of only one CRY isoform. In addition, derivatization of CRY1-selective TH129 by substituting the benzophenone with an azobenzene moiety has enabled reversible regulation of CRY1 function with light (Kolarski et al., 2021a). This shows promise in the field of circadian photopharmacology, where visible light may be used to regulate circadian rhythms by targeting only specific tissues and cells (Kolarski et al., 2019, 2021b). In summary, chemical and structural biology have furthered our understanding of how evolutionarily conserved CRY isoforms can be selectively targeted by small-molecule compounds and provided a rationale for new compound development to optimize interactions with the conformational isomerism present in CRY1 and CRY2 structures.

AUTHOR CONTRIBUTIONS

SM and TH wrote this review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Signaling Mechanisms by Arabidopsis Cryptochromes

*Jathish Ponnu and Ute Hoecker**

Institute for Plant Sciences and Cluster of Excellence on Plant Sciences (CEPLAS), Biocenter, University of Cologne, Cologne, Germany

Cryptochromes (CRYs) are blue light photoreceptors that regulate growth, development, and metabolism in plants. In *Arabidopsis thaliana* (Arabidopsis), CRY1 and CRY2 possess partially redundant and overlapping functions. Upon exposure to blue light, the monomeric inactive CRYs undergo phosphorylation and oligomerization, which are crucial to CRY function. Both the N- and C-terminal domains of CRYs participate in light-induced interaction with multiple signaling proteins. These include the COP1/SPA E3 ubiquitin ligase, several transcription factors, hormone signaling intermediates and proteins involved in chromatin-remodeling and RNA N6 adenosine methylation. In this review, we discuss the mechanisms of Arabidopsis CRY signaling in photomorphogenesis and the recent breakthroughs in Arabidopsis CRY research.

Keywords: cryptochromes, CRY1, CRY2, Arabidopsis, blue light, signal transduction, photomorphogenesis

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Margaret Ahmad,
Université Paris-Sorbonne, France

Reviewed by:
Giorgio Perrella,
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Scott Hayes,
Wageningen University and Research, Netherlands

***Correspondence:**
Ute Hoecker
hoeckeru@uni-koeln.de
orcid.org/0000-0002-5636-9777

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INTRODUCTION

As autotrophic organisms, plants use light as their source of energy through photosynthesis. Light is therefore of uttermost importance to plants and other photosynthesizing organisms. Plants evolved intricate mechanisms to adjust growth, development, and metabolism to the ambient light environment, which varies considerably throughout the days and seasons. Such adjustment optimizes growth under low-light conditions—to increase photosynthesis and competitiveness—as well as under excess light conditions—to prevent oxidative damage caused by high light or UV radiation. Because plants are sessile and, therefore, cannot move to a "better" place when local light conditions are suboptimal, their survival and fitness heavily depend on adequate adaptation to the varying ambient light conditions at their site. Plants, therefore, exhibit enormous plasticity in their growth and development, showing very different phenotypes under different environmental conditions. Hence, genetic developmental programs are not static but are very strongly regulated by light (Kami et al., 2010; Huber et al., 2020).

To sense the ambient light, plants evolved several classes of photoreceptors. These photoreceptors exist in almost every cell of the plant, allowing these cells to individually sense and respond to light. In addition, photoreceptors induce systemic signals that allow communication across cells and tissues to coordinate the growth and development of the entire plant. Photoreceptors enable plants to sense multiple pieces of information beyond the intensity of light, that is color, direction, and daylength (photoperiod). To do so, specialized photoreceptors evolved. These include several classes of blue light receptors: the cryptochromes (CRYs; covered in this review), the phototropins responsible for directional growth towards the light (phototropism), chloroplast movement within the cell and opening of stomata, which are the air pores in plant leaves, and the ZEITLUPE family of photoreceptors (Christie et al., 2015). Red and

far-red light are sensed by phytochromes which enable plants to specifically sense and respond to competing, neighboring plants (Cheng et al., 2021). UV-B light is perceived by the UV-B receptor UVR8, having a major role in photomorphogenesis and providing photoprotection from damaging UV light (Podolec et al., 2021).

While light responses have been studied in multiple plant species including crops, most of what we know about photoreceptor functions and—in particular—signaling events is derived from research in the model species *Arabidopsis thaliana* (Arabidopsis). It is a small, rapid-cycling weed with superior availability of molecular tools, and low-cost access to mutants in almost every gene through the Arabidopsis stock centers. Besides, researchers worldwide can make use of several freely accessible databases for *in silico* DNA, RNA, and protein analyses in Arabidopsis (Garcia-Hernandez and Reiser, 2006; Berardini et al., 2015; The 1001 Genomes Consortium, 2016; Zhang et al., 2020). This review will therefore focus on our current knowledge of Arabidopsis CRYs, with special attention to very recent research progress. For a more comprehensive review of CRY functions, the reader is referred to a previous excellent review (Wang and Lin, 2020a). Our review complements other reviews in this Frontiers collection, such as light-sensing through CRY chromophores (Goett-Zink and Kottke, 2021).

CRYPTOCHROMES REGULATE ARABIDOPSIS GROWTH, DEVELOPMENT, METABOLISM, AND STRESS TOLERANCE

In response to blue light, CRYs regulate almost every stage in the plant life cycle, from seedling establishment to the induction of reproductive growth (**Figure 1**). A most pronounced phenotype is observed in germinating seedlings. This conspicuous phenotype was therefore widely used in mutant screens that allowed the isolation of many genes important for the light response, including the CRY photoreceptors and CRY signaling components.

A germinating seedling is supported by storage reserves in the seed only for a few days after which it needs to start photosynthesis to survive and sustain further growth. This process is called deetiolation and includes the opening of closed embryonic leaves (cotyledons), the differentiation of initially immature chloroplasts into organelles fully capable of photosynthesis, the biosynthesis of chlorophyll pigment as well as the shortening of the embryonic stem (hypocotyl). Deetiolation is induced by blue light through CRYs and by red light through phytochrome photoreceptors. When a seedling grows in continuous darkness, that is, when covered by soil or litter, the default pathway is etiolation: the seedling uses all reserves stored in the seed for elongation to grow through the cover and reach the sunlight. During this period, the hypocotyl elongates to more than 10 times the length of a light-grown seedling—exclusively *via* enhanced cell elongation (Gendreau et al., 1997). Elongation occurs at the expense of most other processes; cotyledons remain closed and chloroplasts do not differentiate nor green. Moreover, a so-called apical hook forms to protect the shoot apical meristem and the cotyledons from damage while the seedling pushes through the soil (Kami et al., 2010; Mazzella et al., 2014).

Deetiolation in the light is achieved by a dramatic change in gene expression involving 10–20% of all genes (Peschke and Kretsch, 2011). These genes are ultimately CRY- and phytochrome-regulated genes and include genes involved in photosynthesis, chloroplast formation, and cell elongation (Griffin et al., 2020). Most of these genes are also under the control of the circadian clock. Recent reports suggest that CRYs play an important role in the entrainment of the circadian clock to the day/night cycle and the burst of gene expression during early dawn (Balcerowicz et al., 2021; Lopez et al., 2021). While phototropins are the blue light receptors for the directed growth of plants toward the light, CRY1 has recently been shown to modulate the phototropic curvature of seedlings in blue light (Boccaccini et al., 2020).

Once a seedling is established, it continues to grow and develop *via* the division of meristematic cells and the production of primordia. Cell division in the shoot apical meristem and leaf primordia requires inputs from light (*via* CRY or phytochrome photoreceptors in blue and red light, respectively) and sugar signals. Hence, seedlings placed in darkness fail to develop any further, not even when supplied with sucrose as an external energy source (Lopez-Juez et al., 2008; Yoshida et al., 2011; Pfeiffer et al., 2016). Leaf primordia subsequently develop into leaves whose thickness is influenced by the light intensity. There is good evidence that the increased thickness of leaves induced by higher light intensities is dependent on CRYs in co-action with phototropins (Weston et al., 2000; Hoshino et al., 2019). During the vegetative phase, Arabidopsis develops a rosette whose leaves are arranged perpendicular to the incoming light, thereby allowing maximal light absorption for photosynthesis. However, when plants are grown under low blue light intensities which limit CRY activity, they undergo a shade avoidance response exhibiting increased elongation of the petioles and a lifting of the leaves (hyponasty). This change in phenotype increases light exposure under plant–plant competition in dense canopies (Keller et al., 2011; De Wit et al., 2016; Pedmale et al., 2016). CRYs are also involved in the protection of plants from excess light by inducing the biosynthesis of substances that protect the photosynthetic apparatus from oxidative damage as well as by enhancing the production of UV-absorbing anthocyanin pigments (Neff and Chory, 1998; Keller et al., 2011; Shaikhali et al., 2012; Alvarez-Fernandez et al., 2021).

CRYs have been implicated in other abiotic stress signaling pathways. CRY1 contributes to UV-B tolerance by regulating the re-dimerization of the UV-B receptor UV RESISTANCE LOCUS 8 (UVR8; Tissot and Ulm, 2020). In addition, stabilization of HY5 by CRYs *via* suppressing the COP1/SPA complex leads to the expression of genes that confer UV-B acclimation and tolerance. Indeed, both CRYs and UVR8 are essential for plant survival under UV-B, as the triple mutant lacking functional

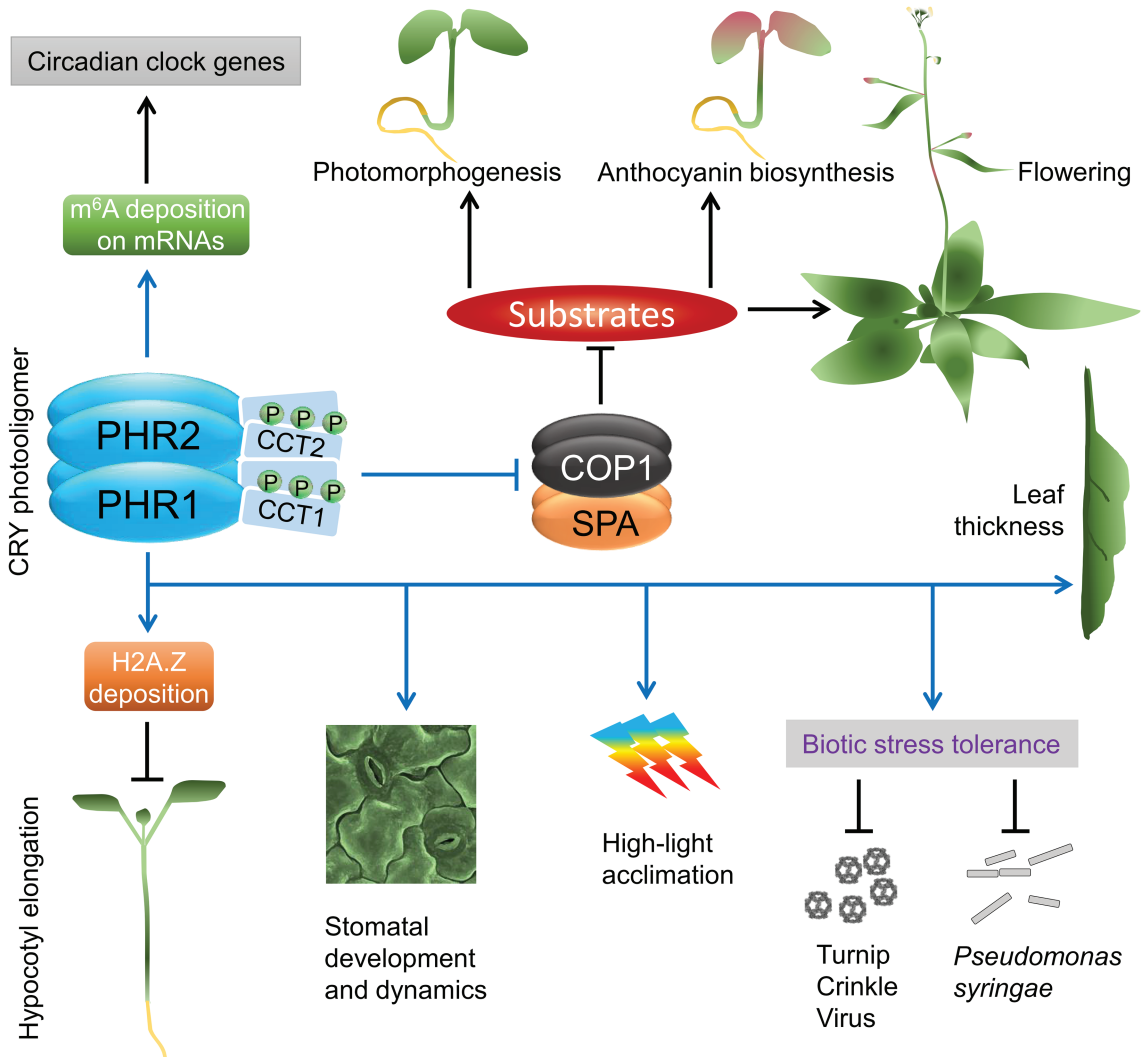

FIGURE 1 | An overview of cryptochrome signaling in Arabidopsis. Blue lines and arrows represent the direct involvement of CRYs. Abbreviations: PHR, photolyase homology region; CCT, CRY carboxy terminus; COP1, CONSTITUTIVE PHOTOMORPHOGENIC 1; SPA, SUPPRESSOR OF PHYA-105 1; m^{6}A, methylation of N6 adenosine; H2A.Z, a variant of histone H2A.

CRYs and UVR8 (*cry1 cry2 uvr8-2*) failed to survive under natural or simulated sunlight containing UV-B (Rai et al., 2019). CRYs were shown to enhance freezing tolerance when plants grown under blue light were acclimated to low, non-freezing temperatures before exposure to subzero temperatures (Li et al., 2021). CRYs are also important for drought tolerance, due to their functions in regulating stomatal aperture in concert with PHOTs and ABA (Mao et al., 2005; Boccalandro et al., 2012; Chen et al., 2012). Besides regulating stomatal aperture, CRYs promote the differentiation of stomata from protodermal cells which is promoted by light; the *cry1 cry2* mutant showed a lower stomatal density in blue light (Kang et al., 2009).

Besides abiotic stresses, CRYs have been implicated in biotic stress signaling pathways. CRYs enhance the resistance against bacterial and viral pathogens that attack the plants. CRY1 activates the systemic acquired resistance (SAR) locally in leaves infected with the bacteria *Pseudomonas syringae*, under continuous light conditions. In line with this, the expression levels of *PATHOGENESIS-RELATED GENE 1* (*PR1*) induced by salicylic acid treatment were enhanced in CRY1-overexpressing plants but decreased in *cry1* mutants (Wu and Yang, 2010). Similarly, Arabidopsis CRY2 and PHOT2 promote resistance (R) protein-mediated defense during Turnip Crinkle Virus (TCV) infection by stabilizing the R protein HYPERSENSITIVE RESPONSE TO TCV (HRT). In addition, HRT may function as a part of the protein complex consisting of CRYs and the E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1; Jeong et al., 2010a,b). CRY1 along with PHOT2 and PHYB was also shown to promote resistance against Cucumber Mosaic Virus (CMV) in Arabidopsis (Zhou et al., 2017).

After vegetative growth, Arabidopsis plants enter reproductive development through flowering, fertilization, and seed set. As an annual species not capable of clonal propagation, seed production is essential for the survival of this species. For successful reproduction, the timing of flowering is extremely crucial and needs to occur during a favorable season. Flowering time is therefore strongly regulated by environmental factors including day length. Long days, as in spring and summer, promote Arabidopsis flowering through a cryptochrome photoreceptor. The mechanisms allowing cryptochromes to sense the length of the day are well-described in (Wang and Lin, 2020a). The flowering of Arabidopsis is associated with the production of multiple, usually branched, inflorescences along which flowers are produced. The extent of shoot branching is highly controlled by many environmental factors including light. While phytochrome B promotes branching, CRY1 inhibits branching and, thus, acts oppositely to phytochrome (González-Grandío et al., 2013; Zhai et al., 2020).

THE CRYPTOCHROME PHOTORECEPTORS CRY1 AND CRY2: STRUCTURE AND FUNCTIONS

The Arabidopsis genome contains three *CRY* genes, two CRYs (CRY1 and CRY2) of the plant CRY subfamily, and one CRY (CRY3) of the CRY-DASH subfamily. CRY1 and CRY2 have proven signaling activities in photomorphogenesis, while CRY3 repairs cyclobutane pyrimidine dimers in UV-damaged single-stranded DNA and is localized in chloroplasts and mitochondria (Kleine et al., 2003; Selby and Sancar, 2006; Pokorny et al., 2008; Kiontke et al., 2020). Plant and animal-type CRYs evolved independently from different types of DNA photolyases; both have lost photolyase activity in the course of their evolution into photoreceptors (Sancar, 2003).

CRY1 and CRY2 have overlapping, but also distinct functions, as defined through the phenotypic analysis of *cry1* and *cry2* mutants. Both CRY1 and CRY2 have important functions during seedling deetiolation in blue light, with CRY1 acting mostly at higher fluence rates of blue light, while CRY2 responds primarily to dim blue light (Ahmad and Cashmore, 1993; Lin et al., 1998). This functional distinction correlates with a difference in photoreceptor stability: CRY2 is very photo-labile; it is degraded within a few hours of exposure to blue light. In contrast, CRY1 is mostly light-stable, though it was recently shown to be partially destabilized at very high fluence rates of blue light (Lin et al., 1998; Miao et al., 2021). Another major functional distinction is the regulation of flowering time: CRY2 is a key inducer of flowering under the long-day conditions that promote flowering in Arabidopsis, while the role of CRY1 is only of minor importance and dependent on the respective environmental conditions used (Guo et al., 1998; Mockler et al., 2003; Exner et al., 2010; Liu et al., 2016).

CRY1 and CRY2 consist of two domains, the highly conserved N-terminal photolyase homology region (PHR) and the more divergent, unstructured CRY Carboxy Terminus (CCT, also called CCE domain). The PHR domains of CRY1 and CRY2 (PHR1 and PHR2, respectively) non-covalently bind the primary chromophore, a flavin, and are therefore responsible for light detection by the photoreceptors (Wang and Lin, 2020a). Besides, PHRs mediate oligomerization of CRYs which is essential for CRY activities (Sang et al., 2005; Rosenfeldt et al., 2008; Wang et al., 2016a; Liu et al., 2020). The 3D structure of monomeric PHR1 was resolved several years ago, demonstrating high structural similarity with DNA photolyases (Brautigam et al., 2004). The crystal structure of PHR2 uncovered that its structure is almost identical to that of PHR1 (Ma et al., 2020b). Recently, three pivotal studies resolved the structure of the active PHR2 conformation either by X-ray crystallography or cryogenic electron microscopy (cryo-EM). All three studies independently demonstrated that the blue light-activated photosensory domain of CRY2 assembles into a tetramer (Shao et al., 2020; Ma et al., 2020a; Palayam et al., 2021).

The CCT domains of CRYs are intrinsically disordered; therefore, no structural information on CCT or full-length CRYs is so far available. The CCT domains of CRY1 and CRY2 share only weak sequence similarities and are of different lengths (Lin et al., 1998). CCTs are not directly involved in the perception of blue light but are important hubs for the blue light-induced interaction with signaling proteins. While the PHR domains were initially thought to solely act in photo-sensing, it has become clear that they also represent important docking domains for signal transduction *via* protein–protein interactions. **Table 1** shows the list of proteins that interact with the PHR domain of CRYs and the respective signaling pathways involved.

EARLY EVENTS IN CRYPTOCHROME SIGNAL TRANSDUCTION

Upon light perception, CRYs undergo a conformational change that leads to oligomerization from a monomer to a tetramer (Ma et al., 2020a; Shao et al., 2020; Palayam et al., 2021; **Figure 2**). Oligomerization of CRY1 and CRY2 is light-dependent *in vivo*, with CRY2 being about six times more sensitive to blue light than CRY1 (Liu et al., 2020). This finding correlates with the phenotypic analyses of *cry1* and *cry2* mutants that identified CRY1 as a "higher blue light intensity" and CRY2 as a "lower blue light intensity" photoreceptor, suggesting that the different kinetics of photooligomerization are causative for the different blue light sensitivity of CRY1 and CRY2, in addition to the differential photoreceptor stability in blue light (Liu et al., 2020). Photooligomerization is indeed required for CRY1 and CRY2 activity *in vivo* (Sang et al., 2005; Rosenfeldt et al., 2008; Wang et al., 2016a). Recent results suggest that photooligomerization is necessary but not sufficient for CRY2 function *in vivo* because the expression of a CRY2 missense mutant ($CRY2^{P532L}$) failed to complement the *cry1 cry2* mutant phenotype despite normal photooligomerization. Hence, the light response likely requires additional conformational or biochemical changes beyond photooligomerization (Liu et al., 2020). Initially, it was assumed that CRY1 and

TABLE 1 | Proteins that interact with the N-terminal domains of Arabidopsis CRYs (PHR1 and PHR2).

Name	AGI code	Identity	Interaction with		Functions in	References
			PHR1	PHR2		
AGB1	AT4G34460	Heterotrimeric G protein beta subunit	Yes	Nd	Organ shape	Lian et al., 2018
ARF6	AT1G30330	Auxin response factor	Yes	Nd	Cell and organ elongation	Mao et al., 2020
ARF8	AT5G37020	Auxin response factor	Yes	Nd	Cell and organ elongation	Mao et al., 2020
ARP6	AT3G33520	Actin-related protein	Yes	Yes	Chromatin remodeling	Mao et al., 2021
BEE2	AT4G36540	Brassinosteroid signaling protein	Yes	Nd	Brassinosteroid signaling, shade avoidance	Wang et al., 2018a
BIC1	AT3G52740	Protein inhibiting CRY function	Yes	Yes	Disrupting CRY dimerization	Wang et al., 2016a; Ma et al., 2020b; Miao et al., 2021
BIC2	AT3G44450	Protein inhibiting CRY function	Yes	Yes	Disrupting CRY dimerization	Wang et al., 2016a; Ma et al., 2020b
BIM1	AT5G08130	bHLH protein	Yes	Yes	Brassinosteroid signaling	Wang et al., 2018b
BZR1	AT1G75080	Positive regulator of brassinosteroid signaling	Yes	Yes	Brassinosteroid signaling	He et al., 2019
BZR2 (BES1)	AT1G19350	Brassinosteroid signaling protein	Yes	Yes	Brassinosteroid signaling	Wang et al., 2018b; He et al., 2019
CIB1	AT4G34530	CRY-interacting bHLH protein	Yes	Nd	Flowering time	Liu et al., 2008; Wang et al., 2018a
CIL1	AT1G68920	CIB1-like bHLH protein	Yes	Nd	Photomorphogenesis	Wang et al., 2018a
CRY1	AT4G08920	Cryptochrome	Yes	Yes	Blue light perception	Sang et al., 2005; Liu et al., 2020
CRY2	AT1G04400	Cryptochrome	Yes	Yes	Blue light perception	Sang et al., 2005; Wang et al., 2016a
GAI	AT1G14920	DELLA protein	Yes	Nd	GA signaling	Zhong et al., 2021; Xu et al., 2021a
GID1	AT3G05120	DELLA protein	Yes	Nd	GA signaling	Zhong et al., 2021; Xu et al., 2021a
HBI1	AT2G18300	bHLH protein	Yes	Nd	Cell elongation and proliferation, plant immunity	Wang et al., 2018a
IAA12	AT3G23050	Auxin response protein	Yes	Nd	Auxin signaling	Xu et al., 2018
IAA17	AT1G04550	Auxin response protein	Yes	Nd	Auxin signaling	Xu et al., 2018
IAA7	AT1G04250	Auxin response protein	Yes	Nd	Auxin signaling	Xu et al., 2018
MTA	AT4G10760	mRNA m6A writer protein	Nd	Yes	N6-adenosine methylation of mRNA	Wang et al., 2021b
PIF5	AT3G59060	bHLH transcription factor	Nd	Yes	Shade avoidance, PHY signaling	Pedmale et al., 2016
RGA	AT2G01570	DELLA protein	Yes	Nd	GA signaling	Yan et al., 2021; Zhong et al., 2021; Xu et al., 2021a
SINAT2	AT3G58040	RING finger domain protein	Yes	Nd	Brassinosteroid signaling, photomorphogenesis	Hu et al., 2021
SINAT5	AT5G53360	RING finger domain protein	Yes	Nd	Brassinosteroid signaling, photomorphogenesis	Hu et al., 2021
SPA1	AT2G46340	Component of COP1/SPA ubiquitin ligase	No	Yes	Photomorphogenesis, flowering time	Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011
SWC6	AT5G37055	Component of SWR1 complex	Yes	Yes	Chromatin remodeling, Flowering time	Mao et al., 2021
TCP17	AT5G08070	TCP family protein	Yes	Nd	Leaf differentiation	Zhou et al., 2019
TOE1	AT2G28550	AP2 family transcription factor	Yes	Yes	Flowering time, innate immunity	Du et al., 2020
TOE2	AT5G60120	AP2 family transcription	Yes	Yes	Flowering time, innate immunity	Du et al., 2020

Nd, not determined.

CRY2 can only homo-oligodimerize, but recently it was shown that CRY1 and CRY2 also form hetero-oligomers. Functional relevance of CRY1/CRY2 heterooligomer action was provided by showing that expression of a truncated CRY2 lacking the functionally important CCT domain (PHR2) in transgenic plants caused a dominant-negative effect by competitively inhibiting homodimerization of both endogenous CRY1 and CRY2. In accordance, overexpression of PHR2 repressed the activities of both endogenous CRY1 and CRY2 during deetiolation (Liu et al., 2020).

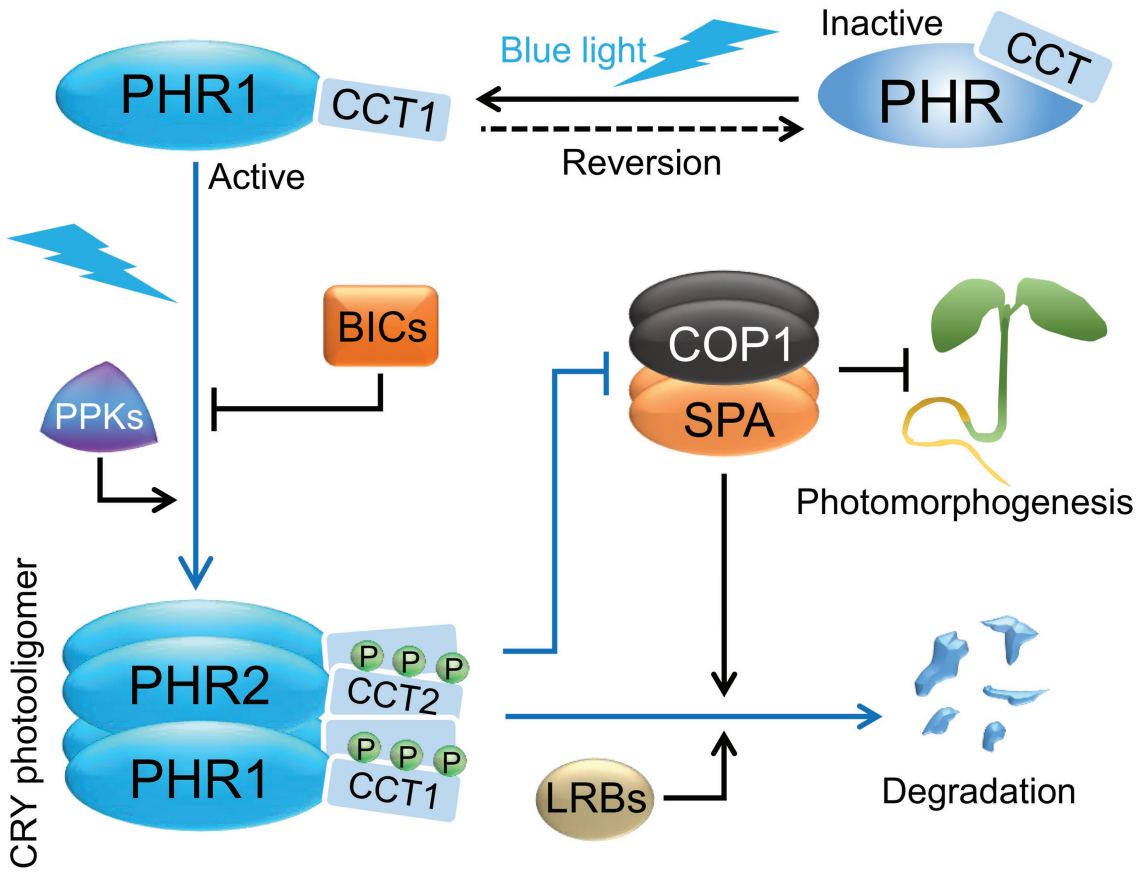

FIGURE 2 | Cryptochrome photoactivation and degradation. Cryptochromes exist as inactive monomers in darkness and are proposed to form a "closed" conformation with the C-terminal domains (CCTs) closely associated with the N-terminal domains (PHRs). In blue light, cryptochromes homo- and heterooligomerize *via* their PHR domains, and serine residues in the CCTs are phosphorylated by PPK kinases. In addition, the CCTs are proposed to dissociate from PHRs forming an "open" conformation. Photoactivated CRYs revert to inactive forms in darkness in a temperature-dependent manner, which may help in retaining their photoresponsiveness. In blue light, BICs suppress CRY photooligomerization and thereby affect CRY function. Exposure to blue light ultimately leads to the degradation of CRY2, and a lesser extent of CRY1, *via* the COP1/SPA and LRB ubiquitin ligases. CRYs promote photomorphogenesis in blue light *via* repressing COP1/SPA activity. Abbreviations: PHR, photolyase homology region; CCT, CRY carboxy terminus; BIC, BLUE LIGHT INHIBITOR OF CRYPTOCHROMES; PPK, PHOTOREGULATORY PROTEIN KINASE; COP1, CONSTITUTIVE PHOTOMORPHOGENIC 1; SPA, SUPPRESSOR OF PHYA-105 1; LRB, Light-Response Bric-a-Brack/Tramtrack/Broad.

The lifetimes of cryptochrome signaling states are limited due to thermal dark reversion (Herbel et al., 2013). Such inactivation mechanisms are important to retain the photoresponsiveness of photoreceptors. Photooligomers of CRY2 revert to inactive monomers when transferred from blue light to darkness in a temperature-dependent fashion. Dark reversion of CRY2 was faster in Arabidopsis plants when compared to CRY2 heterologously expressed in mammalian HEK293 cells, suggesting that additional plant factors enhance CRY2 dark reversion (Liu et al., 2020; Wang and Lin, 2020a; **Figure 2**). Plant factors that reduce CRY1 and CRY2 oligomerization were identified in a genetic screen for negative regulators of CRY activity. Mutations in plant-specific *BLUE LIGHT INHIBITOR OF CRYPTOCHROMES 1* (*BIC1*) and the related gene *BIC2* enhance cryptochrome responses in Arabidopsis, while overexpression of *BIC1* or *BIC2* strongly suppresses CRY functions in transgenic plants (Wang et al., 2016a). BIC1 interacts with PHR2 in a strongly blue light-enhanced fashion in Arabidopsis plants. When recombinantly expressed in mammalian HEK293 cells, BIC1 severely suppresses CRY2 self-association. Moreover, BIC1 leads to an inhibition of blue light-dependent molecular activities of CRY2, such as photobody formation, phosphorylation, degradation, and binding of signaling components, suggesting that BIC1 has a primary function in inhibiting CRY2 photooligomerization (**Figure 2**) which subsequently leads to the suppression of molecular and physiological CRY2 photoresponses (Wang et al., 2016a). Recently, it was shown that BIC1 also interacts with PHR1 in a blue light-dependent manner to inhibit CRY1 self-association and CRY1 degradation under high blue light fluence rates (Miao et al., 2021). Gene expression of *BIC1* and *BIC2* is induced by blue, red, and UV-B light, thereby providing a negative feedback mechanism of CRYs and phytochromes on CRY1 and CRY2 signaling (Wang et al., 2017; Tissot and Ulm, 2020).

The co-crystal structure of PHR2 with the CRY2-interacting domain of BIC2 provided mechanistic evidence for BIC2's inhibitory activity on CRY2 oligomerization (Ma et al., 2020b; Palayam et al., 2021). BIC2-binding did not change the overall conformation of the PHR2 tetramer, but caused structural rearrangements in side chains of amino acids that are considered important for photoreduction of the chromophore. Hence, BIC2 may prevent photoreduction of CRY2 which subsequently would inhibit photooligomerization in blue light (Ma et al., 2020b; Wang and Lin, 2020b). BIC2 might also act by a second mechanism involving competitive inhibition of CRY2 oligomerization. BIC2 occupies part of the oligomeric interaction surface of PHR2 and might thereby prevent photooligomerization of PHR2 (Ma et al., 2020b; Wang and Lin, 2020b; Palayam et al., 2021).

Interestingly, a recent study uncovered an additional function of BIC1 as a co-activator of brassinosteroid-induced gene expression and hypocotyl elongation in blue light. BIC1 interacts with the transcription factors BRASSINAZOLE-RESISTANT 1 (BZR1) and PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) to cooperatively bind and activate BZR1 target genes involved in cell elongation. Hence, BIC1 may integrate cryptochrome and brassinosteroid signaling (Yang et al., 2021). The physical interaction of CRYs with BZR1 and other brassinosteroid signaling proteins provided further support for the direct regulation of brassinosteroid responses by blue light (Wang and Lin, 2020a). A recent finding that CRY2 activates gene expression by directly binding to DNA when expressed in a heterologous non-plant system even points toward a possible role of CRY2 as a transcription factor (Yang et al., 2018). Arabidopsis CRY2 reconstituted in human embryonic kidney cells (HEK293T) could directly interact with many fragments of the *FLOWERING LOCUS T* (*FT*) genomic region, in a blue light-enhanced manner. In contrast, CRY2 was associated only weakly with *FT* in transgenic plants, perhaps due to the presence of endogenous CRY-feedback mechanisms, such as the blue light-dependent degradation and BIC-mediated suppression (Yang et al., 2018). The idea of CRY2 binding DNA is conceivable, as CRYs have evolved from ancestral photolyases that bind pyrimidine dimers in the DNA. In line with this, the physical association of CRY2 with the genomic regions was also shown using ChIP-seq experiments in transgenic Arabidopsis lines (Pedmale et al., 2016). However, as CRY2 lacks the typical features of a canonical transcription factor, further experiments are needed to confirm the transcriptional activity of CRY2 in planta.

Following blue light absorption, CRY1 and CRY2 are phosphorylated (Shalitin et al., 2002, 2003; Yu et al., 2007a; **Figure 2**). There are three serines in the CCT domain of CRY2 and mutations in these serines reduce CRY2 phosphorylation, degradation, and seedling deetiolation in transgenic plants, indicating that light-induced phosphorylation is important for CRY2 activity in the context of a full-length CRY2 photoreceptor (Wang et al., 2015). Experiments expressing deletion-derivatives of CRY2 strongly suggest that light-induced phosphorylation of CRY2 is not important for CRY2 signaling activity *per se* but causes conformational changes that expose the CCT domain of CRY2 to render it accessible for important interacting signaling partners. Phosphorylation of CCT might provide a negative charge that repels CCT from the PHR domain (Yu et al., 2007b). Structural information of full-length CRY2 in its active and inactive conformation will be necessary to prove that blue light converts CRY2 from a "closed" into an "open" conformation (**Figure 2**). A recent work involving in-cell infrared difference (ICIRF) spectroscopy provides further evidence to the idea that blue light illumination causes the dissociation of CCT from PHR. Under darkness, in the full-length CRY from *Chlamydomonas reinhardtii* expressed in bacteria, the CCT was in close association with the only β-sheet of the PHR domain. Blue light illumination caused a re-organization of the PHR domain β-sheet due to the stabilization of flavin neural radical in presence of ATP, implying the dissociation of CCT from PHR (Goett-Zink and Kottke, 2021).

CRY2 is phosphorylated by a family of four related Ser/Thr protein kinases, the PHOTOREGULATORY PROTEIN KINASES (PPK1–PPK4; **Figure 2**). The major phosphorylation sites in CRY2 identified *via* mass spectrometry are located within the CCT domain of CRY2. *ppk* mutants exhibit impaired CRY2 phosphorylation and late-flowering similar to a *cry2* mutant, which supports that blue light-induced CRY2 phosphorylation is important for CRY2 activity (Liu et al., 2017). CRY2 also interacts with SUPPRESSOR OF PHYA-105 1 (SPA1) that was recently shown to have kinase activity (Paik et al., 2019). Whether SPA1 phosphorylates CRY1 or CRY2 is unknown. But CRY1 and CRY2 in *spa* null mutants exposed to blue light retain a normal, retarded migration in protein gels, suggesting that CRY phosphorylation still occurs in the absence of SPA proteins (Holtkotte et al., 2017).

After light-induced phosphorylation, CRY2 is rapidly degraded through polyubiquitination by the COP1/SPA and the Light-Response Bric-a-Brack/Tramtrack/Broad (LRB) E3 ubiquitin ligases. While the COP1/SPA complex particularly mediates the rapid response after 1–2 h of blue light exposure, LRBs are important for CRY2 degradation after more prolonged blue light irradiation (Shalitin et al., 2002; Weidler et al., 2012; Chen et al., 2021; **Figure 2**). Interestingly, CRY2 degradation is enhanced by low ambient temperature (16°C) through the polyubiquitination by LRBs (Ma et al., 2021). In contrast, CRY2 degradation is almost absent at 4°C when plants are acclimated to low temperatures to acquire freezing tolerance (Li et al., 2021). This may be caused by an enhanced shuttling of COP1 out of the nucleus and/or by a generally reduced activity of proteasomes at low temperatures (Catala et al., 2011; Li et al., 2021). Both COP1 and LRBs were also shown to control CRY1 protein stability under high blue light fluence rates (Miao et al., 2021).

SIGNAL TRANSDUCTION THROUGH CRYPTOCHROME-INTERACTING PROTEINS

Cryptochrome signal transduction occurs *via* both the CCT and the PHR domains. Two decades ago, a key experiment had already provided strong evidence that CCT is sufficient to promote photomorphogenesis. Researchers fused the CCT

domains of CRY1 and CRY2 to an artificial dimerization domain. Transgenic plants expressing these fusion proteins exhibited constitutive photomorphogenesis even in darkness, indicating that CCT in the absence of the photosensory PHR constitutively activates the light signaling cascade (Yang et al., 2000). While PHR was initially thought to act exclusively in photoperception, there is mounting evidence that PHR also initiates important signaling processes because expression of CRY1-PHR fused to an artificial nuclear localization sequence complemented the *cry1* mutant phenotype (He et al., 2015). Moreover, many signaling factors interact with PHRs (see also below; **Table 1**). How PHR and CCT coordinate light signaling is so far unknown, but a comparative transcriptome analysis of PHR and CCT-expressing transgenic lines provided insight into the co-regulation of genes by both domains. While approximately 67% of CRY1-responsive genes are regulated by CCT1 alone, others are co-regulated by both CCT1 and PHR1 (Wang et al., 2016b).

The COP1/SPA Ubiquitin Ligase

Loss-of-function missense mutations in the CCT domains of CRY1 and CRY2 confirmed CCTs as signaling hubs (Ahmad and Cashmore, 1993; Ahmad et al., 1995; Guo et al., 1998; Yang et al., 2000). The CCT domains of CRY1 and CRY2 interact with COP1 (Wang et al., 2001; Yang et al., 2001; Ponnu et al., 2019), an E3 ubiquitin ligase that inhibits light signaling by polyubiquitinating multiple transcription factors acting in the various light responses. COP1 is mainly active in darkness, thereby preventing an accumulation of these substrate transcription factors which leads to a suppression of light responses in darkness (Huang et al., 2014; Hoecker, 2017; Ponnu and Hoecker, 2021). Epistatic analyses of *cop1* and *cry1* mutants suggested early on that CRYs initiate light signaling in blue light by inhibiting the activity of COP1 (Ang and Deng, 1994; **Figures 1, 2**). Indeed, substrate transcription factors of COP1 accumulate in seedlings grown in blue light, thereby allowing photomorphogenesis to proceed (Ponnu, 2020).

In darkness, COP1 acts in concert with SPA proteins in a tetrameric COP1/SPA complex consisting of two COP1 proteins and two SPA proteins of the four-member SPA protein family (SPA1-SPA4; Hoecker and Quail, 2001; Saijo et al., 2003; Zhu et al., 2008). Both COP1 and SPA proteins are necessary for the activity of the COP1/SPA ubiquitin ligase since both *cop1* and *spa* quadruple mutants undergo constitutive photomorphogenesis, exhibiting features of light-grown seedlings in complete darkness (Deng et al., 1991; Laubinger et al., 2004; Ordonez-Herrera et al., 2015). The COP1/SPA complex acts as the substrate recognition module in a Cullin 4-based ubiquitin ligase. Substrate recognition occurs *via* the C-terminal WD-repeat domains present in both the COP1 and the four SPA proteins. The WD repeats also act in binding DDB1 in the Cullin 4-complex (Ponnu and Hoecker, 2021). Since the WD-repeat of SPA1 can replace that of COP1 but not vice versa, their functions are likely related but not identical (Kerner et al., 2021). COP1/SPA complex formation is conferred by the coiled-coil domains present in COP1 and SPA proteins. The N-termini of COP1 and SPA proteins are distinct, with COP1 carrying a RING finger domain and SPA exhibiting a kinase-like domain that was recently shown to have kinase activity (Deng et al., 1992; Hoecker et al., 1999; Paik et al., 2019; Ponnu and Hoecker, 2021; Wang et al., 2021a).

CRY1 and CRY2 interact with the COP1/SPA complex in blue light-grown but not in dark-grown seedlings *in vivo* (Holtkotte et al., 2017), which is consistent with the blue light-induced inhibition of COP1/SPA activity by CRYs. Moreover, the COP1/SPA complex preferentially interacts with the phosphorylated isoforms of CRY1 and CRY2 that only exist after blue light exposure (Holtkotte et al., 2017). When examining one-by-one interactions *ex vivo*, CRY1 and CRY2 interact with SPA1 in a blue light-dependent manner, while they interact with COP1 constitutively or in a blue light-enhanced manner (Yang et al., 2001; Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011; Ponnu et al., 2019). This suggests that SPA1 may contribute to the blue light dependency of the CRY-COP1/SPA interaction *in vivo*. Indeed, SPA proteins are required for the CRY1-COP1/SPA interaction *in vivo* because COP1 did not interact with CRY1 in a *spa* quadruple null mutant (Holtkotte et al., 2017).

Domain mapping defined the interacting domains in CRYs and the COP1 and SPA proteins, thereby helping in the elucidation of the molecular mechanisms of CRY activity. The CCT domains of CRY1 and CRY2 interact with the substrate-recognizing WD repeats of COP1 (Wang et al., 2001; Yang et al., 2001; Ponnu et al., 2019). Binding of CRY1 and CRY2 to SPA proteins differs: The CCT domain of CRY1 interacts with SPA1WD (Lian et al., 2011; Liu et al., 2011; Ponnu et al., 2019), while the CCT domain of CRY2 does not interact with full-length SPA1 but with an N-terminally truncated version of SPA1 containing only the WD-repeat domain, suggesting that the N-terminal domain of SPA1 inhibits the CCT2-SPA1WD interaction (Ponnu et al., 2019). CRY2 interacts most strongly *via* its PHR domain with the kinase domain of SPA1 (Zuo et al., 2011).

CRYs inactivate the COP1/SPA complex *via* multiple mechanisms (Ponnu, 2020). Light promotes the nuclear exclusion of COP1, thereby separating it from the nuclear-localized transcription factors targets (Von Arnim and Deng, 1994; Pacin et al., 2014). In blue light, nuclear exclusion of COP1 is dependent on CRY1 (Osterlund and Deng, 1998). Nuclear exclusion of COP1 in white light, but not in blue light, is dependent on SPA proteins (Balcerowicz et al., 2017). Blue light leads to a destabilization of SPA1 and SPA2, but this occurs in a phytochrome A-dependent fashion and is independent of CRYs (Balcerowicz et al., 2011; Chen et al., 2015). Light-activated CRY1 acts to disrupt the COP1-SPA1 interaction (Lian et al., 2011; Liu et al., 2011). CRY2 does not dissociate the COP1-SPA1 complex, but blue light enhances the CRY2-COP1 interaction, which may lead to a stronger inactivation of the COP1/SPA complex (Zuo et al., 2011).

Two recent, complementary studies demonstrated that photoactivated CRY2 inactivates the COP1/SPA complex by competitively displacing substrates from the WD-repeat domain of COP1 (Lau et al., 2019; Ponnu et al., 2019; Ponnu, 2020). This mechanism is based on the sharing of a COP1-interacting

motif between CRY2 and COP1 substrates. COP1 recognizes many diverse substrate transcription factors; despite their sequence divergence, many of them share a conserved Val-Pro (VP) motif that is required for their interaction with COP1 (Holm et al., 2001; Ponnu et al., 2019). The co-crystal structure of COP1 with VP-containing peptides confirmed that the VP directly binds the WD-repeat domain of COP1 (Uljon et al., 2016; Lau et al., 2019). CRY2 also contains a VP-like motif in its CCT domain, suggesting that CRY2 and substrates may compete for binding to the same domain of COP1 (Muller and Bouly, 2015). Indeed, when the VP motif of CRY2 was mutated, the interaction with COP1 was lost (Ponnu et al., 2019). In agreement with this finding, CRY2 carrying a mutated VP motif failed to complement the *cry2* mutant phenotype and was, therefore, inactive in Arabidopsis plants (Ponnu et al., 2019; Liu et al., 2020). In addition, loss of function of CRY2 was observed when the proline residue of the VP motif was mutated ($CRY2^{P532L}$; Ahmad et al., 1995; Liu et al., 2020). A co-crystal structure of COP1WD with a VP-containing peptide of CRY2 confirmed that the VP of CRY2 binds COP1 (Lau et al., 2019). Hence, both COP1 substrates and CRY2 bind COP1WD *via* their respective VP motifs. Competitive binding was investigated using the COP1-substrate PRODUCTION OF ANTHOCYANIN PIGMENT 2 (PAP2), a transcription factor that controls the biosynthesis of anthocyanins in light-grown plants (Maier et al., 2013; Maier and Hoecker, 2014). Co-expression of CRY2 disrupted the COP1-PAP2 interaction, indicating that CRY2 effectively displaced PAP2 from COP1. In contrast, CRY2 carrying the VP mutation was not able to disrupt the COP1-PAP2 interaction (Ponnu et al., 2019). Co-crystals of COP1WD with a VP-peptide from the COP1-substrate CONSTANS (CO) and the corresponding late-flowering phenotype of plants expressing COP1 carrying WD mutations disrupting VP-binding suggest that CRY2 may also outcompete CO from binding to COP1 (Lau et al., 2019). Recently, co-expression of CRY2 was also shown to reduce the COP1-ELONGATED HYPOCOTYL 5 (HY5) interaction in a split-luciferase assay (Li et al., 2021).

Though the CCT motifs of CRY1 and CRY2 share little sequence similarity, CRY1 does contain three potential VP motifs in its CCT domain of which the first one (VP1) is required for COP1-binding (Ponnu et al., 2019). A peptide containing VP1 of CRY1 was also co-crystallized with COP1WD, thus confirming a direct interaction of VP1 with COP1 (Lau et al., 2019). It remains to be determined whether CRY1, like CRY2, inactivates COP1 by substrate displacement. Moreover, it is unknown whether CRYs displace substrates also from the WD repeats of SPA proteins. Considering that both the light-activated CRYs and the COP1/SPA complex act as tetramers, it is conceivable that a cryptochrome tetramer displaces substrates from the COP1/SPA hetero-tetramer.

Cryptochrome-Interacting Transcriptional Regulators

Besides regulating transcription factor activity indirectly *via* the COP1/SPA ubiquitin ligase, CRYs also directly interact with several transcription factors to initiate light signal transduction (Wang and Lin, 2020a). PHR2 of photoactivated CRY2 interacts with a family of CRYPTOCHROME-INTERACTING bHLH (CIB) transcription factors to regulate flowering time in Arabidopsis. Similarly, PHR1 interacts with CIB proteins to regulate seedling deetiolation (Wang and Lin, 2020a). This finding supports the observation that overexpression of PHR1 can initiate light signaling in the absence of CCT (He et al., 2015). CRY1 and the PHR domain of CRY2 also interact with the PHYTOCHROME-INTERACTING FACTORS PIF4 and PIF5 to control hypocotyl elongation under low blue light as it is found under a plant canopy (Pedmale et al., 2016; Xu et al., 2016; **Figure 3**). PIF4 and PIF5 belong to a small bHLH transcription factor family that promotes the expression of cell elongation-related genes in darkness and shade (Balcerowicz, 2020). In the regulation of flowering time, photoactivated CRY2 directly interacts with the floral repressors TARGET OF EARLY ACTIVATION TAGGED 1 (TOE1) and TOE2 to suppress their repressor activities on the floral activator CO (Du et al., 2020).

In seedlings, the hypocotyls do not only elongate in response to darkness or under a canopy but also when exposed to a moderate increase in ambient temperature that is not yet heat stress to the plant (*ca.* 28°C). Blue light inhibits the elongation response to high ambient temperature through an interaction of CRY1 with PIF4, thereby causing a reduction in the transcription activation function of PIF4 (Ma et al., 2016; Xu et al., 2016). CRY1 also controls PIF4 activity in a temperature-dependent manner through the transcription factor TCP DOMAIN PROTEIN 17 (TCP17): PHR1 interacts directly with TCP17 at ambient temperature, thereby inhibiting TCP17 activity (**Figure 3**). At high ambient temperature, the CRY1-TCP17 interaction is reduced and TCP17 can activate *PIF4* gene expression and—after direct binding to the PIF4 protein—the TCP17/PIF4 complex activates cell elongation-related genes, leading to hypocotyl elongation (Zhou et al., 2019).

CRYs mediate blue light control of hormone responses by directly interacting with several hormone signal transduction intermediates (**Figure 3**). Hormones are key regulators in plants as they—similar to light—control growth and development throughout the plant life cycle. Two very recent reports showed that CRY1 directly interacts with the receptor for the hormone gibberellic acid (GA; Zhong et al., 2021; Xu et al., 2021a). GA is known to be important for light responses; it induces cell elongation, and GA biosynthesis is required for the suppression of photomorphogenesis in darkness (Alabadí et al., 2004). In light-grown seedlings, GA biosynthesis and/or the action of GA signaling components are altered (Halliday and Fankhauser, 2003). Both recent studies demonstrate that blue light exposure induces the interaction of CRY1 with the GA receptor GID1 as well as with a family of GA signaling proteins, the DELLAs. DELLAs are repressors of GA signaling whose degradation is induced by GA through the action of the SLEEPY1, (SLY1)/SNE E3 ubiquitin ligases. Such de-repression subsequently allows GA-responsive gene expression and cell elongation (Xu et al., 2014). To analyze the consequences of CRY1-GID1-DELLA interactions, the scientists investigated DELLA degradation and found that blue light and CRY1 reduce the degradation of DELLAs in response to GA. It is expected

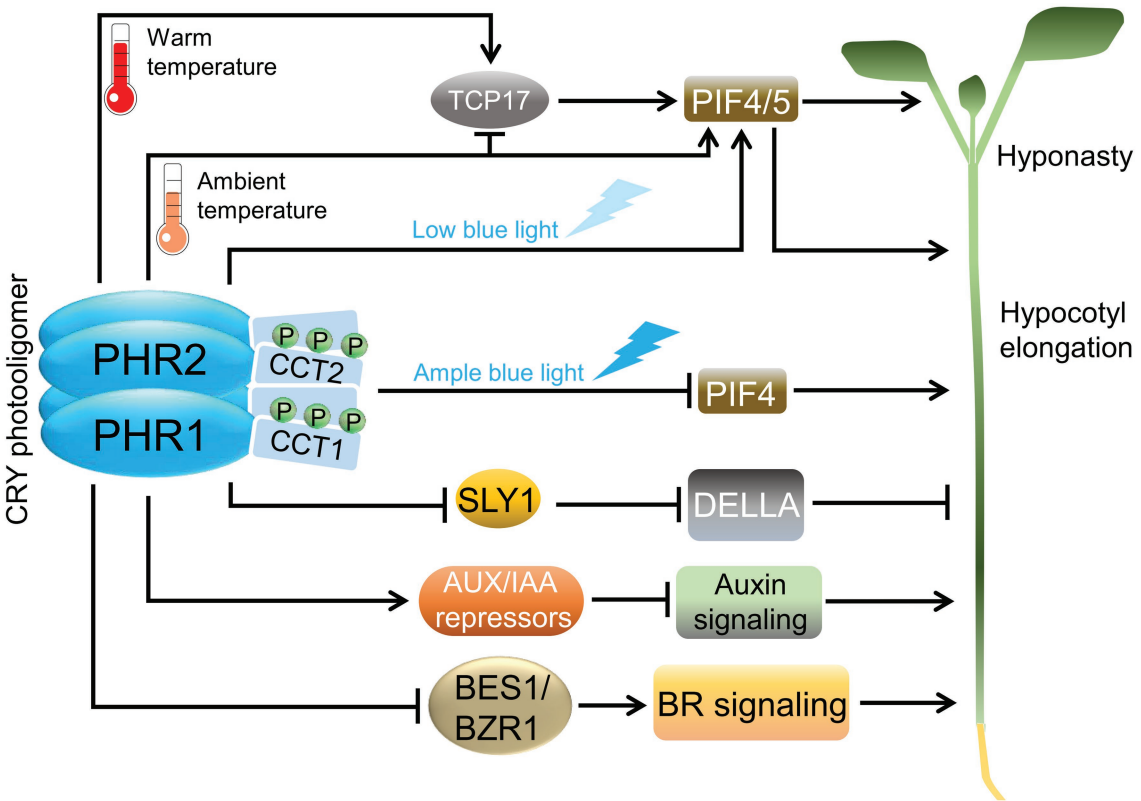

FIGURE 3 | Cryptochromes in warm temperature and hormone signaling. Abbreviations: PHR, photolyase homology region; CCT, CRY carboxy terminus; TCP17, TCP DOMAIN PROTEIN 17; SLY1, SLEEPY1; AUX/IAA, AUXIN/INDOLE-3-ACETIC ACID; BES1, BRI1-EMS-SUPPRESSOR 1; BZR1, BRASSINAZOLE-RESISTANT 1; PIF, PHYTOCHROME-INTERACTING FACTOR; DELLA, DELLA domain-containing protein; BR, Brassinosteroid.

that the increased DELLA levels in blue light contribute to the short hypocotyl phenotype of blue light-exposed seedlings. Consistent with this observation, GID1, DELLAs, and CRY1 genetically interacted in the control of hypocotyl elongation in blue light and GA responsiveness. To elucidate how CRY1 stabilizes DELLAs in blue light, protein–protein interactions were investigated. Two mechanisms were identified: CRY1 inhibits the GID1-DELLA interaction, thereby preventing GA-induced DELLA degradation (Xu et al., 2021a; Zhong et al., 2021). Furthermore, CRY1 disrupts the interaction of DELLAs with the SLY1 E3 ubiquitin ligase that targets DELLAs (Xu et al., 2021a). Taken together, these results show that blue light perceived by CRY1 antagonizes GA-induced cell elongation by enhancing the levels of DELLA repressors. DELLA stability is also controlled by a second E3 ubiquitin ligase (COP1/SPA) in a shade- and temperature-dependent, but GA-independent manner (Blanco-Touriñán et al., 2020). Hence, DELLAs are likely stabilized in light-grown seedlings *via* COP1-dependent and COP1-independent mechanisms.

Auxin is a key hormone in photomorphogenesis and, in particular, in shade avoidance responses. Generally, it promotes cell elongation in darkness and canopy shade (Ma and Li, 2019). CRY1 represses auxin-induced cell elongation in blue light, thereby contributing to the inhibition of hypocotyl elongation in blue light. Treatment of seedlings with external auxin solutions indicated that photoactivated CRY1 inhibits the responsiveness of seedlings to auxin, suggesting that CRY1 inhibits auxin signal transduction (Xu et al., 2018). The nuclear auxin-signaling pathway is quite well-understood (Powers and Strader, 2020): auxin is perceived by a co-receptor complex consisting of an F-box protein of the TRANSPORT INHIBITOR RESPONSE 1/AUXIN-SIGNALING F-BOX (TIR1/AFB) family and a member of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) protein family. Upon binding of auxin, this co-receptor complex assembles, followed by polyubiquitination and degradation of the AUX/IAA repressor protein by the TIR1/AFB ubiquitin ligase. The degradation of AUX/IAA repressors activates auxin-responsive transcription factors (ARFs) to initiate gene regulation and the inhibition of cell elongation in response to auxin. CRY1 signaling directly interferes with the auxin-signaling pathway *via* at least two molecular mechanisms. It was found that the PHR1 directly binds AUX/IAA proteins in a blue light-dependent fashion, thereby inhibiting the interaction of the TIR1/AFB F-Box proteins with AUX/IAAs which causes stabilization of AUX/IAA proteins in the presence of auxin. Hence, blue light-activated CRY1 increases AUX/IAA repressor activity, thereby inhibiting cell elongation in blue light (Xu et al., 2018). In a second mechanism, CRY1 was found to interact with the transcription factors ARF6 and ARF8 in blue light.

This interaction is also conferred by the PHR1 domain. The binding of PHR1 to ARF6 and ARF8 reduces the DNA-binding activity of these transcription factors *in vitro*. Moreover, *in vivo* chromatin immunoprecipitation experiments confirmed that blue light inhibits the binding of ARF6 and ARF8 to known target genes involved in cell elongation (Mao et al., 2020). In summary, these results indicate that CRY1 inhibits auxin signaling by stabilizing AUX/IAA repressors and by inhibiting the activity of ARF6/ARF8 activators of cell elongation genes. Interestingly, AUX/IAAs and ARF6/ARF8 were also found to interact with phytochrome B in red light, suggesting that phytochrome B and CRY1 share these mechanisms in promoting deetiolation in the light (Colón-Carmona et al., 2000; Xu et al., 2018; Mao et al., 2020).

CRYs also alter the response to the hormone class of brassinosteroids (BRs). BRs promote cell elongation and are required for seedling etiolation in darkness and shade (Lv and Li, 2020). For example, a seedling defective in BR biosynthesis or perception undergoes constitutive photomorphogenesis in darkness (Li et al., 1996; Li and Chory, 1997). CRYs inhibit BR signaling by direct interaction with BR signaling intermediates. CRY1 and CRY2 interact with the transcriptional activators BRI1-EMS-SUPPRESSOR 1 (BES1) and BZR1 to inhibit their activity in the BR-activation of gene expression. Moreover, CRY1 interacts with the kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2) which promotes the phosphorylation of BZR1 by BIN2, resulting in reduced BZR1 protein levels in the nucleus (Wang et al., 2018b; He et al., 2019). Hence, CRYs inhibit the levels and activities of two master regulators of BR-response, BES1, and BZR1.

Cryptochrome-Interacting Chromatin Remodeling Proteins

A recent report provided evidence for a direct regulation of chromatin remodeling by CRY1 (Mao et al., 2021). Chromatin-remodeling or multi-dimensional alterations in chromatin architecture regulate gene expression by modifying the access of genomic DNA to the transcriptional regulatory machinery. Histone H2A.Z, a variant of histone H2A, modulates diverse cellular processes largely by regulating nucleosome composition and affecting DNA methylation. In eukaryotes, an exchange of H2A for H2A.Z by the SWI2/SNF2-RELATED (SWR1) chromatin-remodeling complex can lead to activation or repression of gene expression at the respective loci (Jarillo and Piñeiro, 2015; Aslam et al., 2019). CRY1 was shown to interact with two essential components of the SWR1 complex (SWC6 and ARP6) in a blue light-dependent manner, suggesting that chromatin remodeling may be important for photomorphogenesis. Indeed, *swc6*, *arp6*, and *h2a.z* mutant seedlings are hyposensitive to blue light, exhibiting longer hypocotyls than the wild type in blue light but not in darkness (Mao et al., 2021). Hence, H2A.Z histone deposition promotes photomorphogenesis by inhibiting the hypocotyl elongation in blue light. It is therefore hypothesized that CRY1 activates photomorphogenesis in part by promoting H2A.Z deposition. Mechanistically, this may occur by promoting SWR1 complex assembly because CRY1 enhances the interaction between SWC6 and ARP6. To address how SWR1 may be recruited to genes relevant for photomorphogenesis, it was tested whether SWR1 interacts with HY5, a key transcription factor in photomorphogenesis. It was found that SWC6, as well as ARP6, interact with HY5; moreover, CRYs, HY5, and SWC6/ARP6 promote H2A.Z deposition in blue light at selected HY5 target genes that are repressed by HY5 in blue light (Mao et al., 2021). Though HY5 mainly acts in transcription activation (Burko et al., 2020), it can also directly act in gene repression of these cell elongation-related genes (*IAA19*, *expansin*, *XTH*). Hence, the SWR1 complex may be critical in turning HY5 into a repressor of gene expression which subsequently contributes to the inhibition of hypocotyl elongation in blue light (Mao et al., 2021). Interestingly, chromatin-remodeling mutants are also hyposensitive to red and far-red light, indicating that the SWR1 complex is also important for phytochrome signaling. Further studies have shown that phytochrome B interacts with SWC6 and ARP6 (Mao et al., 2021; Wei et al., 2021).

Cryptochromes, RNA Modification by N^6 Methyladenosine, and Liquid–Liquid Phase Separation

The modification of RNAs by methylation of N^6 adenosine (m^6A) has emerged as an important regulatory mechanism. It is quite well-described in animals, while its effects are only beginning to be discovered in plants. In animals, m^6A-methylation can affect RNA metabolism at several levels, including splicing, transcript stability, polyadenylation, RNA export, and translation (Reichel et al., 2019). m^6A is deposited on RNA by so-called "writers," a multiprotein complex with methylase activity. "Readers" interpret the code by binding to the m^6A modification followed by posttranscriptional regulation, and "erasers" remove the m^6A modification. Epitranscriptome analyses in mammals indicated that on average one m^6A modification occurs every 2000 bp and, therefore, is quite frequent (Reichel et al., 2019).

Purification of CRY2-associated proteins by immunoprecipitation and mass spectrometry identified several m^6A writer proteins. These interactions were confirmed by direct protein–protein interaction assays (Wang et al., 2021b). CRY2 interacted with the writers mRNA adenosine methylase (MTA), METHYLTRANSFERASE B (MTB), and FK506-BINDING PROTEIN 12 (FKBP12) INTERACTING PROTEIN 37 (FIP37) in heterologous HEK293 cells and in planta in split-YFP assays and co-immunoprecipitations. Domain mapping indicated that both PHR2 and CCT2 interacted with the writers. Interestingly, CRY1 also interacted with writers, though this interaction was not explored further. To assess the biological relevance of these interactions, the m^6A epitranscriptome between wild-type and *cry1 cry2* mutant seedlings was compared in darkness and light. CRY-dependent m^6A marks were found in particular in the 3′UTR of transcripts which is consistent with a general enrichment of m^6A deposits in 3′UTRs in mammals (Reichel et al., 2019). In particular, RNAs of genes controlled by the circadian

clock, such as *CCA1*, were enriched in blue light- and CRY-dependent m^{6}A modification. The authors, therefore, hypothesized that m^{6}A marks on clock-controlled transcripts may affect mRNA abundance and the circadian clock. Indeed, writer-defective *mta* mutants and *cry1 cry2* mutants exhibited reduced *CCA1* transcript stability in blue light, but not in darkness, when compared to the wild type, suggesting that m^{6}A deposition enhances *CCA1* transcript stability in blue light. Consistent with this finding, *mta* mutants exhibited a longer period length under free-running, continuous white light conditions, similar to the *cry1 cry2* mutant. This phenotype, however, was lost when *mta* mutants were transferred to free-running blue light conditions which may be due to antagonistic functions of phytochromes and CRYs (Wang et al., 2021b).

While there are CRY-dependent m^{6}A depositions on RNAs in blue light, the interaction of CRY2 with writer proteins was not blue light-dependent but also observed in darkness (Wang et al., 2021b). This raised the question of how blue light may control m^{6}A modification through CRYs. Interestingly, it was found that the recruitment of the MTA protein into CRY2 photobodies was blue light-dependent, raising the possibility that MTA concentrations locally increase in CRY2 photobodies under blue light without changing the affinity for interacting proteins (Wang et al., 2021b). Photobodies have been described as membrane-less compartments that concentrate molecules through liquid–liquid phase separation (LLPS; Pardi and Nusinow, 2021). During LLPS, two liquid phases are formed, a denser and a less dense one, thereby locally changing the concentration of biomolecules without the need for membranes. LLPS condensates have certain characteristics (Emenecker et al., 2021; Xu et al., 2021b) that were found also in CRY2 photobodies, such as spherical shape, dynamic nature as shown by rapid photobody recovery after photobleaching, reversibility in darkness, and the capacity of smaller photobodies rapidly fusing to form larger ones. Taken together, these results suggest that CRY2 undergoes LLPS in blue light which leads to a local increase in the concentration of recruited MTA proteins. This may enhance m^{6}A modification of RNAs in blue light (Quail, 2021; Wang et al., 2021b). Interestingly, the RNA-binding, flowering time regulator FLOWERING CONTROL LOCUS A (FCA) that contains prion-like domains shows LLPS behavior *in vivo* (Fang et al., 2019). Another prion-like domain-containing protein EARLY FLOWERING 3 (ELF3) was recently shown to undergo LLPS in response to high ambient temperatures (Jung et al., 2020). These pieces of evidence indicate that LLPS in plants is not limited to photobodies.

CONCLUSION AND PERSPECTIVES

Figures 1, 2 provide overviews of CRY photoactivation and signaling pathways along with the major downstream developmental responses. In addition to the CCTs, the PHR domains interact with many proteins that are integral to CRY functions (**Table 1**). While many fundamental questions starting from the exact mechanisms of CRY photoactivation and dark/thermal reversion remain to be answered, breakthroughs in recent years have immensely expanded our knowledge and presented us with more challenges.

Recent research showed that the active conformation of CRYs involve heterooligomers that are formed upon exposure to blue light. Besides CRY heterooligomers, the functional CRY supercomplexes may include many CRY-interacting proteins. An interesting question related to the dynamics of CRY complexes is: What are the mechanisms that regulate the formation and dissociation of such assemblies? As light responses are dissimilar at tissue and cell levels, the dynamics of the CRY complexes may be controlled at spatial levels. Temporal control of CRY supercomplexes can also be expected as tissues and organs at different maturity levels may respond differently to light signals. Changing light intensities under natural conditions may pose another layer of complexity. In particular, it is not well understood whether cryptochrome signaling enhances the fitness of plants under natural conditions. The LLPS nature of CRY photobodies may hold further clues. As CRY photobodies have been shown to act as hubs for mRNA methylation and gene expression, the influence of CRYs may not be restricted to the circadian genes and may regulate the transcriptional landscape in general. Advanced technologies, such as single-cell epigenomic and epitranscriptomic assays, may bring further breakthroughs in CRY research.

AUTHOR CONTRIBUTIONS

JP and UH wrote the manuscript. JP designed the figure and assembled **Table 1**. All authors contributed to the article and approved the submitted version.

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EDITED BY
Margaret Ahmad,
Université Paris-Sorbonne, France

REVIEWED BY
Patrick Emery,
University of Massachusetts Medical School, United States
Giorgio F Gilestro,
Imperial College London, United Kingdom
Martin Vacha,
Masaryk University, Czechia

*CORRESPONDENCE
Charalambos P. Kyriacou,
cpk@leicester.ac.uk

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Genetic analysis of cryptochrome in insect magnetosensitivity

Charalambos P. Kyriacou* and Ezio Rosato

Department of Genetics and Genome Biology, University of Leicester, Leicester, United Kingdom

The earth's magnetic field plays an important role in the spectacular migrations and navigational abilities of many higher animals, particularly birds. However, these organisms are not amenable to genetic analysis, unlike the model fruitfly, *Drosophila melanogaster,* which can respond to magnetic fields under laboratory conditions. We therefore review the field of insect magnetosensitivity focusing on the role of the Cryptochromes (CRYs) that were first identified in *Arabidopsis* and *Drosophila* as key molecular components of circadian photo-entrainment pathways. Physico-chemical studies suggest that photo-activation of flavin adenine dinucleotide (FAD) bound to CRY generates a $FAD^{\circ-}$ $Trp^{\circ+}$ radical pair as electrons skip along a chain of specific Trp residues and that the quantum spin chemistry of these radicals is sensitive to magnetic fields. The manipulation of CRY in several insect species has been performed using gene editing, replacement/rescue and knockdown methods. The effects of these various mutations on magnetosensitivity have revealed a number of surprises that are discussed in the light of recent developments from both *in vivo* and *in vitro* studies.

KEYWORDS

cryptochrome, *Drosophila*, radical pairs, electron spin, quantum, migration, circadian

Introduction

The magnetosensitivity of migratory birds has long fascinated the biological community with the first demonstration of this phenomenon revealed in the European Robin more than 50 years ago (Wiltschko and Merkel, 1965). It was subsequently discovered that bird magnetic compass navigation was light-dependent and functioned in the blue and green but not red wavelengths (Wiltschko et al., 1993a; Wiltschko and Wiltschko, 1995). However, although the underlying molecular mechanism has proved to be stubbornly elusive, there has been significant progress that can be traced back to the proposal that a class of proteins that were originally identified in plants and animals, the Cryptochromes (CRYs), had the potential to mediate magnetic responses via a radical pair mechanism (RPM) (Ritz et al., 2000). The first CRY-encoding genes to be isolated were the blue light photoreceptors in *Arabidopsis thaliana, AtCry1* (Ahmad and Cashmore, 1993) and *Drosophila melanogaster, dcry* (Emery et al., 1998; Stanewsky et al., 1998). Both molecules were shown to be intimately involved in circadian light responses, in plants and flies respectively (Cashmore et al., 1999). Putative

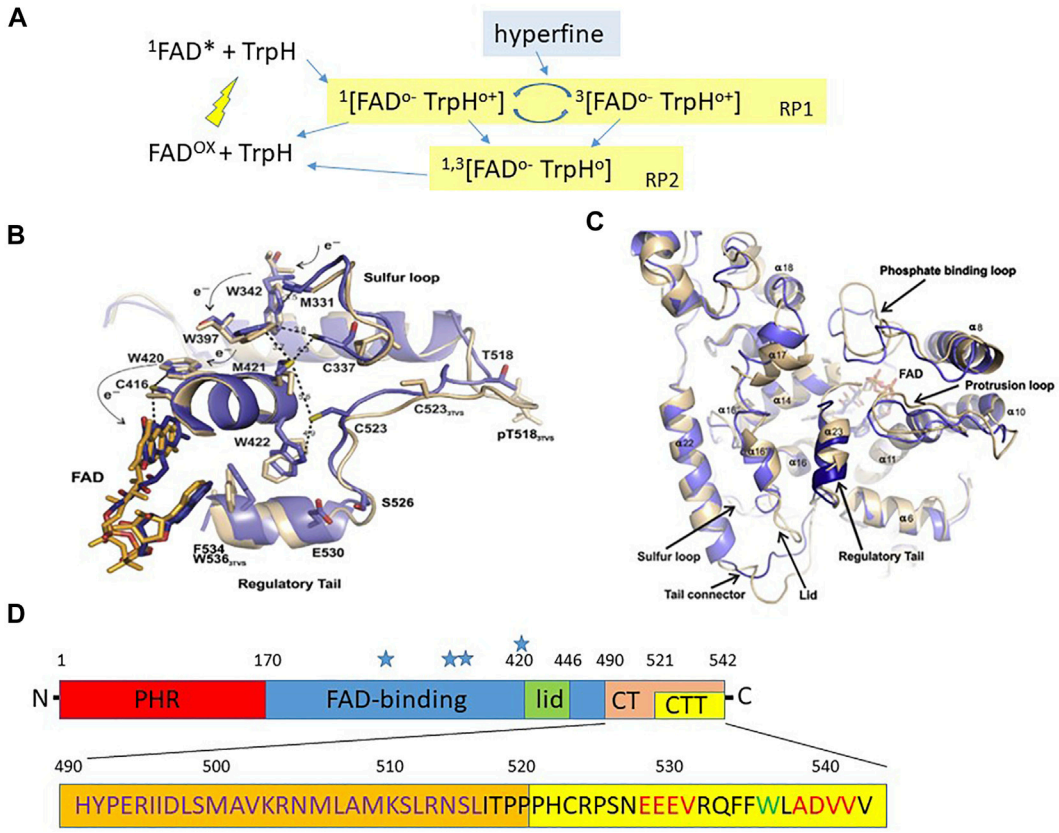

FIGURE 1
Structure of *Drosophila* cryptochrome.**(A)** The framework for a CRY photocycle using *Escherischia coli* photolyase (*Ec*PL) and *Arabidopsis thaliana* CRY1 (*At*CRY1). Photoexcitation excites the oxidised FAD (FAD^{ox}) to the singlet state ($^1FAD^*$) which then receives an electron via a chain of three Trp residues (see Figure 1B) within CRY/photolyase generating a radical pair leaving the terminal Trp minus an electron $^1[FAD^{o-}\ TrpH^{o+}]$ in the singlet state with antiparallel electron spins which can either reverse to the ground state (FAD^{ox} + TrpH) or interconvert to the triplet state $^3[FAD^{o-}\ TrpH^{o+}]$ *via* hyperfine interactions in which the electron spins of the unpaired electrons are in parallel. In *Ec*PL the S and T forms of this RP (RP1) can also convert to a second RP2 by deprotonation (removing H ion) of $TrpH^{o+}$ to the neutral radical, Trp^o which can return to the dark resting state (FAD^{ox} + TrpH) by further redox reactions (redrawn with amendments from Sheppard et al., 2017). **(B)** Crystal structure of *d*CRY regions lying close to the FAD (blue from Czarna et al., 2013, beige Zoltowski et al., 2011). The Trp triad W420, 397 and 342 are shown together with the proposed electron skipping that generates the photoreduced FAD-Trp radical pair. **(C)** Crystal structure of *d*CRY from Czarna et al (2013). The blue represent the structure from Czarna et al. compared to a previous structure in beige from Zoltowski et al (2011). There are some significant differences in the tail structure, later corrected by Zoltowski et al (2011). **(B,C)** reproduced with permission from Czarna et al. (2013). **(D)** Overview of *d*CRY landmarks (not to scale). PHR, Photolyase homology domain; CT, C terminal residues 490–542; CTT C terminal tail, residues 520–542; Blue stars indicate positions of Trp tetrad, Trp 342, 394, 397 and 420. The amino acid sequence of the CT is illustrated below. The calmodulin binding domain is shown in orange, residues ~490–516 in violet. PDZ domain binding motifs in the CTT are in red, and the sole Trp in the CTT is green. The *dCRYΔ* transgene is missing the CRY CTT and the *GFP-CRY CT* transgene encodes GFP fused to residues 490–542 (see text).

CRYs were also identified in humans and initially considered blue light responsive (Hsu et al., 1996).

Sequence comparisons revealed that the CRYs are evolutionary related to the photolyases, which represent an ancient class of enzymes that repair UV-induced DNA damage. The major difference between these two protein classes is in the extended C-terminal that is characteristic of the CRYs and plays a prominent role in both light and magnetic responses (Cashmore et al., 1999; Rosato et al., 2001; Fedele et al., 2014a; Fedele et al., 2014b). CRYs are ubiquitous and found in all branches of life and are part of a large gene family with at least six subgroups in metazoans (Haug et al., 2015). Animal CRYs can be divided functionally into three main classes. The first two encompass the *Drosophila*-like type 1 and the bird specific type 4 CRYs that are photosensitive. The third corresponds to the vertebrate-like type 2 CRYs that in many animals (including birds and insects) act as repressors that negatively regulate the circadian clock and may not be directly responsive to light. The circadian mechanism is an autoregulatory transcription/translation feedback loop that revolves around the rhythmic expression of its positive and negative regulators (reviewed in (Ozkaya and Rosato, 2012). The type 2 CRYs function as negative

regulators together with Period (PER) proteins. However, some animals, such as *Drosophila*, have lost type 2 CRYs so instead they use Timeless (TIM) in partnership with PER as circadian repressors (Yuan et al., 2007; Kotwica-Rolinska et al., 2022).

The radical pair model/mechanism

That radical pairs (RPs) could be sensitive to magnetic fields was proposed by Schulten et al. (1978). The formation of radicals requires energy provided by light that puts into motion an electron transfer from a donor (which, may be organised into a chain) to the excited acceptor molecule. Thus, the donor is left with one less electron and a positive charge, whereas the acceptor has one extra electron and a negative charge, resulting in two paired radicals. Paired electrons can spin in opposite antiparallel (singlet state, S) or in parallel directions (triplet states, T). The interconversion between S and T states is facilitated by the magnetic interaction with nearby atomic nuclei, called the hyperfine coupling, which produces a rapid S to T interconversion (Figure 1A) (Ritz et al., 2009). We can fancifully imagine that the oscillation between S and T states is similar to an elephant precariously and finely balanced on a ball, which can easily flip to the left or to the right. While an elephant standing on the ground cannot be moved easily, the one on the ball only needs a tiny push for it to fall. Using this (ludicrous) analogy, a tiny force (push) such as the earth's ~50 μT magnetic field (MF) can alter the dynamics of the unstable S to T interconversion (the elephant falling left or right) favouring one or the other spin state. This affects the half-life of the RP and of the associated conformational changes of the molecule, with corresponding downstream effects (Hore and Mouritsen, 2016).

D. melanogaster and *A. thaliana* CRYs are flavoproteins and have a FAD (flavin-adenine dinucleotide) binding site to which this catalytic chromophore attaches itself (Figure 1B). A second light-harvesting bound chromophore, pterin, that transfers energy on light activation to FAD is found in *Arabidopsis* *At*CRY1 (Hoang et al., 2008), but not apparently in Drosophila *d*CRY (Selby and Sancar, 2012). In *d*CRY, photoexcitation of FAD in its resting oxidised state by blue light (Figures 1A,B), generates electron transfer across a triad or tetrad (see below) of Tryptophan (Trp) residues (Sheppard et al., 2017). The FAD acceptor ends up with one extra electron (FAD^{o-} the semi-reduced flavosemiquinone radical) whereas the terminal Trp donor is left minus one electron Trp^{o+}, thereby generating the magnetically sensitive RP. From the structure of CRYs the three or four relevant Trps in dCRY are Trp342, 394, 397 and 420, with Trp342 or Trp394 representing the critical terminal residue that generates the radical pair with FAD (Figures 1A,B). The transfer of the electron from FAD^{o-} back to the terminal Trp^{o+} to return to oxidised FAD can only occur from the S state (Hore and Mouritsen, 2016). In CRYs, the earth's MF pushes the S-T interconversion towards the T state, causing the RP to persist for longer. Further light absorption fully reduces FAD^{o-} to $FADH^{-}$ that can be converted back to the oxidised form in darkness by O_2 (Hore and Mouritsen, 2016). This mechanism of reoxidation can generate other radical pairs, which in principle, may be sensitive to a MF even in darkness (Müller and Ahmad, 2011).

Much of the evidence for the existence of RPs within CRYs is based on *in vitro* spectroscopic analyses so it is important to consider whether these physico-chemical and often theoretical approaches can be supported *in vivo*. Assuming the RPM provides the fundamental quantum basis for magnetosensitivity what else would be required to make CRY the cognate receptor for avian navigation? Given the necessary photobiology, CRY should be associated with an organ that is exposed to light and the eye is the obvious candidate. Indeed, the first suggestions of radical pairs in magnetosensitivity were focused on rhodopsins (Hong, 1977; Leask, 1977). A second requirement would be that the RP of CRY should be sensitive to the inclination of the earth's magnetic field as that is the geophysical feature that provides navigational cues (Wiltschko et al., 1993b). One way that might occur is by CRY having a fixed orientation within the containing cells (presumably neurons so that they can generate a behavioural output) and these being spatially organised in a certain fixed orientation within the eye. As the bird turns its head (and its eyes), changes in the orientation of the MF with respect to the bird's body axis alter the spin dynamics of the RP that allows the bird to compensate its direction of flight. Ritz et al. (2000) provide an interesting early theoretical model and simulation of how this might work. How birds might 'see' the magnetic compass lines by dipole interactions within the retina between the RP generating molecule and the opsins has also been discussed (Stoneham et al., 2012).

A RPM underlying navigation is difficult to study in birds because, unlike model organisms such as *Drosophila* they are not easily amenable to molecular and genetic analyses. However, flies are not known for their prowess in navigation and migration so could they provide a model system that is useful to study the RPM and be acceptable to ornithologists and physical-chemists? First, consider that the putatively 'relevant' CRY of the night-migratory European Robin, *Erithacus rubecula* (ErCRY4), shares the relative positions of the four Trps that theoretically mediate the formation of the RP, with both *D. melanogaster* CRY (*d*CRY), and that of the long-distance migrant the Monarch butterfly *Danaus plexippus* (*Dp*CRY1)(Xu et al., 2021). Second, it has been demonstrated repeatedly, by several different laboratories using various paradigms that *Drosophila* and Monarch respond behaviourally to MFs (see later section). Third, while the behavioural output of MF experiments is very different in birds, Monarch and *Drosophila*, given the conservation of the four RP-related Trps in CRYs, the fundamental quantum spin chemistry underlying magnetosensitivity is likely to be the same in these species whereas the downstream effectors of the CRYs

may be different according to species and even cell type. If we accept these premises, then insects such as *Drosophila*, which have the most sophisticated molecular genetic toolkit available, can provide a rigorous and critical experimental analysis of the RP model.

Molecular and structural basis for CRY photoactivation and magnetoreception

While we have discussed the quantum biology and spin dynamics underlying the RPM we have not introduced the molecular/biochemical/structural basis for CRY-mediated magnetosensitivity. Figures 1B,C illustrate the important structural features of CRY (Czarna et al., 2013) relevant to photo and magnetoreception and Figure 1D shows the overall architecture of *d*CRY based on structural, functional and bioinformatics analyses (Rosato et al., 2001; Hemsley et al., 2007). The N-terminal corresponds to the photolyase homology region (PHR) followed by the more central FAD binding region (Figure 1D). Towards the C-terminal (CT, residues 490–542) there is a lid (residues ~420–446) and the tail (CTT, residues 521–542). The C-terminal tail (CTT) that is absent in the *d*CRYΔ mutant (discussed below) distinguishes *d*CRY from the photolyases in that it largely replaces the latter's DNA substrate bound to the FAD pocket (Zoltowski et al., 2011; Czarna et al., 2013; Levy et al., 2013). Without the CTT, dCRYΔ is unstable because it adopts the active ("open") conformation in both light and dark, which makes it constitutively prone to signalling and to degradation (Rosato et al., 2001). Possibly, such instability is due to the unrestricted access by BRWD3 (part of the CRL4 E3 ubiquitin ligase complex) to the FAD pocket, resulting in continuous ubiquitination and proteasomal degradation (Ozturk et al., 2013; Kutta et al., 2018). Within the CTT there are two putative PDZ domain binding motifs and a Trp536 residue. A calmodulin (CaM) binding site is also predicted within a region (residues 490–516) that is a hotspot for protein-protein interactions (Rosato et al., 2001; Hemsley et al., 2007; Mazzotta et al., 2013).

This linear representation of *d*CRY in Figure 1D conceals the folded nature of the protein which is revealed in structural studies showing that the PHR FAD, lid, CT and CTT all lie in close proximity (Figures 1B,C) (Zoltowski et al., 2011; Czarna et al., 2013; Levy et al., 2013). Photoinduction of FAD generates the RP through electron-skipping and mutations Trp397Phe and Trp420Phe obliterate FAD^{o-} formation (Czarna et al., 2013). Mutational analysis of other residues close to each of Trps 420, 397, 342, namely Cys416, Cys337 and Met331 reveals that they are involved in the gating of the electron transfer. In the CTT, Cys523 is important for FAD photoreduction and Phe534, Glu530 and Ser526, anchor the tail to the PHR region in the dark state whereas Ser526 may act as a hinge for the CTT. Consistent with this is the effects of a Ser526Ala mutation that prevents the light degradation of dCRY (Czarna et al., 2013) and the physical interaction of *d*CRY with TIM and PER (Hemsley et al., 2007). Light induced FAD photoreduction displaces the CTT allowing TIM (acidic) and Jetlag (JET, basic, involved in CRY-dependent, light-mediated degradation of TIM; Peschel et al., 2009) to associate with acidic and basic regions adjacent to the CTT. This explains mechanistically why *d*CRYΔ and an almost identical $dCRY^{M}$ mutant that are both missing the CTT are constitutively active in both light and dark (Rosato et al., 2001; Busza et al., 2004; Dissel et al., 2004). The CTT therefore prevents the association in the dark of *d*CRY with the key factors TIM and JET, which is necessary for circadian light responses. Figure 1B shows the location of these functionally important *d*CRY residues in relation to the FAD.

Photoactivation of CRY is a critical step for both circadian entrainment in insects and in RP biology and this relies on bound FAD. Given that Type 1 CRYs are photosensitive and Type 2 apparently not, what is the evidence that FAD binds each CRY type? This is particularly relevant in the debate about whether type 2 circadian repressor CRYs are magnetosensitive, with some studies showing that they are while others that they are not. When purified, dCRY binds to FAD in its oxidised form and on blue light exposure converts to the FAD^{o-} radical (Berndt et al., 2007). The crystal structure of *d*CRY reveals the absence of the characteristic residues to bind pterin (an additional cofactor bound to 6-4 photolyases) in contrast to the well-conserved FAD binding centre (Czarna et al., 2013). The corresponding FAD domain in mouse CRY1 (there are two type 2 CRYs, confusingly, mCRY1 and mCRY2) is also present, so theoretically it can bind FAD. This is in contrast to a study in which mammalian CRYs appeared unlikely to bind physiologically relevant quantities of FAD, as the binding pocket would be too open to 'hold' the cofactor for long enough and this would result in constitutive activation of the protein (Kutta et al., 2017). A further study has introduced another twist, revealing that FAD stabilises mammalian CRYs (Hirano et al., 2017). Ubiquitin ligase FBXL3 binds to the FAD pocket, so competition between cofactor and FBXL3 binding modulates CRY stability and alters the kinetics of CRY ubiquitination and subsequent degradation (Xing et al., 2013). A similar mechanism has been suggested for *d*CRY, with BRWD3 having a similar function to mammalian FBXL3 (Ozturk et al., 2013; Kutta et al., 2018). Interestingly, the first mutant identified in *Drosophila*, cry^{b}, revealed a missense mutation in the FAD binding domain resulting in a highly unstable protein (Stanewsky et al., 1998). Additionally, the protracted light activation of *d*CRY seemingly results in the irreversible opening of the FAD pocket and the expulsion of the cofactor, making activated *d*CRY quite similar in structure to mammalian type 2 CRYs (Kutta et al., 2018). Thus, for both type 1 and 2 CRYs, FAD binding is associated with stability rendering the two types more similar than previously believed. Perhaps then, their photo- and magnetosensitivity differences are more

quantitative than qualitative so that biologically relevant quantities of FAD may bind in an appropriate cellular environment such as an insect cell.

Genetic analysis of magnetic phenotypes in *Drosophila*

Training and testing phenotypes

A demonstration of a magnetic sense in *Drosophila* had flies trained under a wavelength of light of 365 nm in an ambient field facing north and tested in an 8 arm radial maze spanning each point in the compass (Phillips and Sayeed, 1993). Male (but not female) flies showed a strong tendency to choose the northern pointing arm of the maze. Under a green 500 nm light source, this preference was shifted by 90° to the east. These experiments revealed that the magnetic sense can be stimulated by light in the UV/blue to the green wavelengths which fits with the spectral characteristics of *Drosophila* opsins, particularly Rh5 and Rh6 as well as dCRY (VanVickle-Chavez and Van Gelder, 2007; Sharkey et al., 2020).

Subsequent genetic analyses employed flies that were trained in a T-maze by associating a sucrose reward with exposure to a magnetic field (Gegear et al., 2008; Gegear et al., 2010). Under full spectrum light, naïve flies tend to avoid a MF, but after training, they modestly (but significantly) preferred to enter the arm that was exposed to the MF. This behavioural effect could be demonstrated up to a wavelength of 420 nm, which matches the action spectrum of dCRY, and *cry* mutants were defective in this trained magneto-response. A follow-up study revealed that *cry-null* mutant transgenic flies carrying the *D. plexippus* Monarch *Dpcry1* and *Dpcry2* genes could also be trained to move towards a MF up to a wavelength of 420 nm. Furthermore, mutating (what was considered at the time) the terminal Trp to phenylalanine (Phe) that cannot generate a RP in both *Drosophila* dCRY (Trp342Phe) and Monarch *Dp*CRY1 (Trp328Phe) did not disrupt the normal magnetoresponse but did so for the terminal Trp in Monarch *Dp*CRY2. As these mutations should prevent the electron skipping that generate the RP, the results cast some initial doubt on the canonical RP model. However, since these studies, the possibility has been raised that there is a fourth terminal Trp (which would be Trp394 in *d*CRY) that would remain intact in these *d*CRY mutants (Muller et al., 2015; Nohr et al., 2016). However, its role in magnetosensitivity, either as the terminal Trp or as a functional partner to Trp342 remains to be determined (Muller et al., 2015; Nohr et al., 2016; Wong et al., 2021).

One of the surprising results from these experiments is that *D. plexippus Dp*CRY2 also gave a magnetic response in transgenic flies. Using the same assay, human *h*CRY2, which is normally highly expressed in the human retina (Thompson et al., 2003), was also shown to be magnetosensitive in transgenic *Drosophila* (Foley et al., 2011). As CRY2s are not generally believed to be light-sensitive (despite Hsu et al., 1996) it might suggest the possibility that other light-sensitive molecules in the transgenic fly, such as the fly opsins, might be priming CRY2s to mediate the magneto-response, in effect transferring the light signal to CRY2s.

Circadian-based phenotypes

*D*CRY is considered a dedicated circadian photoreceptor in *Drosophila*, so an attempt was made to observe MF effects on free-running circadian locomotor activity cycles (Yoshii et al., 2009). Constant dim blue light (470 nm) that was not intense enough to cause arrhythmicity was used to lengthen free-running circadian period providing a 'sensitised' background on which a static MFs could be applied. The results were quite variable with 50% of the flies showing no effect but of those that did respond to the MF (300 μT), the majority lengthened their free-running period. The net change in period irrespective of direction (longer or shorter) was greater for the exposed flies compared to controls (0 μT). When repeated in red light there was no difference in net period change between experimental and control groups. The use of the nearly null cry^b mutant was not informative as these mutants do not 'see' the light so their period does not lengthen under constant dim blue light - they simply free-run as if in constant darkness (DD) with no period changes on exposure to the MF (see also Figure 2D). A more relevant result was obtained when dCRY was overexpressed in clock neurons. Most flies were arrhythmic in dim blue light, but of those few that were rhythmic, the net change in period was greater than that of wild-type. These results suggested that dCRY might be involved in the fly's magnetic response.

Given that the effects of Yoshii et al. (2009) were quite modest, an attempt to replicate these findings was made using state-of-the-art exposure chambers, a constant dim blue light at 450 nm that lengthened free-running period and exposure to static/low-frequency fields (0, 3 and 50 Hz) under different field intensities (90, 300, 1000 μT) (Fedele et al., 2014a). In all these experiments, although the free-running periods were much longer than 24 h, flies exposed to a MF nevertheless showed a consistent reduction in period length compared to sham controls (Figure 2A). These results were in contrast to those of Yoshii et al. (2009) who generally observed that most flies exposed to a MF tended to have longer periods than sham controls. To examine further this apparent discrepancy, the experiments were repeated under green light at 500 nm, which revealed the opposite phenotype to blue light, namely a further significant period lengthening under MF exposure when compared to sham. This blue-green difference in magnetic responses has been noted several times by Phillips and collaborators in different species and is interpreted as reflecting an antagonistic effect at the two wavelengths (Phillips et al., 2010). Thus, the 470 nm

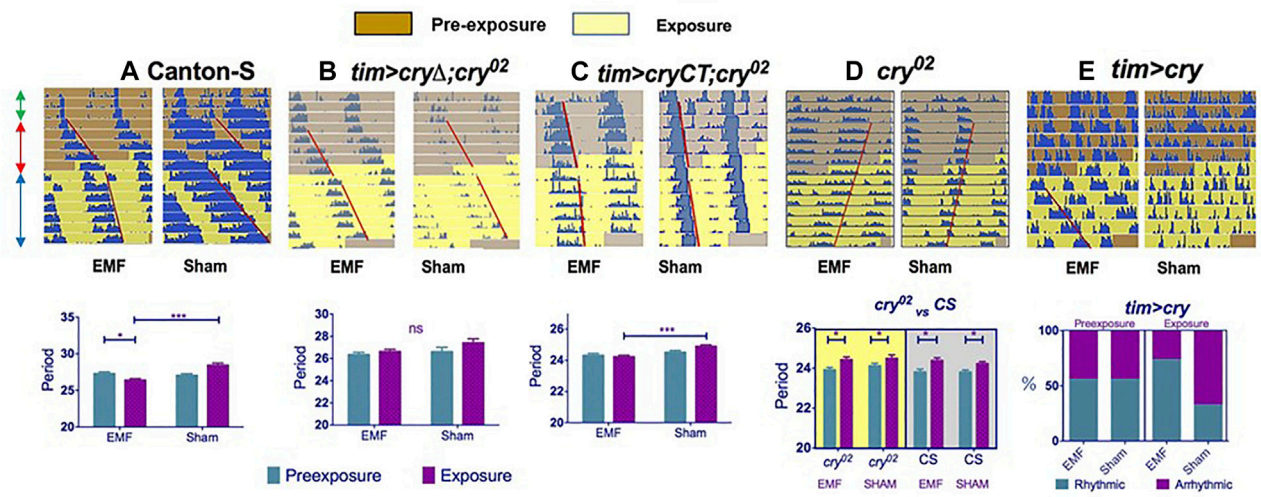

FIGURE 2
Exposure to a low frequency magnetic field has a circadian phenotype in *Drosophila*. Top row **(A–E)**: double plotted representative actograms from a male fly from each genotype. The left hand actogram for each genotype shows the activity of a fly exposed to a MF, whereas the right hand panel shows the activity under sham exposure. Each row of the actogram shows 2 days of activity day1 and day2, below which is represented day2 and day3, then day 3 days 4 and so on. The double headed green arrows on the left of panel A show the first 3 days in a dim blue light-dark BLD12:12 cycle, followed by 5 days in constant dim blue light, BLL (prexposure, red arrows, brown background on actogram), followed by 8 days in the same BLL conditions but with exposure (or sham) to a 3 Hz, 300 μT MF (blue arrows, yellow background). Red lines show offsets of free-running locomotor activity reflecting any change in period after exposure. **(A–C)** The graphs below show the corresponding mean free-running period and sems for each genotype calculated from the pre-exposure and exposure conditions in the sham and experimental groups. **(A)**: Canton-S wild-type **(B)**: *tim > cryΔ;cry^{02}* (*timGAL4; UAScryΔ;cry^{02}*) which means CRY that is missing the CTT (last 20 amino acids) is expressed only in clock cells in a *cry-null* background. **(C)**: *tim > CRYCT;cry^{02}* (*timGAL4; UAS-GFP-CRYCT;cry^{02}*) which expresses only the 52 amino acid CRYCT (fused to GFP for stability) in clock neurons (*tim > CRYCT;cry^{02}*) in a *cry-null* background. **(A,C)** show significant period shortening under exposure (but not sham), whereas the MF has no significant effect on period for the *cryΔ* transgene (see Figure 1). **(D)** The *cry^{02}* null mutant shows no changes in period on exposure to a MF compare to sham (upper panel). The lower panel reveals that the response of *cry^{02}* flies in constant dim BL (shown as actograms in the upper panel and on a yellow background in the lower panel) is no different from the response of *cry^{02}* mutants in constant darkness (gray background in lower panel). Consequently by not showing any period lengthening under BL, *cry^{02}* mutants are not informative in this assay. **(E)** In contrast to *cry^{02}*, overexpression of CRY in clock neurons (*tim > cry*) in a wild-type background leads to high levels of arrhythmicity in the prexposure conditions, but application of a MF significantly rescues this arrhythmicity (upper and lower panels). Furthermore, rhythmic flies on exposure to a MF have shorter periods than those rhythmic flies that are exposed to sham (not shown, see Fedele et al., 2014a). Figure 2 redrawn from Fedele et al. (2014a).

wavelength used by Yoshii et al (2009) would mix both the blue (reduced period length compared to sham) and the green (increased period length) circadian MF responses, almost cancelling out any period phenotype.

Fedele et al (2014a), in agreement with Yoshii et al. (2009), also observed that overexpression of dCRY in clock neurons gave high levels of arrhythmicity under blue LL, consistent with the known role of CRY. However, on exposure to the MF, many of the same flies that were previously arrhythmic in LL now became rhythmic (Figure 2E), as if the MF exposure had suppressed the normal response of CRY to light (Fedele et al., 2014a). This same interpretation, can explain the shorter periods under the MF in blue light (Figure 2). When dCRY is activated by light it drives a longer circadian period and arrhythmicity (depending on light intensity and duration) as well as its own degradation. Perhaps the MF reduces the normal light response, which in dim constant LL, should give a longer and longer period that eventually merges to arrhythmicity? Indeed, under a MF in constant blue LL, dCRY extracted from fly heads appears to be more stable than under sham MF, supporting the view that the MF is somehow curbing the normal CRY-mediated light degradation response (Fedele et al., 2014a). Further experiments revealed that the Trp342Phe mutation of the putative 'terminal' Trp did not prevent the circadian MF response, mirroring the results of the T-maze conditioning assay described above. However, the most unexpected result was observed when a ~50 residue fragment of the CRY C-terminal (CT) was expressed in a *cry-null* background. This transgene has the sequences encoding the CRYCT fused to those for GFP for stability (*GFP-CT*) and does not carry the four canonical Trps of the electron chain nor the FAD binding domain implicated in the RP mechanism. Nevertheless, and quite remarkably, this GFP-CT fragment was sufficient to mediate the circadian MF response when expressed in clock neurons (Figure 2C)(Fedele, et al., 2014a)

A second phenotype also emerged from the locomotor behaviour analysis. The levels of activity were altered under MF exposure so that wild-type flies became hyperactive. Intriguingly, flies carrying a *dcry* transgene missing the

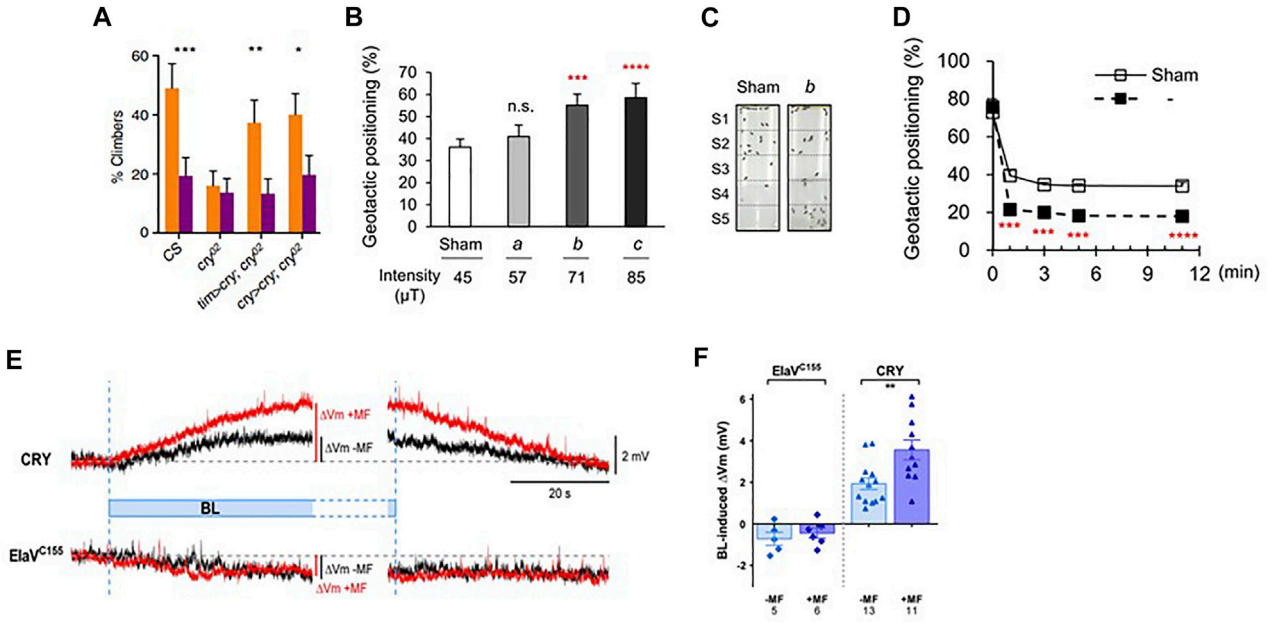

FIGURE 3
MF exposure effects on geotaxis and neuronal firing in *Drosophila*. **(A)** Results of geotaxis assay from Fedele et al. (2014b). Under 450 nm blue light wild-type Canton S (CS) flies walk upwards (negative geotaxis), but application of a MF makes them more positively geotactic generating a significantly lower climbing score as they tend to move downwards. cry^{02} mutants show reduced negative geotaxis, so application of a MF does not change their behaviour, therefore as in circadian behaviour (Figure 2D) this result is not informative with respect to the MF. However, expressing CRY in a mutant cry^{02} background either in all clock neurons (*tim* > *cry*) or just those that normally express CRY (*cry* > *cry*), rescues the normal negative geotactic response in sham exposed flies which is significantly reduced in MF exposed flies. Means and sems shown. Flies were exposed to a static 500 μT MF in dim blue light.*, $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(B,C)**. Results of similar experiment as in A by Bae et al (2016). Geotactic positioning (positive geotaxis) in a tube, very similar to that used by Fedele et al., 2014b (see above, A) and shown in C, at different MF intensities. The graph shows means and sems for flies exposed to the earth's ambient field (sham, 45 μT in Korea) with increasing intensity of exposure. In this case, the results are represented as the flies' positive geotaxis score (% flies in lower S2-S5 sections of tube shown in C, so higher scores means the flies are moving further downwards). As field intensity increases, flies move further downwards generating a higher geotactic score, as observed in Fedele et al, 2014a). ***$p < 0.0005$, ****$p < 0.0001$ compared to sham. **(D)** Geotaxis in a near zero field (Bae et al., 2016). Geotaxis was compared in a zero (labelled 'b' see C) versus ambient 45 μT (sham) field. The ambient (sham, 45 μT) field significantly enhanced positive geotaxis (open squares) compared to zero field (filled squares), so flies moved higher in zero field, in contrast to the downward (positive geotactic) effect of the ambient MF on geotaxis. ***$p < 0.0005$, ****$p < 0.0001$. Figures **(B–D)** reused under open access creative commons license (http://creativecommons.org/publicdomain/zero/1.0/). **(E,F)** Magnetic field effects on neuronal firing in *Drosophila* larval aCC neuron (reproduced from Figure 1 in Giachello et al., 2016 under creative common license). **(E)** Representative electrophysiological recordings from a larval aCC neuron expressing CRY ectopically by using the pan-neuronal *elavgal4* driver driving *UAS-dcry* (labelled 'CRY') compared to control (*elavGAL4* driver only). Blue light produces significant reversible depolarization in membrane potential (ΔV_m, Y-axis, black trace) that is potentiated by a 100 mT MF (red trace). The *elavgal4* driver control generates an increased depolarisation to BL (black) but no enhancement by the MF (red). **(F)** The results from a group of larvae are shown. Means and sems **$p < 0.01$.

C-terminal tail (CTT) of ~20 residues (*dCRYΔ*), did not show circadian-period shortening under MF exposure, but were nevertheless hyperactive, whereas the GFP-CT encoding transgene that produced period-shortening, did not generate hyperactivity. A similar hyperactivity was found for flies carrying the *hCry2* (but not the *hCry1*) transgene. Consequently, the N-terminal region of *d*CRY that contains the four Trps and the FAD binding domain can mediate MF induced hyperactivity but not the period shortening, with the opposite phenotypes generated by the CRY C terminal (GFP-CT) (Fedele, et al., 2014a). *h*CRY2 has a similar N-terminal to *d*CRY but a very different C-terminal, so there exists a correlation between the sequence composition of the CRYs N- and C-terminals and the MF phenotypes observed.

Geotaxis phenotypes

D. melanogaster are negatively geotactic and, when disturbed, walk upwards against gravity rather than walking downwards (positive geotaxis). Negative geotaxis, like most behaviour, is influenced by many genes, and an early microarray analysis revealed that genetic variation in two circadian-related loci, *cry* and *Pdf* [*Pigment dispersing factor*, encoding a neuropeptide that allows clock neurons to communicate with each other (Renn et al., 1999)] could contribute to the geotactic response (Toma et al., 2002). Thus, it was of obvious interest to examine whether this phenotype was sensitive to MFs. Flies showed reduced negative geotaxis under a MF in blue, but not red light (Fedele et al., 2014b) (Figure 3A). Under a sham MF

cry^{02} null mutants did not climb upwards, so not surprisingly (and uninformatively), exposure to a MF had little effect. However, when *d*CRY was expressed with either the *timGAL4 or cryGAL* drivers a normal negative geotaxis was generated under sham, which was reduced on exposure to a MF (Figure 3A). It was also observed that expressing *d*CRY in clock neurons, or eyes or antennae could rescue the geotactic MF response. In contrast, using mutations to remove the eyes or the antennae, disrupted the MF response. These results suggest that at least one of these three tissues, eyes, antennae or clock neurons must express *d*CRY in the presence of intact structures of the other two, in order to mediate the geotactic response to MFs. Furthermore, neither *hCry1* nor *hCry2* could rescue the MF phenotype in a *cry-null* mutant background.

A similar independent but far more sophisticated analysis of fly geotaxis from Chae's group in Korea replicated and significantly extended the work with *d*CRY outlined above, using both a similar tube positioning assay as Fedele et al. (2014b), a vertical choice geotactic Y-maze and free-flight recordings under blue light (Bae et al., 2016) (Figures 3B–D). Under near 0 MF, flies became more negatively geotactic in both maze and flight tests and exposing them to a MF reduced negative geotaxis (i.e., geotaxis became more positive with flies moving downward under a MF). A task was also employed in which food was associated with positive geotaxis in the training condition under a 0 MF. Testing with no food in 0 MF, flies subsequently showed a positive geotactic rather than the normal negative geotactic score. Using various mutants it was also revealed that dCRY, PDF and PYREXIA (PYX) were involved in the geotactic MF response and rescue experiments using *Gal4/UAS* in mutant backgrounds further revealed that *dcry* and *pyx* were required in the antennae to mediate the MF responses, confirming the earlier antennal results of Fedele et al (2014b). The results from both groups suggested that the Johnston's Organ (JO) was the important antennal mechanoreceptor for generating the MF-modulated geotactic responses. Bae et al (2016) also showed indirectly, using a *cryGAL4* driving *UASGFP*, that *d*CRY was expressed in JO, but found no evidence for PDF expression. Yet knocking down PDF in *cry*-expressing cells disrupted the geotactic MF response, suggesting that the geotactic information from JO converges on neurons that express CRY and PDF, namely the (s-LNvs) and large ventral lateral neurons (l-LNvs), two prominent groups of clock cells (Yoshii et al., 2008). This result fits nicely with the work of Fedele et al (2014b) in that the peripheral CRY-expressing MF sensing organs, JO and eyes, require the clock cells (with or without CRY expression) to mediate the MF geotactic phenotype.

A second remarkable study from the Korean group examined 'geomagnetic imprinting' in flies in which developing embryos, larvae and pupae were exposed at different points in their life cycle to a gradient MF that was characteristic of their location in Korea (Oh et al., 2020). Emerging adult flies that were starved and had been pre-exposed to the local MF as embryos, 6–9 h after egg laying, showed more positive geotaxis (moving downwards) to the applied MF gradient compared to control conditions (sham or reversed gradient). Replacing the gradient MF exposure to one characteristic of Vancouver or Madrid had the same effect, but mixing and matching different geographical exposures in the pre-exposure and testing regimes had little effect on geotaxis. In other words, the embryos had become 'imprinted' on the pre-exposure MF gradient. Even more surprising was that these MF-induced effects proved to be transgenerational. The progeny of flies pre-exposed to MFs showed similar geotactic scores to the parents, even though they had not been further pre-exposed to a MF. This downward phenotype required both the male and female parent to have been previously pre-exposed, implying some form of epigenetic inheritance.

In these studies it was starved (but not satiated) flies that reveal these MF-induced geotactic responses suggesting that the phenotype is foraging-relevant. The positive geotactic response under a MF may provide a way for flies to forage more successfully, given that this species preference is for rotting fruit that is located mainly on the ground. The authors also speculate that imprinting might provide a way by which flies may return to a food/mating site from which they successfully emerged, although the ephemeral nature of food sites makes this a bit of a stretch. Nevertheless, these intriguing studies from the Chae group generate some possible answers to why flies might have a functional magnetic sense.

Other MF phenotypes in *Drosophila*

Drosophila larvae convulse if they have been previously stimulated 11–19 h into embryogenesis with pulsed blue (470 nm) light, but not orange (590 nm) light or darkness (Marley et al., 2014). Recovery time from these seizures is dramatically lengthened by exposure to a 100 mT MF and this phenotype is ameliorated in *cry-null* mutants and by anti-epileptic drugs. *d*CRY is optogenetic, so when activated by blue light it generates neuronal firing via changes in the permeability of potassium channels (Fogle et al., 2011; Fogle et al., 2015). This provides a potential explanation for the larval seizure phenotype through increased blue-light stimulated *d*CRY-mediated synaptic activity, which is further modulated by the effects of the MF. Furthermore, expressing *d*CRY ectopically in the larval aCC motor neuron stimulates action potentials under blue light, which is further potentiated by exposure to a 100 mT MF (Giachello et al., 2016) (Figures 3E,F). Once again, the *Trp342Phe* mutation did not compromise the MF effect, in contrast to the *dcryΔ* C-terminal tail deletion, so in this assay both mutants mirrored their circadian period MF phenotype. Several other *dcry* mutants have now been tested in both circadian behaviour and larval physiology and the results reveal parallel effects in the two paradigms (Bradlaugh et al., 2021). While behaviour

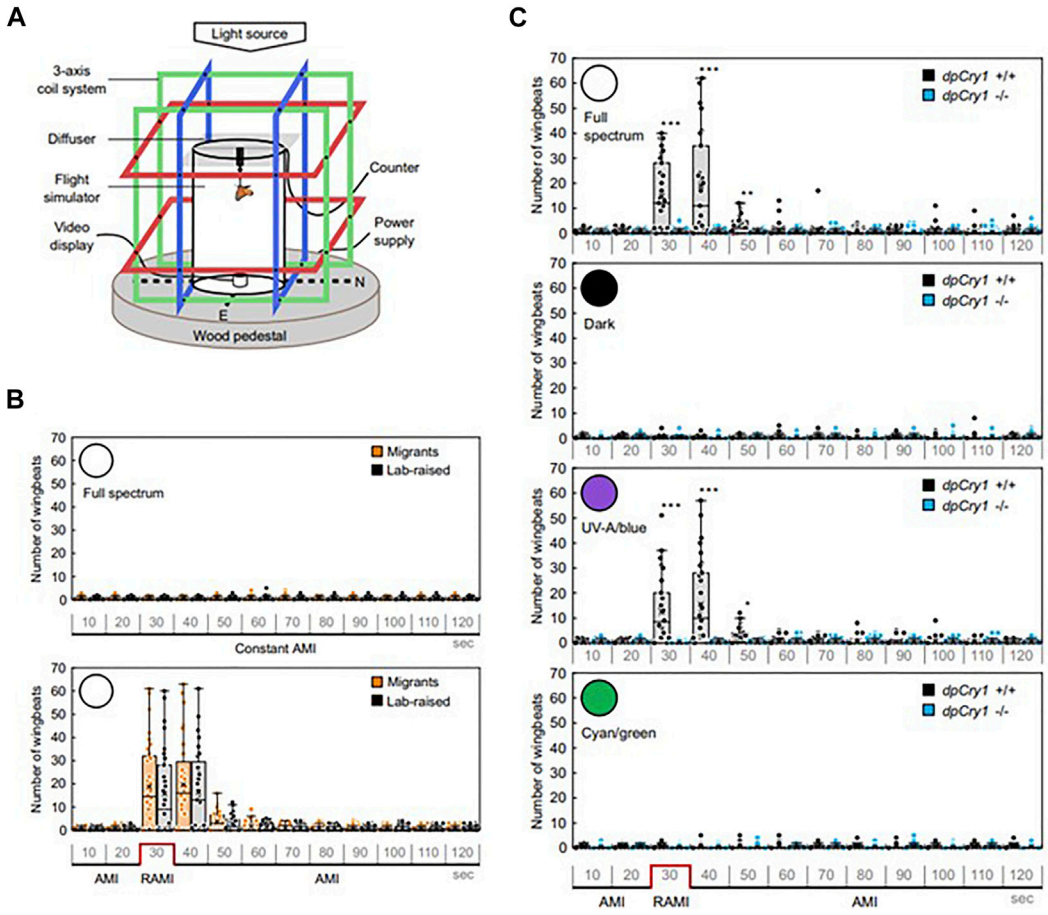

FIGURE 4
Magnetic field effects on Monarch butterfly behaviour. **(A)** Flight simulator with three different axes of Helmholz coils with a Monarch tethered in the middle of the arena. **(B)** Migrant and lab-reared Monarchs do not respond to a constant ambient MF (~45 μT AMI, top panel) unless it is reversed (RAMI, bottom panel), when they immediately increase their wingbeats in response in full spectrum light. **(C)** The wingbeat response to RAMI is observed under full spectrum light but not in darkness in wild-type of *DpCry1*$^{-/-}$ knockout Monarchs. Neither does *DpCry1*$^{-/-}$ show the response in blue/UV light in contrast to the wild-type. Neither wild-type nor the mutant respond to RAMI under cyan/green light (480–580 nm) (Figure redrawn from Figures 1, 2 of Wan et al., 2021 under CC open access (http://creativecommons.org/publicdomain/zero/1.0/). See article for description of statistics).

provides a whole organism readout of responses to a MF, single cell neuronal physiology is several biological steps closer to the initial quantum changes that initiate the MF molecular cascade. It is therefore comforting that the adult fly's behaviour and the single larval neuron show such correlated magnetic responses to *d*CRY manipulations.

Larval behaviour has also been investigated in a crawling assay on an agar covered Petrie dish. Larvae will avoid an area that is exposed to a MF under blue but not red light. Mutant *cry*^{b} or *cry*^{02} larvae do not avoid the MF showing this phenotype is CRY-dependent (Sherrard et al., 2018). In this assay *h*CRY1 can rescue the mutant MF avoidance phenotype, further suggesting that in *Drosophila* vertebrate-like CRY2s can be blue-light sensitive, directly or indirectly. However, the heterogeneous response of CRY2s in rescuing a *dcry-null* background in different behavioural assays discussed so far points to the role played by the cellular context in affording light perception or downstream signalling capabilities to these molecules.

In summary, there is a list of phenotypes in *Drosophila* that respond to MFs that in adults includes, association tasks, circadian cycles, geotaxis, male courtship (Wu et al., 2016), and in larvae, seizures, MF avoidance and synaptic responses, all of which are blue light and CRY-dependent.

MF effects and genetics in other insects

While there are several examples of magnetic field effects on behaviour in insects only in a few have any kind of genetic manipulation been attempted. The Monarch butterfly *D.*

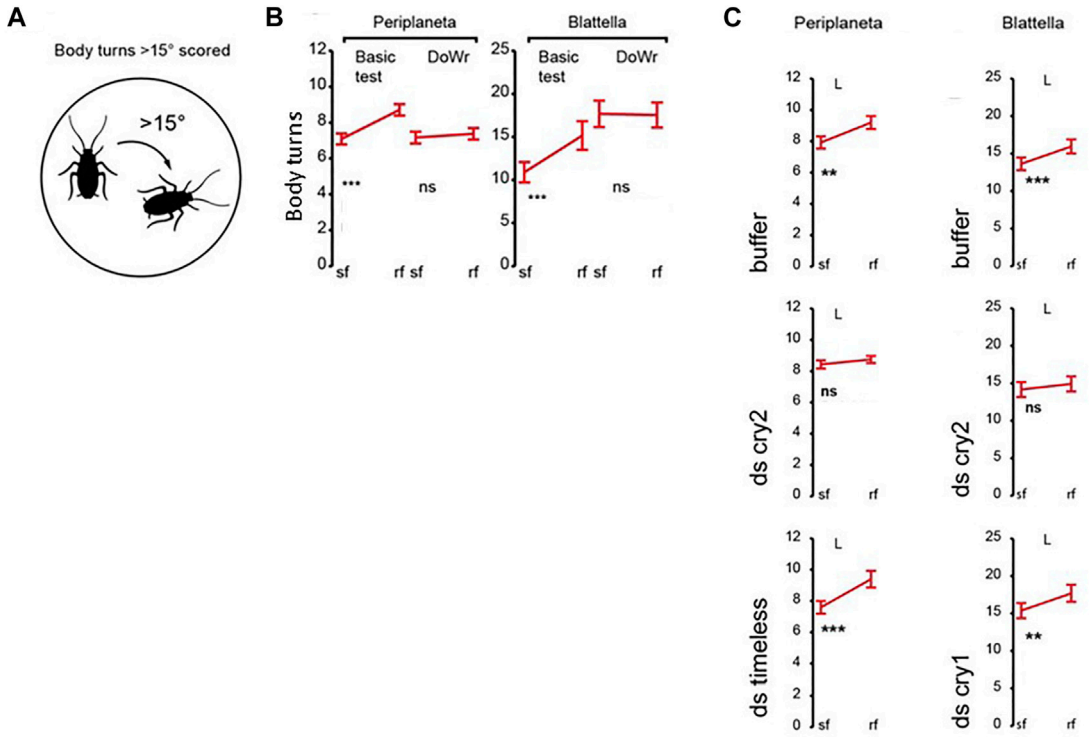

FIGURE 5
Genetic analysis of magnetosensitivity in cockroaches (Bazalova et al., 2016). **(A)** Diagrammatic representation of the body turn phenotype in response to a rotating MF. **(B)** Body turns in response to a MF (~45μT, 18 μT horizontal component) for *Periplaneta americana* and *Blattella germanica*. Sf, steady field, rf rotating field. Basic test—MF exposure, DoWr-sham double wrapped coils. Under a MF (Basic) a significant increase in turning is observed in a rotating field (rf) for both species. Y-axis represents body turns >15°. **(C)**. Transient double stranded (ds)RNAi knockdown of *Pacry2* and *BgCry2* but not *Bgcry1* nor *Patimeless (Patim)* eliminates the MF-mediated turning response. For **(B,C)** $^{**}p < 0.01$, $^{***}p < 0.001$. Figure redrawn and simplified from Figures 1–3 from Bazalova et al (2016) with permission.

plexippus provides a spectacular example of migration from the northern parts of North America to Mexico and back and is therefore the insect of choice for studying long-distance migration (Reppert, et al., 2010). Among various geophysical cues used for navigation, Monarchs also use the earth's magnetic field as an inclination compass as demonstrated using flight simulators under UV and blue light (Guerra, et al., 2014). Using a flight simulator with three sets of Helmholtz coils so that inclination, declination and intensity of the MF could be manipulated (Figure 4A) it was also observed that reversing the natural magnetic inclination increased the butterfly's wingbeat frequency under 380–430 nm but not 480–580 nm wavelengths (Wan et al., 2021) (Figure 4B). Gene editing of *DpCry1* but not *DpCry2* obliterated the magnetically-induced phenotype and covering the eyes or antennae with black paint impaired the wingbeat MF responses (Figure 4C). The fact that *Dp*CRY2 is dispensable for the MF phenotype is inconsistent with some of the reports in *Drosophila* in which *Dp*CRY2 shows MF effects. However, the manipulations employed here were not the typical GAL4-mediated overexpression studies used in *Drosophila*, but mutations generated by CRISPR-Cas9 editing. Also, the MF employed was similar to the earth's, not several or many times more intense as usually (but not always) used in flies. These caveats should be taken into account when attempting to interpret the biological significance of MF effects in *Drosophila* behaviour, although they do not invalidate the fact that MF effects can be observed reliably in flies.

In the cockroach, *Periplaneta americana,* a rotation of the magnetic field generates body turns, which are eliminated in a dsRNAi knockdown of *Pacry2*, the only CRY encoded in the genome (Figures 5A,B). Disrupting circadian locomotor activity rhythms either with constant light or with *Patimeless* knockdown, failed to disturb the MF phenotype reflecting its independence from the circadian clock (Bazalova et al., 2016) (Figure 5B). In another cockroach species, *Blattella germanica,* which encodes both vertebrate-like CRY2 and *Drosophila*-like type 1 CRY, somewhat surprisingly, only knockdown of *Bg*CRY2 disrupted the MF-mediated turning response (Figure 5C). In the latter species, the MF phenotype was observed under wavelengths corresponding to UV through to blue-green but there was a precipitous drop off in effect from 505 to 528 nm, consistent with CRY spectral sensitivity (Bazalova et al., 2016). Covering the eyes with black but not

transparent paint disrupted the MF effect in *P. americana* and immunocytochemistry revealed that *Pa*CRY2 was expressed beneath the retina, probably in glial cells sandwiched between two basement membranes. By being immobilised in this way, *Pa*CRY2 could potentially serve as a direction sensor. Clearly, the focus on the type 2 CRYs here is at odds with the Monarch study outlined above and again underscores once again that the ability of CRY2 molecules to bind FAD effectively may change according to species-specific sequences and cellular environments.

An aversion paradigm where an insect 'freezes' when stimulated with a burst of hot air, paired with exposure to a rotating MF at a 505 nm wavelength, was used in the firebug *Pyrrhocoris apterus*. Males but not females showed the conditioned response to a MF and this was disrupted by CRISPR-Cas9 editing of the insect's *PyaCry2* gene. An unexpected result was that a modest MF response could still be generated even after the animals had been left for 24 h (but not 48 h) in complete darkness after the initial training (Netušil et al., 2021). This is particularly surprising, because the canonical RPM requires light to generate the FAD^{o-} Trp^{o+} radical pair. The implication here is that light activation of *Pya*CRY2 ignites a chain of events resulting in magnetosensitive RPs that are detectable for more than 20 h. It is highly unlikely that an intramolecular FAD^{o-} Trp^{o+} radical pair, may be stable for so long, suggesting that alternative magnetosensitive RPs can occur and operate both in parallel and sequentially within cells. There is some precedent for MF effects occurring after short periods of darkness in both birds (Wiltschko et al., 2016) and plants (Pooam et al., 2018; Hammad et al., 2020). Although such effects occur after tens of minutes of darkness at most, it is still difficult (if not impossible) to incorporate them into a canonical RPM. These 'dark' *P. apterus* results therefore generate a further conundrum for the classic RPM but the modest nature of the effects suggest that independent replication in this or other species would be helpful.

Discussion, speculation and conclusions

The canonical RP hypothesis suggests that the effect of a MF on CRY involves stabilizing the light-induced intramolecular RP. This is because the MF ultimately would decrease the likelihood that the electron acquired by FAD^{o-} through photoactivation may migrate back to the terminal Trp^{o+} (Hore and Mouritsen, 2016) (Figure 1A). The electric charges of FAD^{o-} and Trp^{o+} cause strong intramolecular electrostatic interactions that are believed to modify light-activated CRY into a more 'open' conformation compared to its dark state, which is more amenable to protein-protein interactions for initiating a signalling cascade (Zoltowski et al., 2011; Czarna et al., 2013; Levy et al., 2013). Consequently, the MF would prolong the activation of a signalling pathway such that it would reach a threshold and transduce magnetoreception into complex behaviours like navigation and migration. Under a strict interpretation of such a scenario, natural selection would be expected to optimise the CRYs involved in navigation to become better sensors. This is the rationale of a recent and elegant *in vitro* spectroscopic study that compared the magnetic properties of non-migratory chicken and pigeon CRY4, to the migratory Robin (*Erithacus rubecula*) *Er*CRY4 (Xu et al., 2021). The results revealed that *Er*CRY4 is more magnetically sensitive than CRY4 in the other two species, and that the four Trps of the donor chain were important for magnetic properties as revealed by using Trp-to-Phe mutants. *Er*CRY4 and the FAD^{o-} Trp^{o+} radicals thus fulfilled the physico-chemical requirements of an optimised magnetosensor. However, a 'fly in the ointment' was that one Trp mutant generated a more sensitive magnetic field effect than wild-type *Er*CRY4, which is puzzling. Additionally, the study did not address a number of biological findings that are not easy to reconcile with the canonical FAD^{o-} Trp^{o+} RP mechanism proposed to underlie the magnetosensitivity of *Er*CRY4, for example, studies, in *Arabidopsis* and *E. rubecula* which suggest that reoxidation in darkness may provide the magnetically sensitive RPs (Müller and Ahmad, 2011; Wiltschko et al., 2016; Pooam et al, 2018; Hammad et al., 2020).

The studies performed on insects have been important for discovering the wide range of behaviours that, under appropriate conditions, are sensitive to MFs. These phenotypes rely on different neuronal circuits, so one conclusion is that magnetosensitivity may be a general cellular property, and not just of excitable cells, given the plant literature on MFs. Therefore, it is likely that navigation did not require the evolution of specialised *ad hoc* proteins and signalling pathways but the organisation of general properties into a sensory cascade. Additionally, we have learned that while magnetosensitive RPs are likely sensors of MFs in biological systems, there may be more than one type of magnetosensitive RP, not just the light-activated CRY FAD^{o-} Trp^{o+} (eg Müller and Ahmad, 2011). Radicals are very reactive chemical species and hence short-lived so light-generated RPs cannot persist for tens of minutes (as magnetosensitivity in plants and birds suggest) or many hours (as suggested in *P. apterus*) in darkness. The necessary conclusion from these experiments is that at least in some cells the activation of CRY by light ignites chain-reaction mechanisms that can persist for some time in darkness generating alternative, short-lived magnetosensitive RPs. One example is the mechanism of reoxidation of fully reduced $FADH^{-}$ into oxidised FAD by O_2 that operates both in light and in darkness (Ritz et al., 2009; Müller and Ahmad, 2011). In the same cell, different magnetosensitive RPs may be present in parallel (perhaps even having a synergistic effect) and sequentially, as some RPs do not require the immediate presence of light for their generation.

This brings us to consider the possible mechanisms by which magnetoreception is transduced into behaviour. The first candidate model is the prolonged signalling cascade described above that follows a change in CRY conformation that is central to the canonical RP hypothesis. However, an alternative (or additional) mechanism stems from the redox properties of FAD^{o-} (and potentially of Trp^{o+} see Hong et al., 2018). As mentioned earlier full-length *d*CRY has optogenetic properties. When *d*CRY was expressed in olfactory neurons that are not normally light-sensitive, a similarly enhanced neuronal firing phenotype was

observed under blue light. Pharmacological blockade of K^+ but not Na^+ channels attenuated the response to blue light in clock neurons (Fogle et al., 2011). Furthermore, in *hyperkinetic* (*hk*) null mutants lacking the potassium Kvβ subunit this response was reduced. Structural and functional analyses suggest that Kvβ channels are redox responsive, as they harbour the redox sensitive cofactor NADPH/$NADP^+$ that modulates their function. Indeed, altering the cellular redox environment changes the firing patterns of clock neurons in a HK-dependent manner. Additionally, in *hk* null mutants the blue light response of clock neurons can be rescued by wild-type but not Kvβ variants in which the redox sensor has been impaired. Mutations in other K^+ channel subunits such as EAG (Kvα) that normally co-assemble with HK also disrupted the *d*CRY neuronal response (Fogle et al., 2015).

Thus the potassium channel β subunit (Kvβ) HK (here we use the fly example for simplicity, but it is a general hypothesis) harbours NADPH and HK binds to the α subunits (Kvα) such as EAG, SHAKER (SK) and others regulating the opening of the composite channel according to the redox state of the NADPH/$NADP^+$ cofactor. Consequently, the MF-dependent potentiation of the redox chemistry of FAD could generate a biological response (Arthaut et al., 2017) by requiring FAD^{o-} (hence CRY) to be localised close to NADPH (hence HK). A plasma membrane scaffold protein encoding several modular PDZ domains is Discs-large-1 (DLG1) and is located in the synaptic active zone. Several different classes of proteins including EAG, SH, and HK bind (directly or indirectly) to DLG1 facilitating signalling (Walch, 2013). Potentially, CRY through the PDZ binding motifs in its CTT could also interact with DLG1. Thus, CRY *via* its FAD and its CTT would act both as the sensor and the transducer, enhancing the formation of FAD^{o-} under MFs and then shepherding it to the membrane where it is converted into a change in ion flux (by affecting the opening of potassium channels). A major challenge here is that in several assays, the CRYCT (that contains the CTT) by itself can mediate magnetosensitivity even though it cannot bind FAD directly. Perhaps then, CRYCT is part of a 'magnetoplex' in which one or more components associate with FAD/flavoprotein while the CTT brings such a complex to the vicinity of the neuronal membrane? Under this scenario CRYCT is the transducer whereas the sensor is carried by FAD or another RP competent molecule. In this context, FAD has recently been shown to generate a magnetically sensitive intramolecular RP in HELA cells (Ikeya and Woodward, 2021) confirming *in vitro* studies that revealed magnetosensitivity in solution at physiological pH (Antill and Woodward, 2018). This may help explain why full length CRY encoding the Trp triad or tetrad is sufficient but not necessary to generate a magnetically sensitive RP because FAD may accomplish this alone under certain conditions (somehow with the help of the CRY-CT). Furthermore, in darkness CRY may also intervene as the transducer that could explain magnetosensitivity under non-photic environments.

By analogy, dCRY also plays a functional role in the visual signalling cascade, in addition to its cardinal roles in circadian light resetting and magnetosensitivity (Mazzotta et al., 2013; Mazzotta, et al., 2018; Schlichting et al., 2018). This is accomplished through the CaM binding region in the *d*CRYCT and the PDZ binding motifs in the CTT. *d*CRY associates with Inactivation No Afterpotential D (INAD), a scaffold protein encoding 5 PDZ domains that is located in the photoreceptors (Mazzotta et al., 2013). INAD is part of the 'signalplex', a complex of proteins that mediates phototransduction and several of its constituents, including INAD, have CaM binding domains. Thus, INAD has structural similarities to DLG1 and it is likely that *d*CRY, through PDZ binding and using CaM as a bridge, is recruited by INAD to contribute to the visual signalplex of the fly (Mazzotta et al., 2018). A magnetosensitive *d*CRY acting within this complex in the retina has been speculated to have the potential to allow flies to 'see' the MF (Foley et al., 2011) in the same way as it was been suggested that birds may also 'see' the MF lines of inclination (Ritz et al., 2000; Stoneham et al., 2012).

In summary, using different organisms including our favourite, *D. melanogaster*, we provide an alternative view that builds on the classic formulation of the RPM, but extends it so that it can incorporate some of the more conflicting results within the field. We also extend the RP scenario to the putative downstream effectors, such as DLG1 and HK, that can potentially alter behaviour under a MF. Undoubtedly, the genetic dissection of magnetic phenotypes *in vivo,* combined with physical-chemistry *in vitro,* will continue to stimulate (and puzzle) workers in this area, but ultimately, it will extend our understanding of this remarkable sensory modality.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

CK wrote the ms and prepared the figures. ER reviewed and revised the ms.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Margaret Ahmad,
Université Paris-Sorbonne, France

REVIEWED BY
Lukasz Chrobok,
University of Bristol, United Kingdom

*CORRESPONDENCE
E. Pascal Malkemper,
pascal.malkemper@mpinb.mpg.de

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Cryptochromes in mammals: a magnetoreception misconception?

Li Zhang and E. Pascal Malkemper*

Max Planck Research Group Neurobiology of Magnetoreception, Max Planck Institute for Neurobiology of Behavior—caesar, Bonn, Germany

Cryptochromes are flavoproteins related to photolyases that are widespread throughout the plant and animal kingdom. They govern blue light-dependent growth in plants, control circadian rhythms in a light-dependent manner in invertebrates, and play a central part in the circadian clock in vertebrates. In addition, cryptochromes might function as receptors that allow animals to sense the Earth's magnetic field. As cryptochromes are also present in mammals including humans, the possibility of a magnetosensitive protein is exciting. Here we attempt to provide a concise overview of cryptochromes in mammals. We briefly review their canonical role in the circadian rhythm from the molecular level to physiology, behaviour and diseases. We then discuss their disputed light sensitivity and proposed role in the magnetic sense in mammals, providing three mechanistic hypotheses. Specifically, mammalian cryptochromes could form light-induced radical pairs in particular cellular milieus, act as magnetoreceptors in darkness, or as secondary players in a magnetoreception signalling cascade. Future research can test these hypotheses to investigate if the role of mammalian cryptochromes extends beyond the circadian clock.

KEYWORDS

magnetic sense, chronobiology, magnetic fields, radical pair, spatial orientation

Introduction

Many animals perceive the weak magnetic field of our planet, a modality called magnetoreception (Nordmann et al., 2017). A magnetosensitive organ still awaits discovery, and there have been multiple different hypotheses, including mechanisms based on a physical alignment sensor (magnetite), electromagnetic induction, and a quantum sensor. Thus far, a strong line of evidence supports a quantum sensory mechanism based on light-induced radical pairs in birds (Hore and Mouritsen, 2016). Currently, the most likely candidates for the receptors are proteins from the cryptochrome family (Hochstöger et al., 2020), which is also present in mammals. Are mammalian cryptochromes also possible candidates for a magnetosensor?

Cryptochromes are structural homologs of photolyases (Sancar, 2004), enzymes that repair UV-B light-induced DNA damage using the energy from blue and UV-A light (Sancar, 2003). However, whilst still containing the photolyase homology regions (PHR), cryptochromes no longer have DNA repair functions. Instead, they have developed new signalling roles, mediated through highly dynamic C-terminal extensions (Sancar, 2003; Brautigam et al., 2004; Zhu et al., 2005; Chaves et al., 2006; Huang et al., 2006; Chaves et al., 2011; Zoltowski et al., 2019; Parico et al., 2020).

Modern classification distinguishes plant CRYs, which are blue light photoreceptors (Ahmad and Cashmore, 1993; Wang et al., 2016; Wang and Lin, 2020) and animal CRYs. Animal CRYs are further subdivided into *Drosophila* type CRY (dCRY or Type I CRY), Type II CRYs, and Type IV CRYs (Chaves et al., 2011). Type IV CRYs and dCRY are photoreceptors that mediate light responses such as circadian clock entrainment and putatively light-dependent magnetoreception (Stanewsky et al., 1998; Gegear et al., 2008; Zhang et al., 2017; Hochstöger et al., 2020; Xu et al., 2021). In contrast, Type II CRYs do not appear to fulfil photoreceptive functions but act as light-independent clock genes in the circadian transcription-translation feedback loop (TTFL) that orchestrates cellular and behavioural rhythms (Griffin et al., 1999; Kume et al., 1999; Shearman et al., 2000). While a magnetic sense in mammals is established (Burda et al., 2020), the involvement of mammalian cryptochromes has been questioned because they only express Type II CRYs (Kavet and Brain, 2021). In the following, we look at the evidence for and against a connection between Type II CRYs and the magnetic sense.

Structure and function of mammalian cryptochromes

Mammals possess two CRYs, CRY1, and CRY2, which have been studied mainly in humans and mice (Hsu et al., 1996; Todo et al., 1996; Kobayashi et al., 1998). The two human CRYs share 73% amino acid sequence identity, with the highest sequence divergence in the C-terminal tail (CTT) region (Hsu et al., 1996). CRYs are found across all tissues, but relative expression levels vary between the two proteins (van der Spek et al., 1996; Kobayashi et al., 1998). CRY1 is expressed at high levels in the central circadian pacemaker, the suprachiasmatic nucleus (SCN), where it oscillates in a circadian manner (Miyamoto and Sancar, 1998; Kume et al., 1999). CRY2 levels, on the other hand, are higher in the retina (Miyamoto and Sancar, 1998; Thompson et al., 2003). The mammalian retina is an independent circadian oscillator that entrains to light-dark cycles (Tosini and Menaker, 1996). The photoentrainment is mediated by Neuropsin (Buhr et al., 2015), but CRYs have been suggested to be involved (Vanderstraeten et al., 2020). CRY mRNA was found across all layers of the retina (Miyamoto and Sancar, 1998; Thompson et al., 2003), but immunohistochemistry detected retinal CRY2 exclusively in the photoreceptor layer and CRY1 in cones, amacrine cells and some ganglion cells (Wong et al., 2018).

Mammalian CRYs are central players in the TTFL that controls cellular circadian rhythms across tissues (Figure 1A). At the core of the loop are the transcription factors CLOCK-BMAL1 that activate many genes, including those of CRYs and PERIOD (PER) proteins (Partch et al., 2006; Dardente et al., 2007). In the cytosol, CRYs form heterodimers with PER and translocate back into the nucleus (Smyllie et al., 2022), where they inhibit CLOCK-BMAL1 and their own expression, before CRY and PER degradation starts another 24 h cycle (Kume et al., 1999). CRY1 and CRY2 appear to play similar and partially redundant roles in the TTFL, but different repression potency for the CLOCK-BMAL1 complex results in distinct antagonistic circadian phenotypes in single CRY1 or CRY2 knockout mice (van der Horst et al., 1999; Vitaterna et al., 1999; Anand et al., 2013; Miller and Hirota, 2022). In total darkness, CRY1 and CRY2 knockout mice have 1-h faster or slower free-running rhythms of locomotor activity, respectively, while double-knockouts are arrhythmic (Thresher et al., 1998; van der Horst et al., 1999).

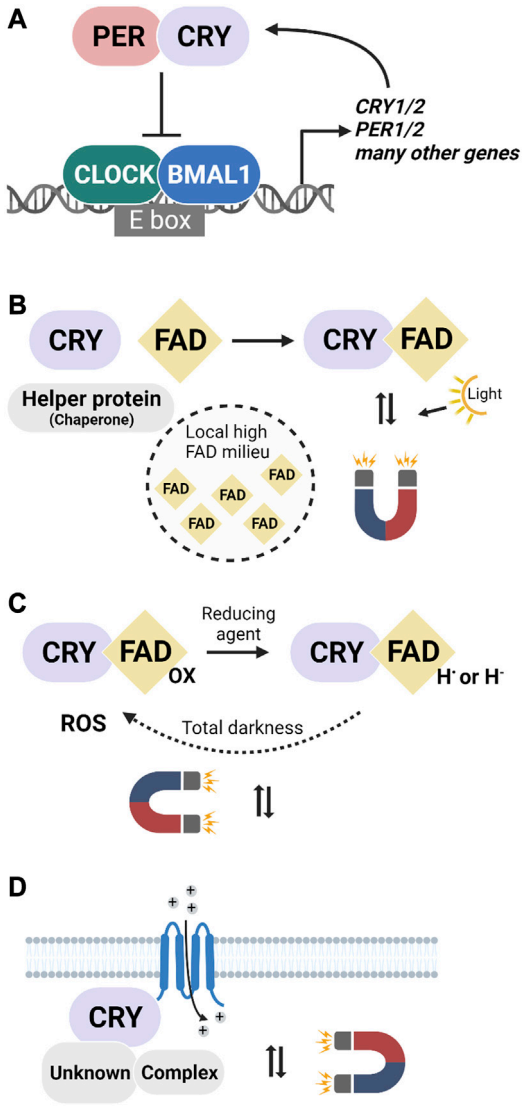

FIGURE 1
The canonical function of mammalian cryptochromes and three hypothetical scenarios for CRY involvement in magnetic sensing. **(A)** The well-known role of CRYs in the transcription-translation feedback loop (TTFL) where they control rhythmic gene expression in a light-independent manner. **(B–D)** Three putative ways in which mammalian cryptochromes could be involved in the transduction of magnetic field effects. **(B)** Light-dependent magnetosensitivity in a specific cellular milieu. FAD-binding could be facilitated by, e.g., helper proteins or high FAD concentrations. **(C)** Light-independent activation by a reducing agent and re-oxidation in the dark accompanied by radical pair production. **(D)** CRYs as secondary (non light-sensitive) components of a magnetosensory signaling cascade that would be initiated by a primary radical pair-forming (photoreceptor) protein that is currently unknown.

The central role of CRYs in the TTFL in nearly every cell of the body has far-reaching consequences. For example, apart from their

role in the central pacemaker, CRYs are involved in the circadian rhythmicity of the autonomous nervous system and insulin metabolism (Ikeda et al., 2007) and the regulation of autoimmunity (Cao et al., 2017). Consequently, perturbations of the CRY genes can manifest in various diseases. Genetic studies in humans have linked CRY mutations with mood disorders (Soria et al., 2010; Kovanen et al., 2013; Geoffroy et al., 2015), sleep disorders (Kripke et al., 2015; Hirano et al., 2016; Parico et al., 2020), and metabolic syndrome (Kovanen et al., 2015). The disruption of circadian rhythms increases the risk of some forms of cancer (West and Bechtold, 2015) and CRYs appear to be a direct link (Chan and Lamia, 2020). Various pro- and anti-cancerogenic effects of CRYs, including the promotion of genomic stability (Hoffman et al., 2010), induction of cell proliferation in human breast cancer cells (Chun et al., 2015), as well as the reduction of tumour formation and growth (Huber et al., 2016; Dong et al., 2019), have been reported. While still in its infancy, the field of circadian medicine, in particular the search for specific small molecule activators or repressors of mammalian cryptochromes (Miller and Hirota, 2022) and photopharmacological regulation of the circadian clock (Kolarski et al., 2021a; Kolarski et al., 2021b), are emerging fields of large potential (Kramer et al., 2022).

The role of cryptochromes in the magnetic sense

The organ(s) that allows animals to detect magnetic fields still awaits discovery, but a line of research on birds supports a quantum sensory mechanism based on radical pairs (Hore and Mouritsen, 2016). The influence of weak magnetic fields on radical pairs is well-known and has been associated with a variety of biological effects (Zadeh-Haghighi and Simon, 2022). The radical pair mechanism (RPM) predicts that the magnetic modulation of a spin-dependent chemical reaction initiates a transduction cascade, ultimately leading to the generation of neuronal signals (Hore and Mouritsen, 2016). Cryptochromes have been proposed as the primary receptors for this mechanism because they form intramolecular radical pairs within their photocycle (Ritz et al., 2000). This photocycle is best studied in *Arabidopsis*, where light excites the flavin adenine dinucleotide (FAD) cofactor and induces an electron transfer along a series of conserved tryptophan residues, resulting in the reduction of FAD and the formation of a radical pair between the terminal tryptophan and FAD (Chaves et al., 2011). The FAD reduction leads to a conformational change of the protein, resulting in a signalling state that initiates downstream effects (Banerjee et al., 2007). In the presence of oxygen, FAD will then be re-oxidised to the ground state, a process that occurs in darkness and involves the formation of a second pair of radicals (Pooam et al., 2019). In non-mammalian vertebrates, CRY4 is a magnetoreceptor candidate, although the exact cellular localization is still debated (Günther et al., 2018; Pinzon-Rodriguez et al., 2018; Hochstöger et al., 2020). It binds stoichiometric amounts of FAD *in vitro*, forms light-dependent radical pairs, and changes its conformation after light excitation (Watari et al., 2012; Mitsui et al., 2015; Zoltowski et al., 2019; Hochstöger et al., 2020; Xu et al., 2021). This provides a compelling case for CRY4, although some experimental findings such as magnetic orientation under monochromatic green light (Wiltschko et al., 2010) or when the magnetic field and light are presented alternatingly (Wiltschko et al., 2016) are not consistent with the canonical RPM model. Independent of the underlying receptor, a diagnostic test for the involvement of radical pairs is investigating magnetic behaviours in the presence of weak radiofrequency (RF) magnetic fields that are expected to interfere with the interconversion of the radicals (Henbest et al., 2004) and migratory birds are disoriented in such fields (Engels et al., 2014; Leberecht et al., 2023).

Sensitivity to magnetic fields has also been reported in mammals such as bats, mole-rats, mice, and humans (Burda et al., 2020). Some mammals, such as subterranean mole-rats, appear resistant to RF, so their magnetic sense is unlikely to involve radical pairs (Thalau et al., 2006). Interestingly, though, in epigeic mammals such as hamsters, mice, and humans, RF effects on magnetic responses have recently been reported (Malkemper et al., 2015; Malewski et al., 2018; Painter et al., 2018; Chae et al., 2022; Phillips et al., 2022; Phillips and Painter, 2023). If these findings are correct, the question of receptor identity arises. It is possible that mammalian magnetoreceptors evolved independently of birds and invertebrates, and alternative receptors such as melanopsin have been suggested (Phillips et al., 2022), but some evidence points to an involvement of CRYs.

Support for magnetic sensitivity of mammalian CRYs is provided by studies of heterologous expression of recombinant Type II CRY in insects. For example, UV/blue light-dependent magnetic behaviours were reported in the fruit fly *Drosophila melanogaster* (Gegear et al., 2008; Yoshii et al., 2009; Bassetto et al., 2023). The response was abolished by knocking out dCRY and rescued by the expression of insect Type II CRY or human CRY2 (but not CRY1; Gegear et al., 2010; Foley et al., 2011; Fedele et al., 2014a). Light-dependent magnetic responses consistent with an RPM were also found in cockroaches and firebugs, although both insects only possess vertebrate Type II cryptochromes (Bazalova et al., 2016; Netusil et al., 2021).

In sum, there is evidence for a magnetic sense in mammals and for a relationship between magnetic field responses and cryptochromes in other animals. At the core of the canonical RPM lies the formation of a light-induced radical pair between the CRY protein and FAD, leading to the question if mammalian CRYs are photosensitive.

Are mammalian cryptochromes photoreceptors?

Due to their similarity to photolyases and the light-sensitive CRYs from *Arabidopsis* and *Drosophila*, it was initially assumed that mammalian CRYs are also photoreceptors. Their role in the circadian clock seemed to support this assertion: To keep in sync with the outside world, the internal clock resets to light stimuli which, in *Drosophila,* is mediated by dCRY (Emery et al., 1998; Stanewsky et al., 1998; Ishikawa et al., 1999). Light input through the retina also resets the mammalian circadian clock (Shigeyoshi et al., 1997) and CRYs are expressed in the retina (Thompson et al., 2003; Wong et al., 2018). Unexpectedly, however, while CRY1/2 double-knockout mice are arrhythmic in total darkness, light pulses still induced phase shifts in the SCN (Okamura et al., 1999) and behavioural entrainment to a light/dark cycle (van der Horst

et al., 1999). Thus, CRYs do not reset the circadian clock. A series of elegant experiments demonstrated that melanopsin, in intrinsically photoreceptive ganglion cells, fulfils that function and CRYs are neither sufficient nor necessary (Berson et al., 2002; Hattar et al., 2002; Hattar et al., 2003). Other non-visual photoreceptive responses in mammals that have been discussed to be related to CRY photoreception—circadian rhythms of electroretinograms, contrast sensitivity and pupillary light responses (Van Gelder et al., 2003; Vanderstraeten et al., 2020)–are also consistent with circadian gating by CRYs, i.e., the modulation of input from other photoreceptors (Wong et al., 2018). Finally, mutation analyses demonstrated that the conserved tryptophan residues essential for photoreception in other species are dispensable for the circadian function in mammals (Froy et al., 2002). In sum, mammalian CRYs do not appear to function as photoreceptors in the circadian clock.

It is possible that vertebrate Type II CRYs have lost their capability to sense light during evolution. The photosensitivity of CRYs depends on FAD that is sequestered non-covalently in a U-shaped binding pocket in the PHR (Zoltowski and Gardner, 2011). The motifs of the pocket that are critical for FAD binding are known from crystal structures of plant CRY (Brautigam et al., 2004), dCRY (Czarna et al., 2013), and pigeon CRY4 (Zoltowski et al., 2019). No full-length crystal structures of mammalian cryptochromes are available to date. Partial structures of mouse CRYs show that in contrast to dCRY where a phosphate-binding loop forms a lid that traps FAD in the binding pocket (Maul et al., 2008), the binding pocket of mouse CRY has a more open conformation that exposes the cofactor and enables dynamic FAD-binding (Nangle et al., 2013; Xing et al., 2013). Accordingly, computer simulations predicted a strikingly reduced binding affinity for FAD in mouse CRYs (Kutta et al., 2017). The authors identified the structural features responsible for the reduced affinity and, based on sequence alignments, concluded that all animal Type II CRYs are only vestigial flavoproteins. They estimated that *in vivo* less than 7% of human CRY2 and 16% of CRY1 have FAD bound (Kutta et al., 2017).

Accordingly, only low (sub-stoichiometric) levels of FAD have been reported for purified animal Type II CRYs (Ozgur and Sancar, 2003; 2006; Kutta et al., 2017; Wang et al., 2018; Hochstöger et al., 2020; Bolte et al., 2021). In contrast, other cryptochromes, such as CRY4, can readily be purified with FAD bound (Hochstöger et al., 2020; Xu et al., 2021).

In sum, all investigated mammals express animal Type II CRYs with very low binding affinity for FAD *in vitro*. Thus, in the absence of evidence for Type I or Type IV CRYs in mammals, it appears unlikely that mammalian cryptochromes are photosensitive. Can the low binding affinity to FAD be reconciled with the findings of Type II CRY as part of a magnetoreception system?

Scenarios for mammalian cryptochrome magnetoreception

We can think of three, not mutually exclusive, scenarios in which mammalian cryptochromes could be involved in magnetoreception: 1) The cellular milieu facilitates FAD binding, 2) Cryptochromes act as magnetoreceptors in darkness, or 3) Cryptochromes are downstream interactors in a magnetoreception signalling cascade.

First, although evidence argues against a general photosensitivity of animal Type II CRYs, it conceivably may occur in a specific cell type or subcellular compartment (Figure 1B). Direct support for FAD-binding to mammalian CRYs is provided by a study on FBXL3, a ubiquitin ligase that binds to the FAD pocket and labels cryptochrome for proteasomal degradation. FAD directly competed with FBXL3 for binding and thereby reduced CRY degradation (Hirano et al., 2017). Further indirect structural evidence is that a fourth tryptophan residue, which is suggested to be fundamental to generating the light-induced signalling state in bird CRY4-mediated magnetoreception (Xu et al., 2021), is also conserved in humans and mice (Muller et al., 2015). Finally, human CRY1 (expressed in flies) undergoes light-dependent proteolysis, indicative of conformational changes and the formation of radicals (Hoang et al., 2008).

Dynamic, rather than static, association with FAD is being recognized as a common phenomenon in flavoproteins (Schnerwitzki and Vabulas, 2022). It follows that, although the affinity for FAD is much weaker in Type II than in Type I/IV CRYs, specific cellular conditions (e.g., redox conditions, high compartmental FAD concentrations or the presence of binding partners) could augment FAD binding to Type II CRYs. In support of this, successful purifications of Type II CRYs with FAD were reported (Hsu et al., 1996; Liedvogel et al., 2007). Interestingly, the very few studies that reported stoichiometric FAD binding supplemented the culture media with FAD, suggesting that high FAD concentrations compensate for the low binding affinity of Type II CRYs (Table 1). Mammals cannot synthesize riboflavin, the precursor of FAD, they must take it up from external sources (Powers, 2003). Intracellularly, a complex of enzymes converts riboflavin into FAD. Consequently, FAD concentrations are not homogenous within a cell, but they vary across time, between cell types or states and across subcellular compartments (Giancaspero et al., 2013; Hirano et al., 2017). A particularly striking example is the 15-fold increase of intracellular FAD during adipogenic differentiation of mouse fibroblasts (Hino et al., 2012). *In vivo*, conditions favouring FAD binding could prevail in specific cell types or compartments, such as the outer segments of photoreceptors in which a Type II CRY (CRY1a) is enriched in some birds and mammals (Niessner et al., 2011; Niessner et al., 2016; Bolte et al., 2021). Within these cells, FAD concentrations would have to be in the higher micro-molar range (CRY1: >16 μM, CRY2: >40 μM) to reach sufficient saturation of Type II CRYs (Kutta et al., 2017). Free FAD concentrations in mammalian cells, however, are only in the nanomolar range, but eight to ten-fold differences were found between cell lines and organs (Hühner et al., 2015). We could not find systematic data on FAD concentrations across different cell types and interspecies differences *in vivo*.

Apart from FAD concentrations, protein interactions could further facilitate FAD binding. The structure of the highly conserved phosphate-binding loop of the FAD binding pocket is sensitive to the local environment, which led to the suggestion that protein-protein interactions might increase the affinity of FAD (Nangle et al., 2013). Specifically, one enzyme of the FAD-producing pathway, FAD synthetase, was suggested to act as a

TABLE 1 Published attempts to purify vertebrate Type II cryptochromes with FAD in heterologous expression systems (in chronological order). (YES) indicates not reported or sub-stoichiometric FAD binding levels.

Host species	CRY1/2	Expression system	Promoter	FAD bound?	FAD supplement?	Stoichiometric?	Method of analysis	References
Homo sapiens	hCRY1, hCRY2	Bacterial	Ptac	(YES)	NO	Not reported	UV-vis absorption spectroscopy	Hsu et al. (1996)
Homo sapiens	hCRY2	Mammalian cell line (Hela)	CMV	(YES)	NO	NO (~30%)	Fluorescence spectroscopy	Özgür and Sancar (2003)
Homo sapiens	hCRY1, hCRY2	Virus/insect cell (Sf21)	CMV	(YES)	NO	NO (<0.1% for hCRY1, ~0.2% for CRY2)	Fluorescence spectroscopy	Özgür and Sancar. (2006)
Sylvia borin	sbCRY1a	Virus/insect cell (Sf9)	CMV	YES	YES (25 μM)	YES	UV-vis absorption spectroscopy	Liedvogel et al. (2007)
Homo sapiens	hCRY1	Virus/insect cell (Sf21)	AcMNPV	(YES)	NO	Not reported	Fluorescence spectroscopy	Hoang et al. (2008)
Mus musculus	mCRY1	Mammalian cell line (HEK293)	CMV	(YES)	YES (500–5,000 μM)	Not reported	Pull-down assay	Hirota et al. (2012)
Homo sapiens	hCRY1	Yeast (Pichia pastoris)	UAS	YES	YES (40 μM)	YES	EPR spectroscopy	Vieira et al. (2012)
Mus musculus	mCRY2	Virus/Insect cell (High5)	CMV	YES	YES (500 μM)	YES	Fluorescence spectroscopy	Xing et al. (2013)
Sylvia borin, *Homo sapiens*	sbCRY1a, hCRY1, hCRY2	Bacterial	T7	NO	NO	NO	UV-vis absorption spectroscopy	Kutta et al. (2017)
Columba livia	clCRY1	Virus/insect cell (Sf21)	AcMNPV	NO	NO	NO	UV-vis absorption spectroscopy	Wang et al. (2018)
Columba livia	clCRY1a, clCRY2a	Virus/insect cell (Sf9)	Polyhedrin	NO	NO	NO	UV-vis absorption spectroscopy	Hochstöger et al. (2020)

chaperone that delivers the flavin cofactor to apoproteins (Torchetti et al., 2011; Giancaspero et al., 2015).

Summarizing this first scenario, cellular and subcellular differences in FAD concentrations and the availability of binding partners could have a significant effect on the fraction of CRYs with FAD bound. Under certain conditions, mammalian CRYs might form light-induced radical pairs.

In a second scenario, mammalian CRYs would be part of a radical pair mechanism independent of light (Figure 1C). Indeed, Type II CRY-dependent magnetic behavioural responses occur after 24 h in total darkness (Netusil et al., 2021). These findings are inconsistent with the canonical RPM and suggest that the radicals formed during the reoxidation of the anionic flavin radical state provide the magnetosensory step of the CRY cycle (Wiltschko et al., 2016; Pooam et al., 2019). In dCRY, this step occurs in darkness and within minutes, but in mammals, the anionic radical appears more stable. Heterologously expressed human CRY1 exists in a mix of redox states with a significant portion of the anionic radical state in the absence of light (Vieira et al., 2012). In *Drosophila*, chemical reduction can replace photoreduction of FAD for dCRY activation (Vaidya et al., 2013). It follows that in a reducing intracellular environment, a significant proportion of CRY would be in the anionic flavin radical state, which, in the presence of oxygen, can revert to the ground state and form radical pairs in darkness (Hoang et al., 2008). A side effect of this reaction is the production of reactive oxygen species (Arthaut et al., 2017). Consistent with this, pulsed magnetic fields were reported to increase reactive oxygen species levels in mammalian cells in a cryptochrome-dependent manner (Sherrard et al., 2018). ROS might even constitute part of a signalling mechanism (El-Esawi et al., 2017). In sum, it is conceivable that chemical, rather than photochemical reduction, could lead to the generation of magnetosensitive radical pairs in mammalian cryptochromes.

Finally, CRYs could play a role in the magnetic sense even without forming radical pairs themselves (Figure 1D). Instead of being the primary receptor molecules, they may be a downstream component of a signalling cascade. After all, mammalian CRYs have highly dynamic C-terminal domains, indicative of evolutionarily optimized protein-protein interactions. Consistent with a secondary role for cryptochromes, studies in *Drosophila* showed that the FAD-binding domain and a series of tryptophans, which are required to form the radicals in the canonical RPM hypothesis, are not necessary for CRY to convey magnetosensitivity (Fedele et al., 2014a; Fedele et al., 2014b; Bradlaugh et al., 2023). The authors speculated that the magnetosensitive radical pairs do not form within CRYs, instead suggesting a role for redox coupling between FAD and a redox-sensitive potassium channel (Fogle et al., 2015). Indeed FAD-dependent magnetic field effects, albeit in mT-strong fields, were reported for mammalian cells (Ikeya and Woodward, 2021). In this scenario, CRYs would be part of a magnetoreceptor complex without forming radical pairs themselves.

Box 1 Future Issues

- Is the finding of an RPM-based magnetic sense in mammals robust?
- Do some mammals express Type I or IV cryptochromes?
- Are there binding partners of mammalian cryptochromes that are not involved in circadian rhythms?
- Do small molecule cryptochrome inhibitors perturb magnetoreception?
- If cryptochromes are not directly responsible for magnetic field sensitivity, what are alternative candidate molecules?

Conclusion

Mammalian cryptochromes are light-independent players in the mammalian circadian clock, but they might also serve functions beyond this. Based on evidence for magnetic field effects in mammals we put forward three hypotheses describing how CRYs might be related to these effects. Although these hypotheses may prove to be incorrect, we believe it is important to test them. Given the many physiological processes in which cryptochromes are involved in mammals, magnetic sensitivity would have profound implications. The right questions (authors' choice in Box 1) and experiments will clarify whether CRY-mediated magnetoreception in mammals is a possibility or misconception.

Author contributions

LZ and EPM designed the study. LZ wrote the first version of the manuscript, EPM reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Brian Crane,
Cornell University, United States

REVIEWED BY
Carlos Marcuello,
Spanish National Research Council (CSIC), Spain
Shinji Masuda,
Tokyo Institute of Technology, Japan

*CORRESPONDENCE
Marootpong Pooam
marootpongp@nu.ac.th

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Cryptochrome and quantum biology: unraveling the mysteries of plant magnetoreception

Thawatchai Thoradit[1], Kanjana Thongyoo[1], Khwanchai Kamoltheptawin[1], Lalin Tunprasert[1,2], Mohamed A. El-Esawi[3], Blanche Aguida[4], Nathalie Jourdan[4], Kittisak Buddhachat[1] and Marootpong Pooam[1]*

[1]Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand, [2]State Key Laboratory for Mechanical Behavior of Materials, School of Material Science and Engineering, Xi'an Jiaotong University, Xi'an, China, [3]Botany Department, Faculty of Science, Tanta University, Tanta, Egypt, [4]UMR CNRS 8256 Adaptation biologique et vieillissement (B2A), Institute of Biology Paris Seine, Sorbonne Université, Paris, France

Magnetoreception, the remarkable ability of organisms to perceive and respond to Earth's magnetic field, has captivated scientists for decades, particularly within the field of quantum biology. In the plant science, the exploration of the complicated interplay between quantum phenomena and classical biology in the context of plant magnetoreception has emerged as an attractive area of research. This comprehensive review investigates into three prominent theoretical models: the Radical Pair Mechanism (RPM), the Level Crossing Mechanism (LCM), and the Magnetite-based MagR theory in plants. While examining the advantages, limitations, and challenges associated with each model, this review places a particular weight on the RPM, highlighting its well-established role of cryptochromes and *in-vivo* experiments on light-independent plant magnetoreception. However, alternative mechanisms such as the LCM and the MagR theory are objectively presented as convincing perspectives that permit further investigation. To shed light on these theoretical frameworks, this review proposes experimental approaches including cutting-edge experimental techniques. By integrating these approaches, a comprehensive understanding of the complex mechanisms driving plant magnetoreception can be achieved, lending support to the fundamental principle in the RPM. In conclusion, this review provides a panoramic overview of plant magnetoreception, highlighting the exciting potential of quantum biology in unraveling the mysteries of magnetoreception. As researchers embark on this captivating scientific journey, the doors to deciphering the diverse mechanisms of magnetoreception in plants stand wide open, offering a profound exploration of nature's adaptations to environmental cues.

KEYWORDS

cryptochrome, radical-pair mechanism, level crossing mechanism, magnetitebased magr, magnetoreception, quantum biology

1 General introduction

The potential physiological significance of plants' geomagnetic response is a multifaceted and intriguing subject that has garnered attention in recent scientific explorations. The geomagnetic field, a natural environmental factor, exhibits considerable regional variations in strength and direction, and its influence on living organisms, including plants, has been a subject of study since the observations of Louis Pasteur in 1862 and Savostine in 1930.

One of the fascinating aspects of this field of study is the ability of plants to perceive and respond to varying magnetic fields. A study by Shine et al. Davies (2023) has shed light on this phenomenon, demonstrating that plants can alter their gene expression and phenotype in response to specific features of the geomagnetic field. This finding opens up new avenues for understanding how geomagnetic triggers could lead to phenotypic changes in plants.

The effects of magnetic fields on plant growth and development have been extensively reviewed, with a focus on the complex nature of magnetic fields' action on plant growth. Silva and Dobránszki (2015) discussed the physiological and biochemical responses of plants to magnetic fields, ranging from nanoTesla to geomagnetic levels. Their review underscored the complexity of this subject and the need for a more robust mathematical framework to understand these effects. Pioneer studies on plant physiological responses to different magnetic fields have explored the effects of static magnetic fields, electromagnetic fields, and various magnet alloys on plants (Abhary and Akhkha, 2023). These studies have laid the groundwork for understanding the intricate relationship between magnetic fields and plant physiology.

The underlying physiological mechanisms of magnetic fields' effects on plants have also been probed. For instance, Xu et al. (2016) and Agliassa et al. (2018a) discovered that the flowering of *Arabidopsis* was suppressed by a near-null magnetic field, a phenomenon related to the modification of cryptochrome. This study also revealed significant decreases in gibberellin levels in plants grown in a near-null magnetic field compared to those in the local geomagnetic field, highlighting the nuanced ways in which magnetic fields can influence plant development. The metabolic processes of plants, including the vital process of photosynthesis, can be altered by geomagnetic disturbance. Davies (2023) observed a decrease in diurnal oxygen production in Elodea plants under high geomagnetic variability, suggesting that geomagnetic disturbance can affect the metabolic process of photosynthesis. Furthermore, the influence of the geomagnetic field on the metabolism of living organisms, including plants, has been supported by a plethora of studies (Erdmann et al., 2017). These investigations have revealed that any weakening or absence of the geomagnetic field can interfere with metabolic processes, adding another layer of complexity to our understanding of geomagnetic effects on plants. Electrical signals in plants, such as action potentials, have also been implicated in physiological processes (Fromm and Lautner, 2006). The presence of these electrical signals in plant cells indicates that plants use ion channels to transmit information over long distances, triggering rapid responses to environmental stimuli and affecting various physiological processes. The physiological significance of plants' geomagnetic response extends to seed germination and growth as well. Studies by Abhary and Akhkha (2023) have found that magnetic fields influence seed vigor, growth, and yield, suggesting that magnetic fields could be harnessed to enhance plant growth and maximize crop performance.

Overall, the potential physiological significance of plants' geomagnetic response is a rich and complex subject that encompasses various aspects of plant growth, development, and physiological processes. The existing body of research has provided valuable insights into how plants perceive and respond to magnetic fields and how these fields influence physiological and biochemical processes. However, the intricate nature of these effects and their significance in plant biology calls for further research to unravel the underlying mechanisms and to explore new frontiers in this fascinating area of study.

In the developing of plant science, the search to comprehend plants' response to the Earth's magnetic field offers an intriguing and multi-disciplinary exploration at the interface of quantum biology and molecular botany. Despite substantial progress in this domain, plant magnetoreception remains covered in scientific doubt, captivating researchers worldwide. The essence of this fascinating mystery lies within the underlying mechanisms that activate such responses, with the radical pair mechanism (RPM) emerging as a dominant candidate. This intriguing phenomenon controls the principles of quantum physics, blurring the lines between these seemingly distinct fields of science. The RPM theory posits that specific light-activated molecules, such as the flavoprotein cryptochrome, create pairs of free radicals whose spin states and hence chemical reactivity may be influenced by Earth's magnetic field (Ritz et al., 2000). This intriguing hypothesis has profound implications for plant biology, suggesting that the cryptochrome-mediated light sensing mechanism in plants could serve as a biological compass, shaping critical processes such as growth, germination, and phototropism (Ahmad, 2016). Moreover, quantum biology's contribution to our understanding of magnetoreception might be further enhanced through the Level Crossing Mechanism (LCM), which proposes that magnetic fields can influence energy level transitions in molecules or atoms, potentially contributing to magnetoreception in plants. However, despite being a novel concept, the LCM has received limited attention compared to RPM, and its potential role in plant magnetoreception remains largely unexplored.

Delving deeper into this enigmatic phenomenon, the magnetoreceptor protein MagR emerges as another promising player (Qin et al., 2016). A recent study by (Parmagnani et al., 2022) postulates a potential role for MagR in plant magnetoreception, with the protein possibly forming complexes with iron-sulfur (Fe-S) clusters in response to geomagnetic fields. This line of thought represents a novel nexus between RPM and MagR theories, introducing the idea of a composite magnetoreceptor that integrates elements of light sensitivity and magnetic field alignment (Parmagnani et al., 2022). However, the MagR theory is not without its challenges. Empirical support is limited, and the relationship between MagR and cryptochrome, as well as their role within the RPM or magnetite-based mechanism, remains elusive.

Notwithstanding these challenges, unraveling the role of MagR could provide an indispensable bridge between the domains of quantum biology and plant magnetoreception (Dodson et al., 2013).

As our understanding of plant magnetoreception continues to unfold, it becomes increasingly evident that we must inspect the complicated involvement between quantum physics and biology. From cryptochrome to MagR, the potential players are numerous, and their intricate interplay paints a complex picture of plant magnetoreception.

However, each mechanism that we mentioned above is still under debate. Thus, the objective of this review is to revisit the current understanding of plant magnetoreception, exploring both well-established and emergent theories, with a particular focus on the RPM and the potential role of quantum biology. By illuminating the potential intersections between these theories and suggesting avenues for future research, we aim to advance our collective understanding of this extraordinary phenomenon. As we embark on this journey, let us marvel at the wonders of nature, where the smallest seed can grasp the vast forces of our planet, driven by the unseen, yet undeniable influence of Earth's magnetic field.

2 Quantum biology and magnetoreception

Quantum biology, an emerging interdisciplinary science, integrates the principles of quantum mechanics into understanding biological phenomena. This integration may initially seem paradoxical, but it possesses the potential to illuminate many complicated biological processes that remain unexplained by classical biology (McFadden and Al-Khalili, 2016). Quantum mechanics, the heart of quantum biology, explores into the science of nanoscopic particles at the atomic and subatomic levels. Governed by rules that often appear counterintuitive in everyday life, these particles form the foundation of quantum theory and its potential applications to biological phenomena (Berman, 2016).

One such application pertains to the understanding of magnetoreception, an elusive sensory mechanism enabling organisms to detect and respond to Earth's magnetic fields. Quantum processes may play a critical role in this phenomenon (Wiltschko and Wiltschko, 2019). The Key aspects of quantum mechanics integral to understanding quantum biology are the concept of *quantum entanglement and quantum coherence*. The concept of *quantum entanglement* denotes a phenomenon where the quantum state of each particle in a pair or group cannot be described independently of the others, regardless of the distance separating them. Providing an accessible metaphor for one of the most confusing yet fundamental concepts in quantum entanglement, imagine two twins who are far apart, one in Bangkok and the other in Paris. If something happens to one twin, the other instantly knows, even though they are thousands of kilometers apart. This illustrates quantum entanglement, where the state of one particle instantly influences the state of another, regardless of their spatial separation. This phenomenon has been confirmed experimentally (Castelvecchi and Gibney, 2022).

In another player, *quantum coherence*, describes the ability of a quantum system to maintain this superposition state over time (Schlosshauer, 2007). To create a clear picture, imagine a coin spinning in mid-air. While it's spinning, you could argue that it could be both heads and tails at the same time, which is similar to a superposition. One might compare quantum coherence to the theoretical scenario in which a spinning coin, suspended mid-air, maintains its rotation indefinitely without committing to a particular orientation, be it heads or tails.

The principle of quantum entanglement and quantum coherence applies to the RPM as a theory in quantum biology. For more detail, RPM involves a photochemical reaction where light excitation creates a pair of radicals, or molecules with an unpaired electron. These radicals are quantum entangled, and their properties show that their spins can mirror each other. The introduction of a magnetic field causes these spins to flip at different rates, disrupting their synchrony. An organism can detect this disturbance, thereby sensing the magnetic field. Thus, RPM is suggested as a candidate mechanism for magnetoreception in living organisms, and cryptochrome proteins are hypothesized to mediate this process (Ritz et al., 2000).

Cryptochromes, proteins that regulate light-dependent physiological processes, are believed to contribute to magnetoreception in various organisms, including plants (Hammad et al., 2020). When exposed to blue light, quantum effects within cryptochromes might trigger a series of biochemical reactions that could potentially influence plant responses to magnetic fields. However, our understanding of plant magnetoreception remains in its beginning, with most experimental evidence derived from studies on *Arabidopsis thaliana* (Pooam et al., 2020). Exposure to magnetic fields elicited responses in these plants, possibly mediated by cryptochromes. Nonetheless, the precise mechanism remains speculative, underscoring the need for further rigorous research. Additionally, not all plant responses to magnetic fields can be explained by the RPM. Another possible quantum-based mechanism in plant magnetoreception has also been reported. The research suggests that *Arabidopsis thaliana* has mechanisms for detecting magnetic fields that do not rely on light and may be more consistent with LCM of magnetoreception than the cryptochrome-associated RPM (Dhiman et al., 2023). LCM is a concept in quantum mechanics that explains how certain systems undergo transitions between different energy levels. It is particularly relevant in the study of magnetoreception. This suggests that multiple pathways may exist for magnetoreception in plants, highlighting the complexity of these processes.

Synthesizing the above, the fusion of quantum mechanics and biology expands our understanding of natural phenomena in unanticipated ways. The intricate dance of quantum particles may be orchestrating more biological processes than we currently comprehend. As we continue to uncover these hidden connections, we could revolutionize our understanding of life's fundamental processes.

3 The quantum-based magnetoreception mechanism

According to the quantum biology concepts we mentioned above, the study of magnetoreception, has spurred a plethora of exciting theories. Significantly, two significant quantum-based theories are proposed, the RPM and the LCM. The subsequent sections will provide an easily understandable introduction to these mechanisms.

3.1 Radical-pair mechanism: illuminating nature's quantum compass

The RPM, an early quantum-based theory proposed over 40 years ago (Schulten et al., 1978), represents a fascinating intersection where quantum physics and biology meet. This intersection opens new possibilities in the field of quantum biology, aiming to crack the mysteries of magnetoreception. The RPM, suggested by Schulten and his team in 1978, explains how Earth's low-energy magnetic fields can interact with biochemical reactions in organisms (Schulten et al., 1978) even though the energy of magnetic fields is lower than thermal energy. This mechanism involves two radical molecules, electron donor and electron acceptor molecule (Figure 1), each produced from a chemical reaction within the organism. Radicals are characterized by an odd number of electrons, contributing to their instability. A defining feature of these radicals is the spin magnetic moment of their unpaired electron. The essential element in this mechanism is the quantum mechanical entanglement of the spins of the unpaired electrons in these two radicals. Intrinsically linked, the state of one instantaneously influences the state of the other, regardless of the distance between them. This entanglement gives rise to the phenomenon of quantum coherence in the radical pair system. Each radical molecule's spin direction can randomly orient itself in either a parallel (triplet) or anti-parallel (singlet) state. The transition between these states, known as singlet-triplet interconversion, is facilitated by hyperfine interaction—an interaction between the nuclear spin (resulting from the spinning motion of protons and neutrons) and the spin of the unpaired electron (Hore and Mouritsen, 2016). This superposition state of singlet and triplet state could refer as the superposition state and it can oscillate between these two states due to the intrinsic properties of quantum mechanics as called, quantum coherent spin dynamics. The rate of conversion between singlet and triplet states tends to remain stable at a specific frequency pattern. Interestingly, the introduction of an external magnetic field interacts with the spin magnetic moments of these radicals, causing them to flip or alter their spins at different rates. This interaction with the unpaired electron, known as the Zeeman interaction, can change the dynamics of the interconversion, affecting the rate and final yield of the reaction. This disruption in their synchrony can be detected by the organism, thus forming the basis for magnetoreception (Hore and Mouritsen, 2016). In summary, the quantum coherence in the RPM is crucial because it allows the radical pair to exist in a superposition of spin states. This superposition enables the radical pair to be sensitive to the surrounding magnetic field. The external magnetic field can influence the relative spin alignment of the radical pair, leading to different reaction outcomes depending on the orientation and strength of the magnetic field. This interference is hypothesized to be sensitive to very weak magnetic fields, such as the Earth's magnetic fields (Lambert et al., 2012). For further detail, we recommend reading one of the most excellent papers in this field: Hore and Mouritsen (2016).

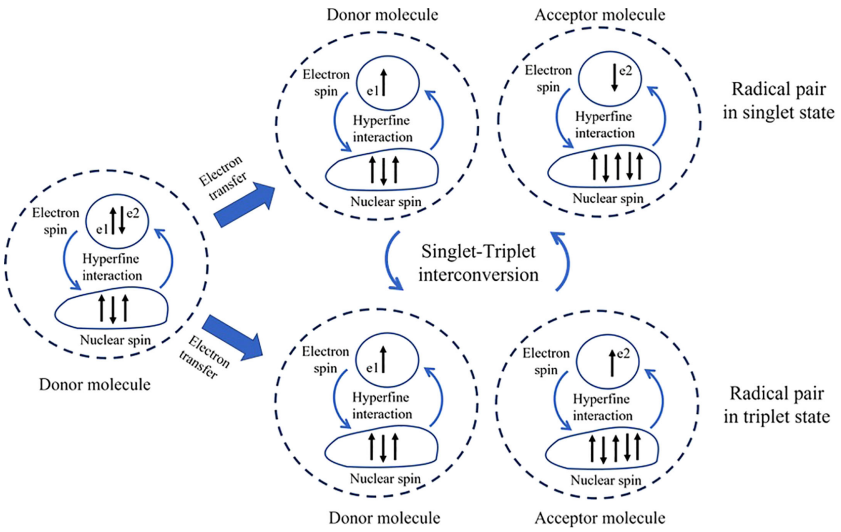

FIGURE 1
Nuclear-spin and the singlet-triplet interconversion. The donor molecule, which has three nuclear spins, transfers one electron (e2) to the acceptor molecule, which has four nuclear spins. The difference in hyperfine interaction resulting from the number of nuclear spins can induce the electron's spin to be in a single or triplet state and facilitate interconversion between them. The dashed line between e1 and e2 represents the correlation between the two electrons.

However, this mechanism is limited by spin relaxation. Over time, these spins undergo relaxation, lose this orientation, and return to a random, equilibrium state. The spin-relaxation time of each radical pair, determined by their hyperfine interactions, varies among different radical pairs. This factor is crucial when considering the potential magneto-sensitivity of a biological molecule. Moreover, another key challenges in quantum biology, and particularly in the RPM, is the issue of maintaining quantum coherence and entanglement in a warm and noisy biological environment. These quantum states are delicate and can be easily disrupted—a process known as decoherence. Overcoming this issue of decoherence to allow for meaningful biological effects is one of the significant hurdles to be crossed in the field of (Schlosshauer, 2007).

The study of quantum effects in biological systems like the RPM in magnetoreception is a relatively new field. However, the insights it offers could revolutionize our understanding of biological processes and necessitate a paradigm shift in the way we approach biological research.

Indeed, magnetoreception in migratory animals and the underlying radical-pair mechanism have been recognized since the 1970s, but the primary magnetosensitive molecule remains elusive. In the next section, we will discuss cryptochrome, the candidate magnetoreceptor from the radical-pair mechanism.

3.2 Cryptochromes: the enigmatic puzzle of magnetoreception

Scientists, in their quest to identify a viable biological molecule for magnetoreception, imagine a molecule within a living organism that can absorb external energy and create a suitable radical pair. This pair should be capable of interacting with external magnetic fields. Light, as one of the most frequent external energies, can induce a biological molecule to produce a radical pair in the living organism. Consequently, this phenomenon leads to the conjecture that photoreceptors could function as magnetosensing sites A significant breakthrough was achieved in 1992 when researchers discovered that migratory birds orient themselves towards the magnetic direction under blue or green light, but not red light. This implies that the photopigment for magnetoreception should be sensitive to these colors (Wiltschko et al., 1993). In more detail, the avian magnetic compass is influenced by light, functioning effectively in bright sunlight and under full-spectrum light (Wiltschko and Wiltschko, 1981). It has been found that short-wavelength light is essential, and the required light intensities are surprisingly low. Orientation experiments with birds have shown sensitivity to wavelengths such as 373 nm (UV), 424 nm (blue), 501 nm (turquoise), and 565 nm (green). However, wavelengths such as 568 nm and 585 nm (yellow) and 617 nm, 635 nm, and 645 nm (red) led to disorientation (Wiltschko et al., 1993; Wiltschko and Wiltschko, 1995; Wiltschko and Wiltschko, 1999; Pinzon-Rodriguez and Muheim, 2017). The sensitivity of migratory birds to specific wavelengths, particularly in the blue and green regions of the spectrum, underscores the intricate relationship between light and navigation. This phenomenon may be linked to the birds' ability to perceive the Earth's magnetic field, with blue and green light potentially playing a role in enhancing this perception. For navigation, birds may need to have photoreceptors that can perceive short-wavelength light such as 373 nm (UV), 424 nm (blue), and 501 nm (turquoise), allowing them to respond to these specific wavelengths. The observed disorientation under yellow and red light suggests a finely tuned mechanism that relies on specific wavelengths for orientation.

Concurrently, the blue-light photoreceptor cryptochrome was discovered in plants (Ahmad and Cashmore, 1993). Cryptochrome was subsequently incorporated into the radical-pair mechanism by Schulten and colleagues, which was in line with their theory. In 2000, Schulten and Ritz proposed that cryptochrome is a potentially magnetically sensitive molecule in the radical-pair mechanism, implying a crucial role for eyes and light in the magnetic compass (Ritz et al., 2000).

For more information, cryptochromes are flavoproteins fundamental to a multitude of biological processes in various organisms, notably in *Arabidopsis* thaliana, where they were first discovered (Ahmad and Cashmore, 1993). They regulate numerous functions in plants, such as circadian rhythms, photoperiodism, flowering, and hormone signaling (Chaves et al., 2011). These proteins bear a structural resemblance to photolyases, enzymes involved in DNA repair, thereby suggesting a common ancestral origin (Chaves et al., 2011). Chaves et al. (2011) reviewed the primary structure of cryptochromes which consists of an N-terminal photolyase-related region and a divergent C-terminal domain. The photolyase-related region encompasses the chromophore binding site: the flavin adenine dinucleotide (FAD). It is within this region that the crucial radical pair interactions central to the proposed magnetoreception mechanism occur. The FAD molecule is thought to be the site where the radical pairs form in the magnetoreception process. Following the absorption of a photon, an electron from the FAD molecule is excited into a higher energy state, resulting in the formation of a radical pair involving FAD and a nearby tryptophan (Trp) residue (Hore and Mouritsen, 2016). These radical pairs, entangled and subsequently existing in a quantum superposition of singlet and triplet states, can be influenced by Earth's magnetic field. This influence, in turn, affects the interconversion between these states and the overall yield of reaction products. The C-terminal domain of cryptochromes, which is essential for signal transduction, contains a series of protein-protein interaction motifs that enable cryptochromes to participate in various signaling pathways. Thus, once the magnetic information is perceived and processed via the RPM, the C-terminal domain aids in translating it into a physiological response (Ahmad, 2016). The structural complexity of cryptochromes, with their chromophore binding site and interaction motifs, emphasizes their versatility in sensing and responding to environmental cues, including magnetic fields. The unique capability of cryptochromes to form radical pairs, thereby facilitating magnetoreception, underscores their importance in the domain of quantum biology.

The activation of cryptochromes (Figure 2), particularly the photoreduction of FAD via the Trp triad pathway, induces a conformational change that allows cryptochrome to interact with

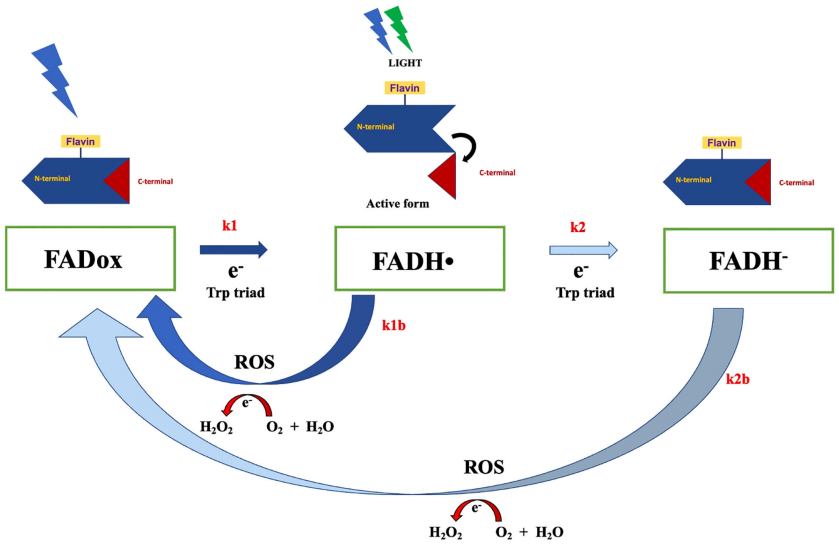

FIGURE 2
The *Arabidopsis* cryptochrome photocycle. In the dark, cryptochrome remains in an inactive state, with flavin in its oxidized form. Illumination with blue light can trigger flavin photoreduction, whereby FADox is reduced to FADH• (rate constant k1) through a forward electron transfer via the Trp triad pathway. This process induces conformational changes and the unfolding of the c-terminal domain, the signaling state of cryptochrome, allowing the protein to interact with signaling partners. Subsequent illumination of FADH• with blue or green light can prompt further photoreduction, wherein flavin in FADH• is reduced to the fully reduced state FADH-, at a constant rate k2. However, the reduced state of flavin can revert back to FADox via a reoxidation reaction, generating reactive oxygen species (ROS) and H_2O_2.

protein partners for signal transduction response (Ahmad, 2016). This process also leads to alterations in the C-terminal extension, facilitating interaction with signaling partners such as CIB1 (Liu et al., 2017). As these interactions can result in protein phosphorylation during the activation state, they can significantly influence plant development. Interestingly, cryptochrome reoxidation generates reactive oxygen species (ROS), which could potentially contribute to magnetoreception response (Ahmad, 2016). Cryptochromes have also been proposed as potential magnetoreceptors in animals (Fedele et al., 2014a; Fedele et al., 2014b; Wiltschko et al., 2021), particularly in avian navigation (Ritz et al., 2000; Wiltschko and Wiltschko, 2019). Experimental evidence aligns cryptochromes with the radical-pair mechanism, positioning them as integral to magnetoreception (Gegear et al., 2008; Foley et al., 2011; Bazalova et al., 2016). Despite the compelling evidence supporting the role of cryptochromes in animal magnetoreception, their function in plant magnetoreception is not yet thoroughly established. Experimental results in this context are insufficient to provided comprehensive insight into plant magnetoreception mechanisms mediated by cryptochromes. Further studies are, therefore, warranted to elucidate this understudied aspect of plant physiology.

3.3 Exploring the potential of cryptochrome as a key player in plant magnetoreception

Magnetoreception in plants, although a relatively underexplored domain, has sparked considerable interest due to its complex interplay with the Earth's magnetic fields. Certain effects on plant development, as a result of magnetic field exposure, have been documented (Maffei, 2014). For example, pea epicotyls were found to be longer under low magnetic field conditions, potentially due to increased cell elongation and osmotic pressure (Yamashita et al., 2004). These findings suggest that magnetoreception plays a role in plant growth and development, although the specific mechanisms require further investigation. The influence of magnetic fields, both weak and strong, on plant physiology opens exciting new avenues for research in plant sciences (Maffei, 2014).

Regarding the mechanism, magnetic fields affect cryptochrome-dependent responses, including hypocotyl growth and anthocyanin accumulation, as well as influencing on the expression of related genes (Xu et al., 2012; Xu et al., 2014; Xu et al., 2017; Xu et al., 2018; Pooam et al., 2019). Unquestionably, experiments conducted in some studies also demonstrate the direct effect of magnetic fields on the phosphorylation on the C-terminal domain of cryptochromes (Pooam et al., 2019; Albaqami et al., 2020; Hammad et al., 2020). To evaluate the response of C-terminal phosphorylation to the magnetic field, we conducted phosphorylation experiments utilizing Western blotting techniques. Cryptochrome phosphorylation was visualized on the Western blots, evident through an upward mobility shift of the phosphorylated protein. We analyzed the intensity of the phosphorylated band using ImageJ software, following the methodologies described by Pooam et al. (2019) and Albaqami et al. (2020). Our findings revealed that the phosphorylation of cryptochrome was modulated in response to the applied magnetic field (Pooam et al., 2019; Albaqami et al., 2020). This increase in phosphorylation serves as a direct indication of enhanced cryptochrome biological response and activation and substantiates the role of cryptochrome in magnetoreception.

Nonetheless, other photoreceptors, such as phytochromes, might also be influenced by geomagnetic fields (Agliassa et al.,

2018b; Dhiman and Galland, 2018). While the evidence points towards a significant role of cryptochromes in magnetoreception, their exact contribution, whether as primary magnetoreceptors or functioning in tandem with other molecules, remains to be definitively established. Future research is essential to fully elucidate this complex process.

To find a resolution to this debate, another approach to provide evidence that cryptochromes function as magnetoreceptors involves the use of radiofrequency (RF) as a diagnostic tool. The application of RF fields to investigate the role of cryptochromes in magnetoreception is based on their capacity to interfere with the dynamics of radical pairs. Specifically, radiofrequency fields can induce additional flips in the spins of the electrons within the radical pairs, thereby disrupting the magnetic information (Ritz et al., 2009). Within the framework of the RPM, the alignment of electron spins in the radical pair is susceptible to external magnetic fields. These fields can induce the flipping of spins, which modifies the interconversion rate between singlet and triplet states. The organism can detect these changes, which form the basis for magnetoreception. The introduction of an RF with a suitable frequency - known as the Larmor frequency, which is determined by the gyromagnetic ratio of the electron and the local magnetic field strength - can trigger additional spin flips, essentially scrambling the magnetic information. This occurs because RF, like the Earth's magnetic field, contain a magnetic component that can interact with the electron spins (Engels et al., 2014). If the application of the RF impairs an organism's ability to sense magnetic fields, it suggests that magnetoreception is likely mediated by a RPM. Experiments using radiofrequency fields have been performed on migratory birds, and the results strongly suggest that the birds' magnetic compass operates based on a RPM, potentially involving cryptochromes (Ritz et al., 2009; Engels et al., 2014).

Moreover, findings from the study demonstrate a significant decline (up to 24%) in response to blue light in *Arabidopsis* seedlings exposed to radiofrequency (RF) fields. This provides compelling evidence to support the notion that cryptochromes are likely candidates to act as plant magnetoreceptors, within the context of the radical-pair mechanism (Albaqami et al., 2020).

3.4 Diving into the depths: decoding the dance of the radical-pair mechanism and photocycle in cryptochromes

RPM presents a compelling model for comprehending magnetoreception in cryptochromes, a process contingent upon the formation of specific radical pairs and their prospective sensitivity to magnetic fields. Despite extensive research focusing on this mechanism, uncertainty remains concerning the radical pairs most critical for magnetic sensitivity within the cryptochrome photocycle. Two primary radical-pair hypotheses, namely the conventional and alternative radical-pair mechanisms (Figure 3), have emerged, each proposing distinct radical pairs as the primary magnetically sensitive entity (Player and Hore, 2019).

In order to elucidate this, we offered a comprehensive explanation of the photocycle of plant cryptochromes. In this context (Player and Hore, 2019), two radical pairs, termed conventional mechanism (Figure 3A) and alternative mechanism (Figure 3B), were identified that fulfil some, if not all, the criteria of the radical-pair hypothesis. The *conventional mechanism* emerges from the forward photochemical cycle and necessitates an FADox ground state, wherein the photoreduction of FAD produces a [FAD•− TrpH•+] radical pair. Conversely, the *alternative mechanism* relies on flavin reoxidation and requires the creation of FADH− via either of two methods: light-dependent photoreduction of FADH•, or light-independent reduction through cellular processes. The ensuing reactions between FADH − and oxygen give rise to a semiquinone-superoxide [FADH• O2•−] radical pair.

Consequently, the RPM, both conventional and alternative, offer distinct methods for examining and understanding

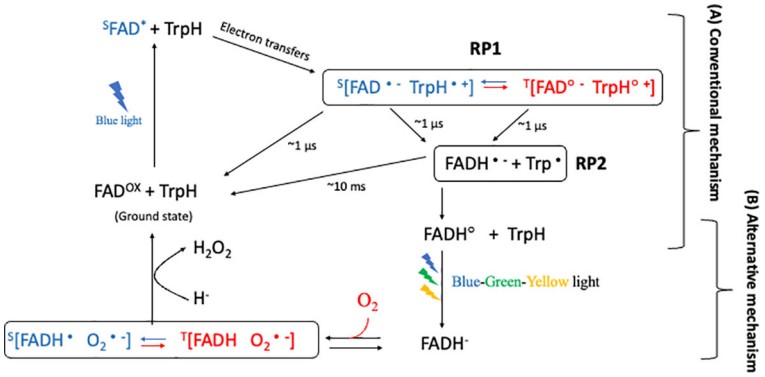

FIGURE 3
Conventional and Alternative radical-pair mechanism in cryptochrome. After blue-light illumination, the first radical pair, RP1, is generated, with a microsecond-scale lifetime. RP1 is proposed to be the magnetically sensitive radical pair in **(A)** the conventional mechanism. The blue color represents the singlet product, and the red color represents the triplet product during the interconversion between singlet and triplet product. RP2, which is generated from RP1. Subsequent illumination with blue-green-yellow light causes flavin to fully reduce (to FADH-) in the triplet state. In this state, the fully reduced flavin can revert back to the ground state in the presence of oxygen in the dark, generating a superoxide radical pair. The radical [FADH• O2•−] pair, which has a lifetime of several minutes, is therefore suggested to be the magnetically sensitive radical in **(B)** an alternative mechanism.

cryptochrome magnetoreception. However, the intricacy of the photocycles implicated in these mechanisms, coupled with the variability among different cryptochrome types, implies that a comprehensive understanding of magnetoreception will necessitate continued investigation and multifaceted research approaches (Karki et al., 2021).

3.5 The conventional radical-pair mechanism: quantum pioneer in the magnetoreception

This mechanism (Figure 3A) posits that the crucial step in magnetoreception involves the photoreduction reaction of the cryptochrome photocycle, culminating in the formation of radical pairs. This process generally encompasses the involvement of a photoactivated molecule, often a protein, and an adjacent electron donor, together constituting the radical pair. The FAD and Trp are regularly proposed as the radical pair constituents within cryptochromes. Upon light absorption, the cryptochrome-embedded FAD molecule is excited and accepts an electron from a neighbouring Trp residue, leading to the formation of the radical pair [FAD•− TrpH•+; RP1]. In this radical pair, the unpaired electron spins on the FAD and Trp radicals can assume either a triplet or singlet configuration. The quantum entanglement and quantum coherence of these spins enables the radical pair to subsist in a superposition of these states, with the local magnetic field influencing the interconversion between the singlet and triplet states (Ritz et al., 2010). Additionally, the radicals in the singlet state can revert to the ground state [FAD and TrpH]. However, the radicals in both singlet and triplet states can also advance and become [FADH•Trp•; RP2], which could be referred to as RP2 (Müller et al., 2015; Nohr et al., 2016). The lifetime of RP1 and RP2 is approximately 1 μs and 10 ms, respectively (Maeda et al., 2012). The RP2 appears to have a longer lifetime than RP1 and is more suitable as the potential radical pair for magnetoreception. Nevertheless, the quantum yields of FAD•− in RP1 has strongly anisotropic 14N, therefore, RP1 is presumed to be determined by the Earth's magnetic fields and can be the basis for magnetic sensing in migratory birds for this mechanism more than RP2 (Solov'yov et al., 2007; Hore and Mouritsen, 2016). In support of this mechanism, several experiments also demonstrated the effects of the magnetic field on forwarding electron transfer of cryptochrome's (Maeda et al., 2012; Mouritsen and Hore, 2012; Dodson et al., 2013; Sheppard et al., 2017). However, most of the experiments supporting this mechanism are *in-vitro* experiments. For instance, the finding supports the light-dependent RPM postulate that light triggers the formation of radical pairs (Maeda et al., 2012). The presence of a magnetic field influenced these radicals, enhancing the recovery of the FAD ground state, which aligns with the theoretical predictions of RPM. Moreover, the absorption signal of AtCry1 below 420 nm decayed rapidly due to the protonation of FAD•− to FADH•, further underscoring the light-dependent RPM (Maeda et al., 2012). These *in vitro* studies on AtCry1 unambiguously substantiate the conventional light-dependent mechanism of RPM (Maeda et al., 2012).

In addition, the application of RF at the Larmor frequency has emerged as a promising tool for characterizing the properties of the magnetically-sensitive radical pair. Hore and Mouritsen (2016) conducted calculations suggesting that if RF at 1.4 MHz disrupts the orientation of migratory birds, one of the radicals in the pair should be devoid of hyperfine interactions. To explore this, they simulated a system of radical-pair reaction in which the first radical had a strong hyperfine interaction. For the second radical, they varied the number of hyperfine interactions from 0-4. Their findings suggested that if both radicals have several hyperfine interactions, the effect of RF should manifest at a variety of different frequencies. However, if one of the radicals has only one or no hyperfine interaction, the RF effect could be prominent at only the appropriate frequencies. Especially if one radical has no hyperfine interaction, the sensitive effect could present only at 1.4 MHz, the same frequency that showed the effect in the behavioural experiment. In the context of the conventional radical-pair mechanism, the [FAD-Trp] pair aligns with Hore & Mouritsen's model in which both radicals have significant hyperfine interactions with the nuclei more than 10. Thus, Lee et al. (2014) proposed that this pair might not be sensitive to the stimulation of RF. Furthermore, Deviers et al. (2022) demonstrated that the radical triad system, as previously mentioned, could offer enhanced magnetosensitivity, which seems irreconcilable with the classical RPM, suggesting a shift in our understanding of the mechanism involved in cryptochrome's magnetoreception (Deviers et al., 2022). This has profound implications for our comprehension of cryptochrome function and its potential role in magnetoreception.

While the existing evidence offers substantial support for the light-dependent RPM, the majority of these insights are derived from *in vitro* studies. This leaves a critical gap in our understanding as *in vivo* validation of this mechanism has yet to be conclusively demonstrated. The complex interplay of biological and environmental factors in a living organism could significantly influence the operation of the RPM. Thus, comprehensive *in vivo* studies are indispensable for fully corroborating the light-dependent RPM and establishing its relevance in real-world biological contexts.

3.6 The alternative radical-pair mechanism: the unsuspected *in-vivo* triumph

Contrary to the conventional mechanism, the alternative radical-pair mechanism posits the radical pair [FADH• O2•−] as the principal magnetosensing candidate (Ahmad, 2016). Lee et al., (2014) postulated that this pair might exhibit greater sensitivity to Earth's magnetic fields than the [FAD•− TrpH•+] pair. This radical pair endures for several minutes (Herbel et al., 2013), a duration suitable for magnetic interaction, an idea supported by numerous studies (Maeda et al., 2008; Ritz et al., 2009; Müller and Ahmad, 2011).

However, despite the extended lifespan of the [FADH• O2•−] pair, the spin-relaxation time of the superoxide radical is exceptionally short, which could impede its potential as a magnetically sensitive molecule (Karogodina et al., 2009; Karogodina et al., 2011; Kattnig and Hore, 2017). To counteract

the rapid spin relaxation issue, Kattnig and Hore (2017) suggested an enhanced model of the radical-pair mechanism incorporating a radical scavenging system. Nevertheless, this model remains theoretical, awaiting empirical validation. Recently, Hong and Pachter (2023) conducted spin dynamics simulations to investigate the influence of inter-radical interactions and scavenging radicals on magneto-sensitivity. They explored various radical pairs and examined how the presence of scavenging radicals affects the spin dynamics of these pairs. Through their simulations, they assessed the impact of scavenging radicals on the spin relaxation rates and overall magneto-sensitivity of the radical pairs. By analyzing the spin dynamics of the proposed radical pairs and considering the effects of scavenging radicals, Hong and Pachter (2023) offer valuable insights into the mechanisms that could enhance magneto-sensitivity. Their theoretical framework provides an understanding of the interplay between inter-radical interactions and scavenging radicals, which could potentially address the rapid spin relaxation issue in the enhanced model proposed by (Kattnig and Hore, 2017). It is important to note that the study by Hong and Pachter (2023) is based on theoretical spin dynamics simulations. While their findings are valuable, further empirical validation is necessary to confirm the effectiveness of the proposed mechanisms. Therefore, their paper serves as a foundation for future experimental studies to investigate the role of scavenging radicals in enhancing magneto-sensitivity and addressing the rapid spin relaxation issue in the radical-pair mechanism. Intriguingly, a more recent study by Deviers et al. (2022) proposed a radical triad system, consisting of the flavin semiquinone, the superoxide, and a tyrosine or ascorbyl scavenger radical, could provide superior magnetosensitivity under realistic conditions. The spin-selective recombination reaction with a third radical in this system may enhance magnetosensitivity, offering new insights into the RPM and the potential role of cryptochrome in light-independent magnetoreception (Deviers et al., 2022). Player and Hore (2019) demonstrated that specific restrictive conditions are necessary to mitigate the rapid relaxation time of superoxide. To address the problem of rapid spin relaxation time, as discussed by Kattnig and Hore (2017), several strategies can be explored. One approach is the chemical control of spin-lattice relaxation in molecular qubits, as demonstrated by Amdur et al., (2022). By understanding and manipulating molecular vibrations and relaxation times, it may be possible to enhance spin relaxation time in superoxide-containing radical pairs. Amdur et al., (2022) investigated this control to discover a room temperature molecular qubit, highlighting the potential of molecular electronic spins as a novel platform for qubit structure and control. Additionally, the study by Nicolaides et al. (2023) provides insights into spin pumping from organic radical films, presenting another avenue to explore. They demonstrated spin current emission from a Blatter-type radical with outstanding stability and low roughness, indicating the possibility of realizing spin pumping even in the absence of long-range ferromagnetic order (Nicolaides et al., 2023). These approaches, along with the deposition of highly-ordered arrays on solid surfaces to create 2D-lattices and doping with controlled amounts of spin-free radicals, can contribute to solving the rapid spin relaxation issue and foster the development of suitable molecular qubits with potential industrial applications (Xu and Ping, 2023).

Additionally, some *in-vivo* studies also reported results supporting the alternative mechanism of RPM. For instance, some reports demonstrated that migratory birds could also orient to Earth's magnetic direction in green light at about 565 nm. In this case, the flavin cofactor of cryptochrome should be able to absorb the light at 565 nm (Green light). One possible flavin state that can absorb light at around 565 nm is the neutral radical redox FADH° state of cryptochrome, which reduces to FADH- after green light absorption (Nießner et al., 2013; Nießner et al., 2014). From this state, there is no possibility for the radical pair [FAD•– TrpH•+] to form and interact with magnetic fields. Additionally, from the behavioural experiment where birds were exposed to an intermittent light/dark cycle and a magnetic field present only in the dark interval, birds were found to orient in the magnetic direction, even when the magnetic field exposure was only during the dark period (reoxidation step). These findings support the radical-pair that formed during flavin reoxidation of cryptochrome in the dark as the possible magnetically-sensitive radical-pair for magnetoreception, known as the alternative radical-pair mechanism (Wiltschko et al., 2016).

Interestingly, in the plant model, results from Xu et al. (2014) showed the effect of magnetic fields on the dephosphorylation of *Arabidopsis* cryptochrome in darkness. Also, Agliassa et al. (2018b) demonstrated the impact of geomagnetic fields on the photomorphogenic-promoting gene expression in etiolated seedlings, which they linked to the light-independent mechanism. Recently, similar results from Agliassa and Maffei (2019) reported the expression of plant endogenous clock genes that was altered after exposure to the near-null field in the dark condition (Agliassa and Maffei, 2019). These magnetically responsive results in the dark imply that the reoxidation step might be the magnetically-sensitive reaction in the cryptochrome photocycle. Therefore, the potential magnetic sensitivity radical-pair for this mechanism should be [FADH- O2•–].

Significantly, our own laboratory work has been instrumental in advancing our understanding of the alternative RPM. Our research with *Arabidopsis* cryptochrome-1, as presented in Pooam et al. (2019) and Hammad et al. (2020), offers compelling evidence for the role of magnetic fields in cryptochrome activity. We have observed that exposure to a static magnetic field can enhance cryptochrome responses to light, a phenomenon that is especially noticeable when the magnetic field is applied during dark intervals between light exposures. These findings suggest that flavin reoxidation, potentially involving reactive oxygen species (Procopio et al., 2016), could be the magnetically sensitive step in the cryptochrome photocycle. This alternative mechanism contrasts with the traditional focus on the forward electron transfer reaction involving Trp• and Tyr• radicals (Mouritsen and Hore, 2012; Dodson et al., 2013; Hong and Pachter, 2015; Lüdemann et al., 2015; Worster et al., 2016) and instead emphasises the importance of dark-state reactions.

Our research results suggest a shift in the narrative surrounding cryptochromes and their role in magnetosensing. We have uncovered the significance of the flavin reoxidation reaction and

its interaction with a magnetic field in the *Arabidopsis* cryptochrome photocycle, a subject thoroughly reviewed (Chaves et al., 2011; Ahmad, 2016b; Hammad et al., 2020). Our comprehension of the cryptochrome photocycle has evolved to recognise that the flavin reoxidation – the conversion of FADH• back to FADox – plays a pivotal role in cryptochrome magneto-sensitivity. This conversion reaction, likely involving the superoxide radical (O2•−), is susceptible to magnetic field effects, thereby introducing a new pathway for magneto-sensitivity in cryptochromes. This perspective significantly expands our understanding of the cryptochrome photocycle and its role in magnetosensing, highlighting that much remains to be learnt about the exact mechanisms at play.

The recent study by Deviers et al. (2022) lends credence to this hypothesis, demonstrating that the reoxidation reaction of the reduced FAD cofactor in cryptochrome, potentially involving a radical triad system, can display remarkable magnetosensitivity under realistic conditions. This study thus reinforces the concept that the radical triad system, comprising the flavin semiquinone, the superoxide, and a scavenger radical, might play a crucial role in cryptochrome's magnetosensitivity (Deviers et al., 2022). Our understanding of the radical-pair mechanism and its role in magnetoreception continues to evolve. The long-established model of the [FAD•− TrpH•+] radical pair serving as the primary magnetosensing agent is being contested by newer findings that highlight the potential of the [FADH• O2•−] pair, and even more so the radical triad system (Deviers et al., 2022).

Our own research has contributed to this shifting perspective, shedding light on the possibility that the flavin reoxidation reaction, involving the [FADH• O2•−] pair or a triad system, could be a magnetically sensitive step in the cryptochrome photocycle. This expanded understanding could have profound implications for our understanding of cryptochrome activity, magnetoreception, and, by extension, navigation in migratory animals.

Although the light-independent variant of the RPM provides a promising alternative for explaining magnetoreception in scenarios like plant growth where light may not be readily available, it also faces several challenges and limitations, inherently tied to the complexities of quantum mechanics and the nature of the radicals involved.

Firstly, as with the light-dependent RPM, the light-independent variant faces the challenge of maintaining quantum coherence in biological systems. Decoherence – the loss of quantum coherence – is a ubiquitous problem in quantum systems and is thought to occur rapidly in the warm, wet, and noisy environment of biological cells (Schlosshauer, 2007). A crucial question is how long quantum coherence must be maintained in radical pairs to influence biological processes. Current research suggests that the timescales could be surprisingly long, requiring mechanisms to protect the coherence (Hore and Mouritsen, 2016). Moreover, our theoretical understanding of how magnetic fields could influence the recombination rates or product yields of radical pairs remains underdeveloped. Current quantum mechanical models, although useful, do not provide comprehensive insight into these effects, particularly for complex biological systems with many interacting components (Kominis, 2009).

Secondly, this light-independent RPM proposes the involvement of biologically ubiquitous, yet highly reactive molecules like superoxide radicals. Superoxide, a byproduct of cellular metabolism, is suggested to form radical pairs whose spin states could be influenced by magnetic fields, facilitating magnetoreception (Buchachenko, 2016). However, superoxide is a potent ROS, and its levels are tightly regulated within the cell due to its potential to cause oxidative damage. How organisms might harness superoxide for magnetoreception without disturbing the delicate redox balance is a significant open question. Yet, we believe that the level of ROS production from the cryptochrome photocycle is at a mild level and might not disrupt the redox balance.

Thus, while the light-independent RPM opens new avenues for understanding magnetoreception, it also poses significant challenges. The interplay of quantum coherence, the role of radicals like superoxide, and the theoretical modelling of radical pair dynamics under magnetic fields represent key areas for future research.

In conclusion, both the conventional and alternative RPMs have their merits and limitations, but the alternative RPM provides a compelling avenue for future research. Further studies are needed to expand our understanding of this complex biological phenomenon, potentially leading to novel insights into magnetoreception in plants and other organisms.

3.7 Quantum yield in conventional and alternative mechanisms of radical pair formation in magnetoreception

In the realm of magnetoreception, the quantum yield of radical pairs holds significant importance. Quantum yield refers to the efficiency of generating radicals through a photochemical process, with higher values indicating greater efficiency (Hiscock et al., 2016). Several studies have delved into the quantum yield of radical pairs in plant cryptochrome, such as [FAD•− TrpH•+], [FADH•Trp•], and [FADH• O2•−], revealing their magnetic sensitivity (Maeda et al., 2012; Müller et al., 2014; Procopio et al., 2016).

For instance, Maeda et al. (2012) conducted a significant study on the kinetics and quantum yields of photo-induced flavin-tryptophan radical pairs in cryptochrome, finding them to be magnetically sensitive. The research clarified the mechanistic origin of the magnetic field effect and determined the rates of various processes involved. While the study indicates that the quantum yield of radical pairs in plant cryptochrome can be influenced by the magnetic field, it does not provide specific quantum yield values for different states or the exact value for the formation of a secondary species (RP2) from the magnetically sensitive radical pair RP1 in the conventional mechanism. The findings suggest an influence of the magnetic field on quantum yield but leave room for further exploration to pinpoint exact values. Furthermore, Müller et al. (2014) estimated the quantum yield of the FADH· Trp· radical pair formation in isolated Arabidopsis cryptochrome 1 (At CRY1) to be approximately 2%. Procopio et al. (2016) further linked the quantum yield of FADH° formation to the

biological function of plant cryptochrome. However, the specific quantum yields of each radical pair and their modulation by the magnetic field are often not directly provided. This gap in knowledge extends to the probability of simultaneous radical formation in cryptochromes, a complex issue that warrants further exploration.

In the context of the Alternative Mechanism, the quantum yield of radical pair formation should be lower at certain light intensities, reflecting its formation from the radical pair in the Conventional Mechanism. This aspect, along with the probability that two or three radicals are formed simultaneously in all cryptochromes in a plant cell, presents intriguing questions for future research.

Understanding the quantum yield of radical pair formation in magnetoreception, particularly in the Conventional and Alternative Mechanisms, is an intricate and essential aspect that warrants further exploration. The existing studies, while providing valuable insights, leave certain questions unanswered, such as the specific quantum yield values for various radical pairs and the probability of simultaneous radical formation in cryptochromes.

Future research could focus on designing controlled experiments to measure the quantum yield of different radical pairs at varying light intensities. By utilizing advanced spectroscopic techniques and computational modeling, researchers could investigate the formation and interconversion of radical pairs in both the Conventional and Alternative Mechanisms. Special attention could be given to understanding how the quantum yield of radical pair formation in the Alternative Mechanism might be lower than that in the Conventional Mechanism at certain light intensities. Additionally, the study of the triad system and the probability that two or three radicals are formed simultaneously in all cryptochromes in a plant cell could be explored through targeted experiments. This might involve the development of new methodologies or the adaptation of existing techniques to specifically probe these aspects. Furthermore, collaboration between experimentalists and theorists could foster a more comprehensive understanding of how magnetic fields influence the quantum yield of radical pairs. This interdisciplinary approach could lead to the development of new theoretical models that more accurately represent the complex dynamics of magnetoreception.

Thus, the quantum yield of radical pair formation in magnetoreception presents a promising and rich area for further investigation. By focusing on the specific aspects mentioned above and employing a combination of experimental and theoretical approaches, researchers could unravel new dimensions in the study of magnetoreception. This exploration could not only enhance our understanding of this complex biological phenomenon but also pave the way for innovative applications in various fields.

3.8 Cracking the code of cryptochrome: advanced approaches and future directions in plant magnetoreception research

Evidence from some studies have already confirmed that the light-independent reaction of cryptochrome is a crucial step for magnetoreception. Nonetheless, further corroboration from independent laboratories, and the application of diverse experimental techniques, could be essential for the validation of this mechanism. Therefore, the fascinating field of plant magnetoreception presents several opportunities for future experimental studies. These could potentially resolve the ongoing debate between the conventional and alternative radical-pair mechanisms. Here are some research directions that plant biologists might contemplate.

Initially, investigations into the structure and dynamics of cryptochromes could be of great value. Structural investigations, such as those employing X-ray crystallography or cryo-electron microscopy, might provide critical insights into the binding of superoxide and other radicals to cryptochromes (Brautigam et al., 2004). This could help to determine whether the alternative RPM has a structural foundation. Molecular dynamics simulations could supplement these structural investigations by providing a dynamic perspective on radical interactions within the cryptochrome (Solov'yov and Schulten, 2012). These structural techniques could furnish us with precise, high-resolution images of the radical (superoxide, for instance) binding to the cryptochrome. If the radicals bind and interact in a way that is consistent with the conventional RPM, then that model is likely accurate. If the interaction aligns more with the alternative RPM, then that model might be the valid one.

Moreover, another promising starting point is absorption spectroscopy. This method can illuminate the potential light-dependent behavior of the cryptochrome photocycle. By subjecting cryptochrome to light of various wavelengths and intensities and observing the resultant absorption spectra, we can gain insights into the photocycle's response to light. In the conventional RPM, the formation and recombination of radical pairs are directly linked to the absorption of light. Therefore, if we observe changes in the absorption spectra that align with the predicted behavior of the radical pairs under this model, that will provide evidence supporting the conventional RPM. For instance, we might see absorption peaks corresponding to the energy differences between the singlet and triplet states of the radical pairs. Conversely, if the alternative RPM involves additional light-triggered steps or a different mechanism, we might expect to see different patterns in the absorption spectra. For example, there might be additional absorption peaks or variations in peak intensities that cannot be explained by the conventional RPM. To unravel the temporal dynamics of this response, one could consider using pulsed light instead of continuous light. Conversely, to probe the light-independent reactions, a parallel set of observations could be carried out under complete darkness, providing a contrasting backdrop to the light conditions (Ozturk, 2017). Complementing these experiments, electron paramagnetic resonance (EPR) spectroscopy could be employed to detect the presence of radical pairs within cryptochrome under different light conditions. This technique, capable of detecting unpaired electron spins, offers a direct probe into the RPM (Sheppard et al., 2017).

Additionally, *in vivo* studies using plants could be conducted. For instance, genetically engineering plants with altered cryptochrome structures could help reveal the critical components for magnetoreception. Genetic manipulation techniques, such as CRISPR/Cas9, could be employed to create

these modified plants (Ma et al., 2015). Cryptochrome, the protein hypothesized to mediate magnetic sensing, has a specific structure. Certain parts of this structure are thought to be critical for its function. Using genetic engineering tools like CRISPR/Cas9, we could alter the structure of cryptochrome in the plants. These alterations could target the specific parts of cryptochrome that are believed to be important for its magnetic sensing function. If the conventional RPM is correct, then these parts of the cryptochrome structure would interact with radicals in a particular way to sense magnetic fields. The conventional RPM suggests that the radical pair is formed between a FAD molecule, which is the chromophore of the cryptochrome, and a Trp residue (Trp-triad) within the protein structure. In the alternative RPM, it's suggested that a superoxide radical, or another similar radical, binds to the FAD molecule to form the radical pair. This would mean that the binding site for this radical would be located on the FAD molecule. In terms of genetic engineering, it might be possible to modify the Trp residues in the Trp-triad, or the binding site on the FAD molecule, to test the predictions of the conventional and alternative RPMs. Nevertheless, this would require a highly detailed understanding of the cryptochrome structure and function, and the ability to make precise modifications to the protein sequence. To date, the specific site of each radical pair in the cryptochrome structure is not clearly understood.

Finally, it might be worthwhile exploring the potential influence of other environmental factors on magnetoreception. Could variables such as temperature or light intensity influence the efficiency of magnetoreception? Answering these questions could shed light on whether the conventional or alternative RPM is more viable under different environmental conditions. For example, in the case of temperature, Temperature effects on singlet-triplet interconversion: In the context of the RPM, a key feature of radical pairs is their ability to interconvert between singlet and triplet states, which is a quantum mechanical process that can be influenced by magnetic fields. The rates of singlet-triplet interconversion could potentially also be influenced by temperature, although the details of this are likely to be complex and could depend on specific characteristics of the radical pair and its environment.

A notable reference discussing the impact of temperature on the RPM in birds is a paper by Henrik Mouritsen and colleagues (Mouritsen et al., 2016). In this study, the authors argue that the key radical pair in avian cryptochrome (FADH•-TrpH•+) is indeed temperature-sensitive, which could provide an explanation for the experimentally observed effects of temperature on bird magnetoreception. To definitively determine the effects of temperature on magnetoreception, and to help differentiate between the conventional and alternative RPMs, one could design experiments in which the temperature is systematically varied while monitoring magnetoreception. Comparing the experimental results to the predictions of the conventional and alternative RPMs, taking into account their respective expectations for temperature effects, could provide further insights into which model is more likely to be correct. However, it's important to note that interpreting these experiments may be challenging, due to the complex and potentially varied ways in which temperature can influence radical pair dynamics.

In the case of light, the conventional and alternative RPMs, both would theoretically be affected by light intensity, but potentially in different ways. For the conventional RPM, light is necessary to form the radical pair. Therefore, under low light conditions, the formation of radical pairs might be limited, potentially reducing the strength or reliability of the magnetoreception signal. Conversely, under high light conditions, more radical pairs would be expected to form, possibly enhancing magnetoreception. For the alternative RPM, if it involves some sort of light-triggered enzymatic process or additional light-sensitive step, then its dependence on light intensity might be more complicated. For example, there might be an optimal light intensity that maximizes the magnetoreception signal, with either too little or too much light reducing efficiency. By conducting experiments with varying light intensities, we could compare the resulting magnetoreception abilities to the predictions made by each RPM. For instance, if we find that magnetoreception abilities increase linearly with light intensity, this might support the conventional RPM. Alternatively, if we observe a more complex relationship between light intensity and magnetoreception, this could provide evidence for the alternative RPM.

Each of these suggested experiments could potentially provide critical insights into the mechanisms of plant magnetoreception. They each represent a piece of a larger puzzle, and together, they could lead us towards a more comprehensive understanding of this intriguing biological phenomenon. Nevertheless, experiment on plant magnetoreception frequently yields inconsistent results that prove challenging to replicate. Such inconsistencies might originate from the intricate dynamical degrees of freedom influencing magnetic compass sensitivity in cryptochromes, as depicted by the recent study by Grüning et al. (2022). In their groundbreaking paper, Grüning et al. (2022) examined the magnetic compass sensitivity of cryptochromes from plants (*Arabidopsis* thaliana AtCry1) and avian species (European robin Erithacus rubecula ErCry4a, and pigeon Columba livia ClCry4a). The researchers utilized molecular dynamics simulations and density functional theory-derived hyperfine interactions to scrutinize the impact of thermal motion and fluctuations in the dihedral and librational angles of FAD and Trp radicals on radical pair recombination dependent on the geomagnetic field direction. Their findings disclose that librational motions of TrpC•+ in avian cryptochromes are considerably smaller than in AtCry1. This suggests that the variations in the dynamical properties of cryptochromes across species might contribute to the inconsistent results frequently observed in plant magnetoreception studies. Additionally, they demonstrated that the magnetic sensitivity of cryptochromes isn't solely dependent on singular snapshots of protein structure, but should also consider the ensemble properties of hyperfine interactions. This revelation provides insight into why research in this field often produces inconsistent and hard-to-replicate results.

In conclusion, the validation of the magnetoreception mechanism in plants necessitates rigorous multidisciplinary investigation. Studies must delve into cryptochrome structures, their light-dependent behavior, and the impact of environmental factors. Further, the inconsistencies often seen in magnetoreception

research underscore the complexity of this biological process. By exploring these proposed research directions, we may garner insights into this fascinating phenomenon, ultimately clarifying the underpinnings of plant magnetoreception.

4 Beyond boundaries: exploring magnetoreception's hidden dimensions:

While the RPM has led magnetoreception research, new theories emerge. LCM proposes that magnetic fields affect molecular energy transitions, potentially explaining plant magnetoreception. Meanwhile, the Magnetite-based theory suggests the protein complex MagR and biogenic magnetite offer insights into non-avian magnetoreception. These fresh perspectives could revolutionize our understanding of how organisms navigate Earth's magnetic field.

4.1 Level crossing mechanism: the quantum vanguard unraveling magnetoreception's mysteries

The LCM is a budding quantum-based theory of magnetoreception offering an engaging alternative to the RPM (Binhi, 2019). Contrary to the RPM's assertion that magnetic fields modulate radical pair-involved reactions, the LCM posits that these fields could influence transitions between energy levels of magnetic moments in molecules or atoms (Binhi, 2019).

At its core, the LCM is based on a principle of quantum mechanics known as "level crossing," a phenomenon where the energies of two states within a quantum system become identical under the influence of an external field, leading to the states' merging. In the context of magnetoreception, this external field is the geomagnetic field, and the quantum system may be represented by certain magnetic molecules or atoms within a biological entity. This suggests that changes in the geomagnetic field could instigate level crossings, thus influencing biological processes (Binhi, 2016).

To better grasp this concept, visualize a busy train station with each train symbolizing different energy levels of magnetic moments in molecules or atoms. Each train travels at a distinct speed, indicating different energy states. Sometimes, two trains (energy levels) move side by side at the same pace, a brief synchronization akin to the "level crossing" in quantum mechanics. The stationmaster, representing the geomagnetic field, can alter the trains' speeds by manipulating the track switches. As the stationmaster alters these tracks, trains initially moving at different speeds might end up side by side, their speeds synchronized, thereby creating a "level crossing." In this metaphor, the brief synchronization of the trains' speeds symbolizes the energy levels of magnetic moments in molecules or atoms within a biological system becoming identical under the geomagnetic field's influence. These changes in the trains' speeds and routes could then affect the passengers (biological processes), potentially inciting changes in their behaviors or conditions. Just like the stationmaster's ability to influence the trains' speeds and paths, causing occasional synchronization that impacts passengers, the geomagnetic field can affect certain magnetic molecules or atoms' energy levels, potentially influencing biological processes.

Experimental evidence supporting the LCM as a feasible magnetoreception mechanism stems from studies on the model plant organism, *Arabidopsis thaliana*. Researchers noted magnetic effects in various processes like cryptochrome- and blue light-independent seed germination, red-light elicited anthocyanin accumulation, and hypocotyl elongation in darkness (Agliassa et al., 2018b; Dhiman and Galland, 2018) These phenomena could not be adequately explained by the cryptochrome-mediated RPM, thereby aligning more closely with the LCM's predictions, which anticipate multiple maxima in stimulus-response curves and the effects of magnetic field reversals (Binhi, 2019).

A thorough investigation by Dhiman et al. (2023) further underscored the potential relevance of the LCM in understanding magnetoreception. The researchers scrutinized the magnetoreception of *Arabidopsis thaliana* by analyzing several developmental responses in weak static magnetic fields. The study determined that a magnetic field of 50 μT could accelerate seed germination by approximately 20 hours, consistent across the wild-type strain and the cry1cry2 double mutant, which lacks cryptochromes 1 and 2 (Dhiman et al., 2023).

Interestingly, the study revealed that the magnetic field-induced germination acceleration was obscured in the presence of exogenous sucrose. In addition, the researchers identified maxima near 9 and 60 μT in the stimulus-response curves for hypocotyl elongation. Moreover, the photo-accumulation of anthocyanins and chlorophylls could also be modulated effectively by magnetic fields when exposed to low-irradiance red and blue light, respectively (Dhiman et al., 2023).

These findings suggest the existence of light-independent mechanisms of magnetic field reception in *Arabidopsis thaliana*, mechanisms that remain unidentified. Furthermore, the study outcomes align more closely with the LCM's predictions than those of the cryptochrome-associated RPM. This alignment provides additional support for the potential significance and applicability of the LCM in explaining magnetoreception phenomena in plants (Dhiman et al., 2023).

In summary, while the LCM's capacity to explain the intricate responses of biological systems to magnetic fields suggests a substantial role in our understanding of magnetoreception, further research is required. This research aims to fully explore and validate the LCM's implications, especially concerning the established RPM and cryptochrome-mediated processes in plants. However, it is crucial to recognize that the LCM, as a relatively new theory in the field of magnetoreception, has certain limitations and challenges that have yet to be fully addressed.

One of the key limitations of the LCM is the absence of direct experimental evidence. While studies such as those conducted by Dhiman et al. (2023) and Agliassa et al. (2018b) have yielded results aligned with LCM predictions, they do not conclusively demonstrate the operation of the LCM in magnetoreception. The observed responses could potentially be explained by other, as-yet-unknown mechanisms. Thus, while the LCM can explain certain

observations, direct proof of its operation in biological organisms remains elusive.

Another limitation relates to the identification of molecular candidates that can undergo level crossing under the Earth's magnetic field. As of 2021, no specific molecule or atomic structure has been identified in biological organisms that could serve this function. This stands in contrast with the RPM, where cryptochromes have been identified as potential magnetoreceptors (Ritz et al., 2000).

Moreover, maintaining quantum coherence in a biological environment, a significant challenge for any quantum-based theory, is of particular concern for the LCM. Quantum coherence refers to the maintenance of the phase relationship between different states in a quantum superposition, a key requirement for quantum phenomena like level crossing to occur. However, biological systems are typically warm and wet, conditions that can cause rapid decoherence, disrupting the delicate quantum states (Schlosshauer, 2007).

Lastly, the LCM, like any scientific theory, is subject to change and refinement as new evidence becomes available. Currently, the LCM is a promising but not yet fully established theory of magnetoreception. Further empirical research is needed to validate and potentially expand upon this theory.

Building upon the LCM as a plausible theory for plant magnetoreception necessitates rigorous experimentation (Binhi, 2019). An ideal starting point is investigating the LCM's prediction of a resonant response to specific frequencies of magnetic field oscillation, although exact frequencies are yet to be identified. These can be experimentally determined, with plants subjected to a range of frequencies and subsequent changes in biological processes, such as seed germination or root growth direction, being meticulously recorded. If particular frequencies induce substantial effects, it would corroborate the LCM (Binhi, 2019).

Another intriguing aspect of the LCM is the requirement of quantum coherence (Schlosshauer, 2007). Quantum coherence, in the context of the LCM, refers to the maintenance of specific phase relationships between quantum states in a superposition, enabling the system to exhibit behaviors uniquely quantum mechanical in nature. Detecting quantum coherence under biological conditions, therefore, is a significant step in affirming the LCM. Techniques such as electron spin resonance or quantum beat spectroscopy, capable of detecting unpaired electron spins or interference patterns in fluorescence following excitation respectively, could be employed for this purpose (Lambert et al., 2012). If quantum coherence can be convincingly demonstrated in plant samples, it would further reinforce the LCM's standing.

Lastly, the LCM predicts a distinct temperature dependence compared to the RPM (Binhi, 2019). To understand why temperature could be a significant factor, it's crucial to realize that both the LCM and the RPM are quantum mechanical processes, and temperature can influence quantum states. In the case of the LCM, magnetic field influences can cause certain energy levels in a system to cross, leading to changes in the system's behavior that could affect biological processes. How much these levels cross, and the biological consequences of these crossings, could well depend on temperature. However, the specific temperature points that would validate the LCM are not yet clear. This area requires further theoretical development and experimental testing. An experimental approach might involve subjecting plants to a range of different temperatures, then measuring their responses to magnetic fields under these different conditions. If the changes in responses at certain temperatures align with the predictions of the LCM but not the RPM, this could provide evidence to support the LCM. By cultivating plants across a temperature spectrum and monitoring alterations in their magnetic responses, it could be ascertained which model's predictions better coincide with empirical observations. If the observed temperature dependence matches the LCM's predictions, it would bolster its acceptance.

In summary, these experimental strategies, focusing on frequency specificity, quantum coherence, and temperature dependence, provide promising paths to validate and refine the LCM, thereby advancing our understanding of plant magnetoreception.

4.2 MagR: magnetite's maverick transitioning from foe to ally in magnetoreception

In the captivating realm of magnetoreception, two predominant theories have garnered significant attention: the RPM, focusing on cryptochromes that have been previously discussed, and the Magnetite-based theory, which hones in on the protein complex MagR (Kirschvink and Gould, 1981; Dodson et al., 2013). The Magnetite-based theory postulates that magnetoreception transpires through biogenic magnetite, a ferromagnetic mineral pervasive in numerous organisms, analogous to a natural compass system at the molecular level. The protein complex, MagR, is envisaged as part of this magnetite-based receptor complex, with magnetite crystals within the MagR complex aligning to Earth's magnetic field, thereby generating a mechanical force that might trigger a biochemical response (Qin et al., 2016). This theory has been implicated in non-avian species such as Drosophila and mice; however, its role in plant magnetoreception is less understood, with cryptochrome-based mechanisms predominantly at the forefront (Ahmad, 2016; Qin et al., 2016).

Parmagnani et al. (2022) have unveiled fresh insights into this complex landscape through their exploration of the formation and function of iron-sulfur (Fe-S) clusters in plants, particularly focusing on how geomagnetic fields could influence them. Their paper posits that GMF reduction affects the amplitude of certain clock genes in *Arabidopsis thaliana*, thereby influencing processes such as plant growth and development, and possibly modulating the expression of various genes. The protein MagR, familiar from the magnetite-based theory, is postulated as a potential biomagnetic sensor in plants due to its capacity to form complexes with other proteins and Fe-S clusters. Furthermore, they hint at a potential integration of MagR and cryptochrome theories, suggesting a complex mechanism that marries elements of light sensitivity and magnetic field alignment (Qin et al., 2016; Xu et al., 2018). In this model, cryptochrome serves as a light sensor, inducing a

conformational change in the MagR protein upon activation, which could alter the orientation of the embedded magnetite crystals within the MagR complex. The involvement of the blue-light receptor cryptochrome in magnetic sensitivity is also invoked to support this integrated theory (Parmagnani et al., 2022).

Nevertheless, the empirical evidence buttressing this composite magnetoreceptor is scant, and it remains ambiguous whether the MagR-cryptochrome complex operates via the RPM or a magnetite-based mechanism (Dodson et al., 2013). Despite potential advancements, the MagR theory exhibits certain shortcomings compared to the RPM in the context of plant magnetoreception, primarily due to the more comprehensive understanding and extensive study of cryptochrome-based mechanisms (Ahmad, 2016; Qin et al., 2016; Parmagnani et al., 2022).

In order to solidify the MagR theory, often termed as the 'Ancient Rival' of RPM in the realm of magnetoreception, several experimental approaches are possible. We could scrutinize the presence and distribution of magnetite in plants using methods like magnetic force microscopy or transmission electron microscopy. Altering the MagR genes in plants and subjecting them to different magnetic fields under controlled light conditions could shed light on MagR's impact on magnetoreception. This 'old adversary' seems increasingly like a 'new ally', potentially contributing to our understanding of magnetoreception in organisms. A sophisticated understanding of the interplay between these two mechanisms—cryptochrome-based and MagR-based—is pivotal for advancing our knowledge of plant magnetoreception.

5 Conclusions

In conclusion, this review synthesizes our current knowledge of the magnetoreceptive abilities of plants, underscoring the fascinating interplay between quantum and classical biology. The profound implications of these mechanisms, ranging from influencing plant growth and development to potentially impacting broader agricultural practices (Galland and Pazur, 2005), serve to emphasize the significance of this field of study. As we navigate the labyrinth of plant magnetoreception, we are reminded of the complexity of nature's adaptations to environmental cues (Brautigam et al., 2004). The exciting prospect of delving further into the relationship between the RPM, LCM, and the MagR theory invites a multi-faceted exploration of the magnetoreception phenomenon. Future research will undoubtedly benefit from adopting an integrative approach, simultaneously probing the individual contributions and interactions of these theories. Moreover, an exciting dimension of this exploration is the potential industrial applications involving cryptochromes. The recent study also provides valuable insights into this area. It explores the use of photogenerated spin-correlated radical pairs (SCRPs) as molecular qubits for quantum sensing, highlighting the potential for resolution-enhanced imaging tools, magnetometers, and magnetic switches. The synthetic tunability in molecular systems offers promising avenues for the design of next-generation selective magneto-sensing devices and molecular qubits for quantum technologies (Mani, 2022).

While there is still a vast expanse of the plant magnetoreception landscape yet to be traversed, the direction provided by this review, coupled with relentless scientific curiosity, promises intriguing discoveries. As we continue to unravel the mysteries of this elusive sense, the doors to understanding how life has evolved to sense and respond to our planet's magnetic field remain wide open. We are only beginning to scratch the surface, and the journey ahead is teeming with untold promise and potential. Thus, this review does not signify an end but rather serves as an inspiring prelude to the thrilling journey of discovery that lies ahead in the realm of plant magnetoreception, including the innovative industrial applications of cryptochromes in quantum technologies.

Author contributions

TT: Writing - original draft, Writing - review & editing. KT: Writing - review & editing. KK: Writing - review & editing. LT: Writing - review & editing. ME-E: Writing - review & editing. BA: Writing - review & editing. NJ: Writing - review & editing. KB: Writing - review & editing. MP: Conceptualization, Supervision, Validation, Writing - original draft, Writing - review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Győző Garab,
HUN-REN Biological Research Centre, Hungary

REVIEWED BY
Andras Lukacs,
University of Pécs, Hungary
Jathish Ponnu,
University of Cologne, Germany

*CORRESPONDENCE
Margaret Ahmad
margaret.ahmad@sorbonne-universite.fr

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'Seeing' the electromagnetic spectrum: spotlight on the cryptochrome photocycle

Blanche Aguida[1], Jonathan Babo[1], Soria Baouz[1], Nathalie Jourdan[1], Maria Procopio[2], Mohamed A. El-Esawi[3], Dorothy Engle[4], Stephen Mills[5], Stephan Wenkel[6], Alexander Huck[7], Kirstine Berg-Sørensen[8], Sotirios C. Kampranis[9], Justin Link[10] and Margaret Ahmad[1,4]*

[1]Unite Mixed de Recherche (UMR) Centre Nationale de la Recherche Scientifique (CNRS) 8256 (B2A), Institut de Biologie Paris-Seine (IBPS), Sorbonne Université, Paris, France, [2]Department of Biophysics, Faculty of Arts and Sciences, Johns Hopkins University, Baltimore, MD, United States, [3]Botany Department, Faculty of Science, Tanta University, Tanta, Egypt, [4]Biology Department, Xavier University, Cincinnati, OH, United States, [5]Chemistry Department, Xavier University, Cincinnati, OH, United States, [6]Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, Umeå, Sweden, [7]DTU Physics, Technical University of Denmark, Kongens Lyngby, Denmark, [8]DTU Health Technology, Technical University of Denmark, Kongens Lyngby, Denmark, [9]Biochemical Engineering Group, Plant Biochemistry Section, Department of Plant and Environment Sciences, University of Copenhagen, Frederiksberg, Denmark, [10]Physics and Engineering Department, Cincinnati, OH, United States

Cryptochromes are widely dispersed flavoprotein photoreceptors that regulate numerous developmental responses to light in plants, as well as to stress and entrainment of the circadian clock in animals and humans. All cryptochromes are closely related to an ancient family of light-absorbing flavoenzymes known as photolyases, which use light as an energy source for DNA repair but themselves have no light sensing role. Here we review the means by which plant cryptochromes acquired a light sensing function. This transition involved subtle changes within the flavin binding pocket which gave rise to a visual photocycle consisting of light-inducible and dark-reversible flavin redox state transitions. In this photocycle, light first triggers flavin reduction from an initial dark-adapted resting state (FADox). The reduced state is the biologically active or 'lit' state, correlating with biological activity. Subsequently, the photoreduced flavin reoxidises back to the dark adapted or 'resting' state. Because the rate of reoxidation determines the lifetime of the signaling state, it significantly modulates biological activity. As a consequence of this redox photocycle Crys respond to both the wavelength and the intensity of light, but are in addition regulated by factors such as temperature, oxygen concentration, and cellular metabolites that alter rates of flavin reoxidation even independently of light. Mechanistically, flavin reduction is correlated with conformational change in the protein, which is thought to mediate biological activity through interaction with biological signaling partners. In addition, a second, entirely independent signaling mechanism arises from the cryptochrome photocycle in the form of reactive oxygen species (ROS). These are synthesized during flavin reoxidation, are known mediators of biotic and abiotic stress responses, and have been linked to Cry biological activity in plants and animals. Additional special properties arising from the cryptochrome photocycle include responsivity to electromagnetic fields and

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their applications in optogenetics. Finally, innovations in methodology such as the use of Nitrogen Vacancy (NV) diamond centers to follow cryptochrome magnetic field sensitivity *in vivo* are discussed, as well as the potential for a whole new technology of 'magneto-genetics' for future applications in synthetic biology and medicine.

KEYWORDS

cryptochrome, photoreceptor, flavoprotein, redox, photomorphogenesis, circadian clock, magnetic fields, ROS

1 Introduction

Cryptochromes are members of the photolyase/cryptochrome superfamily of highly conserved flavoprotein receptors which can undergo photochemical redox reactions in response to light (Ozturk, 2017; Vechtomova et al., 2021). They are found throughout the biological kingdom, from archaebacteria to man, and participate in diverse physiological responses such as plant development and in entrainment of the circadian clock in plants and animals. Since their first identification in plants (Ahmad and Cashmore, 1993), the structural and biochemical properties of isolated cryptochrome proteins from many organisms have been extensively characterized *in vitro*, including photoreactions to the picosecond time scale (see eg. Chaves et al., 2011; Zoltowski et al., 2011; Ahmad, 2016; Schroeder et al., 2018; Hore and Mouritsen, 2016; Kondoh and Terazima, 2017; Schelvis and Gindt, 2017). At the other end of the scale, the physiological and behavioral consequences of cryptochrome activation *in vivo*, both as light sensors and as signaling molecules have been extensively studied, including those involved in magnetic orientation in birds (Wiltschko et al., 2021) and those of importance to medicine, such as in clock-related pathologies and as potential therapeutic substrates in neurobiology and other diseases (eg Michael et al., 2017; Chan and Lamia, 2020; Damulewicz and Mazzotta, 2020; Lohof et al., 2022). In addition, physiological effects of cryptochromes in crop improvement (e.g. Fantini and Facella, 2020; Wang and Lin, 2020), as well as their usefulness for the creation of optogenetic tools (Seong and Lin, 2021; Huang et al., 2022), also have significant economic potential.

In this review, we focus primarily on the question of how light absorption by certain cryptochromes mediates a biological signaling response *in vivo*. This causal connection, between cryptochrome photoreactions observed *in vitro* with biological responses observed *in vivo*, is what distinguishes these Crys from classes of photoactive proteins that simply use light as an energy source (such as photolyases, or proteins of the photosynthetic apparatus). First and foremost, a light-driven chemical transformation in the receptor must be induced by light which leads to the initiation of biological signaling activity. This results in a conformational change enabling the receptor to interact with cellular signaling proteins (see eg higher plant phytochromes and phototropins as well as the animal opsins (Galvão and Fankhauser, 2015; Shichida and Matsuyama, 2009).

A second critical feature of a functional photoreceptor is that there must be a mechanism in place to return it to the inactive state once the light signal is gone. This must furthermore occur on a time scale consistent with its biological function. Otherwise, the receptor remains permanently in the 'lit' state and visually blind after the initial photon absorption event; or else returns to the dark-adapted state before it has a chance to initiate biological signaling responses.

These two features comprise the so-called photocycle – the mechanism by which a biological receptor is activated by light to achieve the 'lit', or physiologically active, state, and then returns to the inactive 'resting' state once the light signal is removed. This photocycle, enables the system to respond to the light input intensity, and is outlined in generic form in Figure 1. In this review we will discuss the origins of the photocycle, the means by which the photocycle responds to environmental signals, and the *in vivo* physiological implications of this photocycle in the control of cryptochrome responses. Finally, we will discuss emerging topics related to novel methodology for Cry analysis using NV diamond centers, and development of novel optogenetic tools using plant Crys in yeast.

The review focusses primarily on the plant sensory Crys (Cry1 and Cry2 of *Arabidopsis*), as these are among the best characterized; however some pertinent comparisons to other cryptochromes such as avian and *Drosophila* Crys will be drawn, as well as to algal Crys.

2 Origins: in the beginning, there was photolyase.

At the time of the origins of life, the earth had a particularly inhospitable atmosphere consisting primarily of hydrogen sulfide, ammonia, methane, and CO_2 (Zahnle et al., 2010). In the absence of oxygen, there was no protective ozone layer to prevent ionizing radiation from reaching the earth's surface and the DNA-damaging effects of UVB and UVC radiation posed a major challenge to

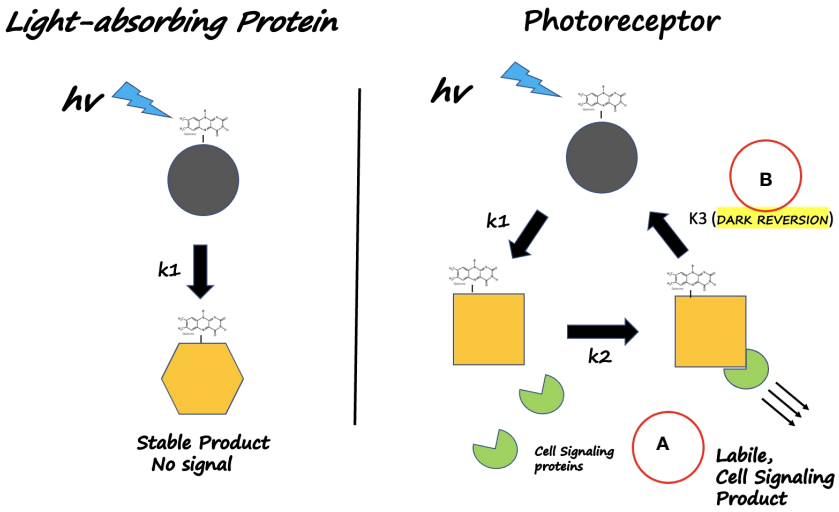

FIGURE 1
What defines a Photoreceptor? Left Panel: Light - absorbing NON- VISUAL proteins (Eg. *E. coli* photolyase) use light as an energy source. The dark-adapted state is partially oxidized *in vitro* and undergoes flavin reduction and conformational change (rate k1); however it does not interact with cellular signaling proteins and does not revert to the dark-adapted form on a physiological time scale. Instead, the reduced state remains stable and harvests additional light energy for catalytic repair of DNA. Right Panel: Light-absorbing Photoreceptors (plant Crys) undergo a photocycle on a time scale that reversibly triggers a physiological response. **(A)** Light absorption (rate constants k1, k2) triggers receptor conformational change that enables detection by downstream signaling proteins. **(B)** Return to darkness (rate constant k3) restores the inactive resting state on a time scale of minutes. In this way, biological activity is triggered by light but also responds to light intensity and duration (via k1, k2), relative to the rate of dark reversion (k3).

unicellular organisms. It was in this reducing, ionizing environment, around 3 billion years ago, that the first members of the DNA photolyase/cryptochrome gene family are thought to have arisen. Photolyases, or PHR, are DNA repair enzymes that use visible light to catalyse the repair of damaged DNA (Sancar, 2004; Vechtomova et al., 2021). Photolyase variants can repair each of the two principal types of DNA damage; 6-4 photoproducts and cyclobutane pyrimidine dimers (CPD). Since strongly ionizing radiation always occurred in combination with visible light on this early earth, using light as a catalyst for DNA repair was an effective survival strategy and, as a result, members of the photolyase gene family are today widely dispersed throughout the biological Kingdoms.

2.1 DNA repair came first

The mechanism by which photolyases use light to repair DNA is well understood. Structurally, photolyases are globular proteins that non-covalently bind two cofactors: an antenna pigment bound near the amino terminal close to the surface of the protein, and a flavin, FADH-, bound within a hydrophobic cavity at the C-terminal alpha-helical domain (see Sancar, 2004; Sancar, 2016; Zhang et al., 2017; Vechtomova et al., 2021). Photolyases recognize structural changes in DNA caused by CPD or 6-4 photoproduct damage lesions and bind to them via ionic interactions. A stable enzyme/substrate complex is then formed by flipping the damaged base pairs of the DNA into the central cavity of the enzyme proximal to the flavin. Both of these events occur in the absence of light. To initiate DNA repair, an antenna pigment, which is generally either methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deaza- riboflavin (8-HDF), first absorbs a photon of light and subsequently transfers this energy via FRET to the reduced flavin cofactor ($FADH^-$). The resulting high energy excited state flavin (FADH*-) transfers an electron to the DNA lesion which repairs the DNA (Sancar, 2004). The electron subsequently returns to the flavin to restore the fully reduced resting state of the enzyme. This process lasts less than a microsecond and is fully catalytic.

2.2 'Photoactivation reaction' and characterization of the 'Trp triad'

The reaction mechanism of photolyases has important consequences on the resting redox state of the catalytic flavin cofactor. Exclusively the reduced flavin redox state ($FADH^-$) of photolyases is catalytically active, as no other redox form can catalyse DNA repair. Therefore, a photolyase enzyme (either *in vitro* or *in vivo*) in which the flavin becomes artifactually oxidized loses all DNA repair function (Sancar, 2004; Brettel and Byrdin, 2010). However, purified preparations of isolated photolyases under atmospheric oxygen typically occur in various oxidized redox states and must first be reduced in order to provide functional enzymes for studies *in vitro*. This requirement for reduced flavin led researchers to discover and characterize the process of 'photoactivation', in which the flavin can be photoreduced into the catalytically active form.

As an example, in the case of *E.Coli* photolyase, blue light illumination of the catalytic flavin in the oxidized redox state will result in formation of the excited state flavin (FAD*), which will

then extract a nearby electron from a conserved W residue (W382) within the protein. This is followed by further electron transfer from W382 to a suitably positioned conserved W359 residue, which in turn extracts an electron from surface-exposed W306. This process of intraprotein electron transfer is accompanied by the transient formation of the FAD•- anionic radical, followed by its protonation to the neutral radical FADH• state.

To achieve the catalytically active state, a second round of illumination is required. In this round, the photon is absorbed by the neutral radical FADH• to again generate an excited state (FADH*). Subsequently, intraprotein electron transfer to the flavin (FADH* <– W382 <– W359 <– W306) will yield the fully reduced, catalytically active redox state ($FADH^-$). Although variations exist among different photolyases and/or cryptochromes in the ultimate electron acceptors being used (eg. a fourth terminal Y and/or W residue), the minimal electron transfer pathway consisting of the three W residues is widely referred to as the 'Trp triad'. It is conserved throughout the cryptochrome/photolyase gene family, and is essential for generating active photolyase (PHR) in cases where the flavin has become oxidised *in vitro* (Sancar, 2004; Brettel and Byrdin, 2010; Kavakli et al., 2017; Kavakli et al., 2019; Maestre-Reyna et al., 2022).

There are two important features of the photoactivation pathway that distinguish it from DNA repair. Firstly, unlike for DNA repair, the catalytic flavin itself can serve as an efficient photon acceptor, excluding the necessity for an antenna pigment. This is because oxidized or radical state flavin can efficiently absorb visible light. In particular, the neutral radical redox state (FADH•) absorbs in UV, blue, green and yellow light, making it responsive to all wavelengths with the exception of red light (Brettel and Byrdin, 2010; Bouly et al., 2007).

2.3 Photolyases absorb light for DNA repair primarily via their antenna pigment

The photochemical reactions undergone by photolyases harness incoming light energy for DNA repair, and are highly efficient (quantum yield of up to 0.8) (Figure 2). However, this process requires that the resting (dark-adapted) state of photolyase contains fully reduced flavin ($FADH^-$), which does not efficiently absorb visible light (cutoff above 410nm). Instead, DNA repair is initiated primarily through photon absorption by the antenna pigment (Sancar, 2004), with peak absorption in the near-UV range (around 380nm) in the case of folate derivatives. Therefore many photolyases are essentially 'blind' to visible light (400 – 500nm range) and only respond efficiently to near-UV light. Exceptions occur in the case of the so-called long wavelength photolyases which repair DNA efficiently in blue light (peak near 440nm); however, these 'long wavelength' photolyases also absorb light primarily through an antenna pigment (deazaflavin derivatives) (Sancar, 2004). In sum, most photolyases have peak activity in the UV/A region, they function less well in the visible range, and none respond at longer wavelengths above 500nm (to turquoise, green, or red light).

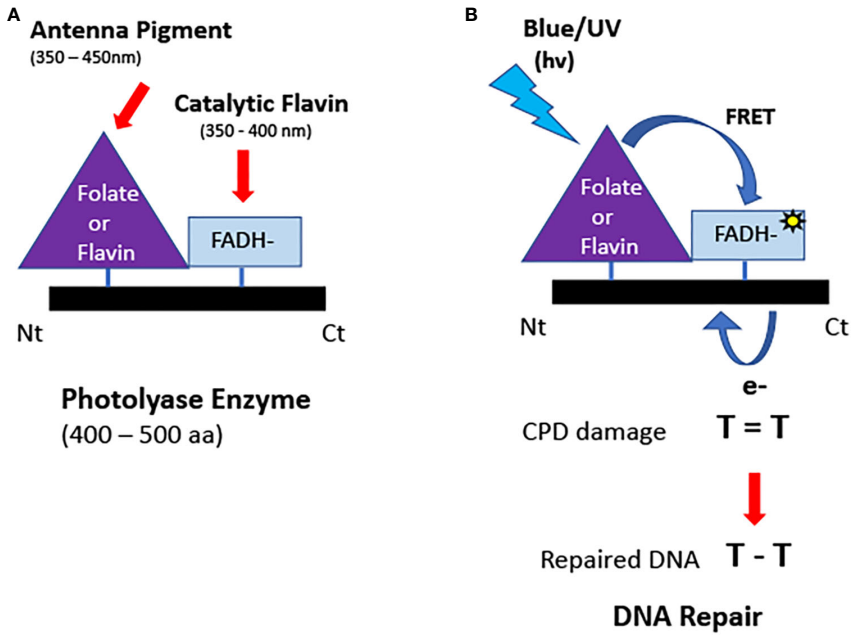

FIGURE 2
Structure and function of photolyases. **(A)** Domain structure. Photolyases typically bind two chromophores: a light-absorbing folate or deazaflavin antenna pigment and the catalytic $FADH^-$ cofactor. **(B)** Enzymatic repair of DNA. Light energy is absorbed by the antennae cofactor bound to the N terminal of the PHR and transferred by FRET to generate the excited state reduced flavin. Reduced flavin transfers an electron to repair the DNA lesion (CPD or 6-4 photoproducts). The electron is restored to the enzyme following repair. The catalytically active flavin ($FADH^-$) does not absorb light in the visible spectral range and therefore relies on antenna pigments to capture light energy.

2.4 Photolyases do not undergo a photocycle *in vivo*

One important biological requirement for efficient DNA repair is that the flavin of photolyases is stably maintained in the reduced redox state *in vivo*. The cellular resting flavin redox state is imposed by a number of parameters including protein structure of the flavin pocket in photolyase, accessibility to oxygen, and the cellular midpoint potential (Wen et al., 2022). Current evidence suggests that flavin is indeed maintained in the reduced state for photolyases *in vivo*. For example, photolyases in their purified state are notoriously difficult to oxidize, even when exposed to atmospheric conditions (oxygen concentrations orders of magnitude greater than within the cell). In the case of purified *E. coli* photolyase, full oxidation can only be achieved using added chemical oxidants (Sancar, 2004; Wen et al., 2022). This indicates that at the low cellular oxygen concentrations *in vivo* PHR is likely to remain reduced. Additional evidence that PHR is perpetually maintained in the reduced redox state comes from mutant analysis (Kavakli et al., 2017; Kavakli et al., 2019). Therefore, although it has been reported that photolyases undergo conformational changes in response to photoreduction (Cellini et al., 2022; Maestre-Reyna et al., 2022) these do not appear to be reversible *in vivo* and do not interact with the cellular signaling machinery (Figure 2, left panel).

In sum, photolyases are exquisitely sensitive to light but use light exclusively for a catalytic function. They do not appear to have a photosensory signaling role. They do not efficiently absorb visible light, do not interact with signaling proteins in plants, and do not undergo a redox photocycle consistent with a physiological time scale (seconds to minutes).

3 Cryptochromes: making the transition from photolyases to photoreceptors

A major milestone in the evolution of life occurred about 2 billion years ago when the accumulated actions of the first photosynthetic organisms resulted in the so-called 'great oxygenation event' giving rise to the present-day atmospheric conditions of a primarily Oxygen/Nitrogen atmosphere (Lyons et al., 2014). This event had a two-fold impact on the photolyases. First, there was less selection pressure for their DNA repair function thanks to the development of a protective ozone layer screening out the worst (>98%) of the ambient ionizing radiation (Cockell, 1998). Second, even relatively minor mutations affecting the photolyase flavin pocket now resulted in loss of enzymatic DNA repair function due to spontaneous oxidation of the catalytic flavin cofactor in an oxygenating environment. In other words, the stage was set for the development of novel functions in this widespread, ancient gene family and, indeed, phylogenetic analysis shows that the earliest likely cryptochromes, seem to have emerged around this time (Vechtomova et al., 2020).

Currently, many so-called cryptochromes have no known light signaling function, so it cannot be said that evolution of this gene family is exclusively driven by novel functional requirements. Indeed, some so-called cryptochromes designated from phylogenetic data have since been shown to retain a degree of photolyase function and, vice versa, some so-called photolyases have since been shown to have signaling roles (Vechtomova et al., 2020). Currently, there is consensus that there exist at least 6 major categories of the cryptochrome/photolyase gene family, based on evolutionary relatedness and function (Figure 3). These include the Type I and Type II photolyases, which are efficient in the repair of CPD DNA lesions; the Type 6-4 photolyases efficient in the repair of 6-4 photoproducts; the plant type light signaling cryptochromes Cry1 and Cry2 found in green plants and algae; the Cry-DASH cryptochrome superfamily which retain single stranded DNA repair activity; the animal Type I cryptochromes from insect; and the animal Type II cryptochromes in vertebrates and other animals including insects (For an abbreviated schematic, see Figure 3). This diverse array of cryptochromes results from their having evolved independently multiple times in the course of evolution, in many different organisms and from different ancestral photolyase lineages. A definition that fits most cryptochromes is that they are photolyase-like proteins with impaired dsDNA repair function, of which some have evolved novel roles in signaling.

Currently, light-sensing roles have been shown in the case of certain Cry-DASH cryptochromes (fungal and algal) (Kiontke et al., 2020), as well as cryptochrome subgroups related to 6-4 photolyases (Petersen et al., 2021). The plant-like cryptochromes related to Cry1 and Cry2, as well as the Drosophila Type I cryptochromes (Chaves et al., 2011; Deppisch et al. 2023) are among the best studied photosensory Crys.

3.1 Differences between plant cryptochromes and photolyases

Plant Cry1 and 2 are closely related to the Type I CPD photolyases, particularly within their N-terminal, photolyase-like domain (Figure 3). The most striking difference structurally between photolyases and plant type cryptochromes is the existence of a C-terminal extension of variable length (CCT). This domain, which is poorly conserved among different cryptochromes, consists of random coil sequences lacking defined structural features. The C-terminal domain is folded into the flavin binding pocket and undergoes conformational change upon light activation. In addition, it contains a nuclear localization signal as plant Cry1 and Cry2 are localized to the nucleus, although Cry1 also is found in the cytosol where it plays different developmental functions. A further structural distinction from photolyases is oligomerization of the N-terminal domain of Crys subsequent to illumination. This photo-oligomerization is necessary for Cry function and precedes interaction with downstream cellular signaling proteins (Ponnu and Hoecker, 2022). In support of their role, both oligomerisation and blue-light signaling can be perturbed by small Cry1 protein-isoforms which competitively inhibit formation of homo-oligomers (Dolde et al., 2018; Kruusvee et al. 2022). In sum, plant Crys have evolved structural modifications consistent with a signaling role.

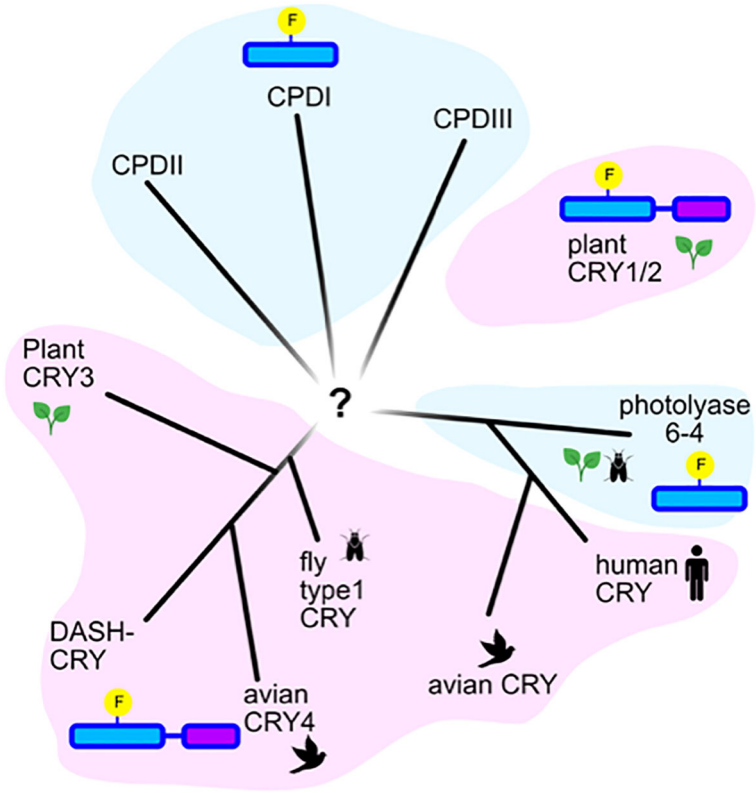

FIGURE 3
Phylogenetic relatedness of cryptochromes and photolyases. Representation of an unrooted tree showing the evolutionary relatedness of the major families of photolyases in blue shading (Types I, II, and III CPD photolyases, Type 6-4 photolyases), and of the major classes of cryptochromes (plant Cry, animal Type II Cry (human and avian), animal Type I Cry (fly), Cry-Dash, Cry4 and Cry3. According to (Ozturk, 2017).

Photochemically, plant cryptochromes undergo the same photoreduction reaction as photolyases (the so-called 'photoactivation reaction', see Section 2.2). This involves a sequential series of electron and proton transfer events, via the conserved Trp triad pathway similarly to E. coli photolyase, albeit with varying rate constants and quantum efficiency (see eg. Thöing et al., 2015). However, plant Crys do not catalyse light-triggered repair. This has been related to the poor conservation of the amino acid residues necessary for dsDNA binding and, even more significantly, to the fact that dark-adapted Cry flavin *in vivo* is in the fully oxidized redox state (Chaves et al., 2011; Burney et al., 2012). Thus, the flavin is not maintained in the catalytically active state required for DNA repair.

Additional differences relate to the flavin binding cavity, where plant Crys have an aspartic acid located close to the flavin which protonates the radical in the course of photoreduction (e.g. Burney et al., 2012; Müller et al., 2014). The resulting negatively charged aspartic acid renders the fully reduced state (FADH-) unstable. As a consequence, Crys occur stably in their oxidized redox state. They can absorb blue/UV-A light directly through their flavin cofactor and do not rely on antenna pigments as do photolyases. In further distinction to photolyases, Crys also bind ATP and other small molecule metabolites which stabilize the radical (FADH•) redox state (Müller and Bouly, 2014; Müller et al., 2014; Kottke et al., 2018).

In sum, although structurally very similar to photolyases, plant Crys do not catalyse DNA repair and instead undergo rapid flavin reoxidation after illumination; a C-terminal extension promotes oligomerization that contributes to signaling reactions (Müller and Ahmad, 2011; Van Wilderen et al., 2015).

3.2 The plant cryptochrome photocycle

Cumulatively, all of these changes have led to the evolution of a light-inducible, dark - reversible photoreduction pathway which is at the basis of Cry photoreceptor function. This property of reversible response to light is known as a photocycle (Figure 4).

The plant cryptochrome photocycle has been derived from a variety of physiological and spectrochemical approaches, including the direct detection of flavin redox states *in vivo* using whole-cell EPR and Infrared spectroscopic methods (summarized in Chaves et al., 2011; Ahmad, 2016; Procopio et al., 2016; Goett-Zinc and Kottke, 2021). Briefly, the photocycle is as follows: cryptochrome occurs in the dark with flavin in the oxidized redox state (see Figure 4, INACTIVE at top left). In this dark-adapted state, the C-terminal domain is folded within the flavin binding cavity and Cry is biologically inactive. Absorption of blue or UV light by FADox induces flavin photoreduction to the neutral radical state (FADH•) at a rate (k1) that is proportional to the light intensity (Procopio

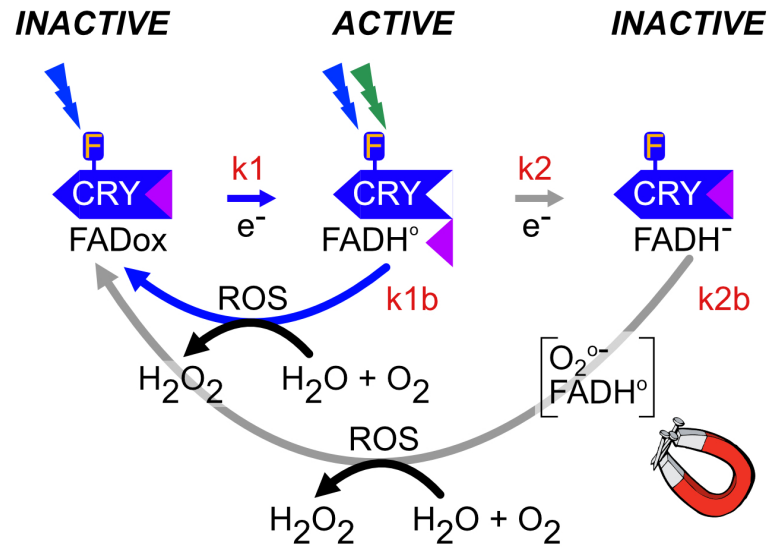

FIGURE 4
Plant Cry1,2 photocycle. In the dark, flavin is in the oxidized redox state and the protein adopts a biologically inactive conformation (*INACTIVE*). Upon illumination (blue or UV light), the anionic flavin radical is formed (FAD•-),which is then protonated to a relatively stable neutral radical redox state (FADH•), with a half-life of over a minute, which is shown in the figure. These redox state transformations trigger a structural change in the protein in which the C-terminal domain flips out from the flavin binding pocket and becomes accessible to biological sigaling partners (*ACTIVE*). This represents the biologically active ('lit') state. Further illumination of the flavin radical (which can absorb either UV, blue or green light) results in formation of the excited radical state (FADH*) followed by reduction to the fully reduced ($FADH^-$) redox state shown in the figure. This state is biologically inactive (*INACTIVE*). Both radical (FADH•) and reduced ($FADH^-$) flavin undergo slow reoxidation to the resting (FADox) redox state. Biological activity is defined by the equilibrium concentration of FADH•, which is determined from the sum of both light-dependent (k1 and k2) and light-independent (k1b and k2b) rate constants (Procopio et al., 2016). The magnet symbol indicates reactions potentially subject to modulation by magnetic field exposure (flavin reoxidation – Section 5.1).

et al., 2016). Photochemically, flavin reduction occurs via intraprotein electron transfer through a chain of conserved Trp and Tyr residues by the process of 'photoactivation' as in photolyases (see 2.2). The redox state transition induces a conformational change at the C-terminal domain (Goett-Zink et al., 2021) and renders the protein biologically active (Figures 4, 5). Further illumination induces the formation of the fully reduced redox form (FADH-) (rate constant k2), which is short-lived and biologically inactive in plants.

To restore the resting state, reduced flavin (FADH• and $FADH^-$) reacts with molecular oxygen (k2b, k1b) to restore oxidized FADox (*INACTIVE*). This reoxidation reaction occurs independently of light and is accompanied by the formation of Reactive Oxygen Species (ROS) and H_2O_2 byproducts. The Cry photocycle can therefore be considered as a dynamic equilibrium between distinct flavin redox states whose relative concentration is governed both by the light intensity (k1 and k2) and by the dark reversion rate (k1b and k2b) (Procopio et al., 2016).

3.2.1 The dark reversion reaction: mechanism of flavin reoxidation

As stated above, one of the unique features of Crys as distinct from photolyases is that they undergo efficient flavin reoxidation *in vivo*. Oxidation of reduced free flavin in solution (FAD(H)•, $FADH^-$) to the oxidized state (FADox) is a well-understood reaction that can result in the formation of multiple ROS (reactive oxygen species) intermediates including superoxide ($0_2^{-\circ}$), peroxide derivatives and H_20_2 (Romero et al., 2018). Whereas the kinetics for re-oxidation of free FAD flavin are relatively slow (250 $M^{-1}s^{-1}$), the oxygen reactivity of protein-bound flavins may be literally orders of magnitude greater (e.g. 2×10^6 $M^{-1}s^{-1}$ for glucose oxidase, or 4×10^4 for D-amino acid oxidase) (Massey, 2002). The basis for these astounding differences in efficiency is far from established. However, properties mediated by the protein component such as metal ion binding, stabilization of radical intermediate states, organic ligand binding, oxygen diffusion rates, hydrophobicity, and the charge within the flavin pocket can all have critical effects on reoxidation rates in different flavoenzymes (Massey, 2002).

In the case of plant cryptochromes, protein-bound flavin is reoxidised from the $FADH^-$ and/or FADH• radical state by a relatively slow process (k = 50 $M^{-1}s^{-1}$) involving the formation of superoxide, flavin hydroperoxide and flavin anionic radical reaction intermediates as well as hydrogen peroxide (H_20_2) as an end product (Müller and Ahmad, 2011; Van Wilderen et al., 2015). Importantly, this relatively slow reoxidation rate (half-life on the order of several minutes) has also been observed to occur *in vivo* using whole-cell EPR spectra of insect cells overexpressing plant crys (Herbel et al., 2013), confirming its biological relevance.

3.2.2 The cryptochrome photocycle is responsive to multiple environmental signals

Light: the photocycle explains how Cry is an effective, reversible light sensor, as biological activity is proportional to the equilibrium concentration of activated (FADH•) redox form, which the light intensity controls through flavin reduction reaction rate constants k1 and k2 (Figure 4) (Procopio et al., 2016). Most studies on

biological light sensing activity of plant cryptochromes have therefore focused on light-driven forward reaction intermediates either in isolated proteins or *in vivo*.

Other signals: However, as mentioned above, an additional feature of the photocycle is a relatively slow dark reversion rate (k1b, k2b) of several minutes, which is orders of magnitude slower than the light activation step (milliseconds). Since the dark reversion rate (k1b) governs the lifetime of the active form (FADH•), reoxidation rate has a disproportionate effect on biological activity of Crys. This is how external forces that affect reoxidation rates will modulate Cry biological activity, even independently of light. Such tuning forces can include temperature (Pooam et al., 2021); ligand binding (Eckel et al., 2018); localized changes in the midpoint potential of the cellular environment (Romero et al., 2018) or, most intriguingly effects of applied magnetic fields (see 5.1 below).

ROS Signaling: A second potential signaling feature conferred by the reoxidation step is that it generates Reactive Oxygen Species (ROS) as a byproduct of illumination. ROS, and in particular hydrogen peroxide (H_20_2) and superoxide radicals, are versatile signaling intermediates that promote resistance to biotic or abiotic stress, regulation of senescence (ageing), repair of oxidative damage to DNA and proteins, and many other responses. ROS dependent signaling mechanisms are highly conserved, and can include both enzymes and transcription factors that are directly regulated by ROS (e.g. FOXO in C. elegans and humans). They may also stimulate enzymatic activity and ROS scavenging pathways that decreases cellular ROS concentration (e.g. catalase) (Storz, 2011; Schieber and Chandel, 2014). In the case of plant cryptochromes, ROS is formed directly in the nucleus as a byproduct of illumination of both Cry1 and Cry2 (Consentino et al., 2015; Jourdan et al., 2015) and this mechanism has been implicated in the modulation of genes for pathogen defense, and resistance to biotic and abiotic stress (El-Esawi et al., 2017).

In sum, the cryptochrome photocycle confers an exquisitely tunable, versatile biological signaling mechanism that is responsive not only to light wavelength and intensity, but also to extraneous factors that can modulate redox reaction rates. Thus, Crys respond to factors including temperature, pH, cellular metabolites, and even exposure to electromagnetic fields (see section 5). In plant Crys, the primary signaling mechanism is well established to arise from conformational change triggered by FADox to FADH• redox state transition. However, there is also a secondary signaling role for ROS synthesized in the course of the Cry photocycle, which may have important roles in response to stress.

4 Biological relevance

The real challenge in assigning any photocycle deduced from *in vitro* photochemical properties is to demonstrate the actual validity of the proposed mechanism *in vivo*. Evidence in support of the biological validity of the plant Cry photocycle has been obtained from *in vivo* spectroscopic, genetic, biochemical, and physiological studies in plants, yeast, and other experimental systems (Chaves et al., 2011; Ahmad, 2016; Hammad et al., 2020).

Here we review the evidence in support of the radical (FADH•) redox state of Crys as the biologically active state, and that any factors that alter the concentration of the FADH• form (such as light) will correspondingly modify Cry biological activity. In addition, we explore the connection between Cry redox state interconversion and the physiological changes that occur in plants.

4.1 Evidence that the flavin radical is the 'lit' biologically active state *in vivo*

An action spectrum of the physiological response of Cry1 to different wavelengths of light *in vivo* showed the characteristics of oxidized flavin (peak at 450, shoulders near 420 and 480nm, and no significant response above 500nm - green light) (Ahmad et al., 2002). This is consistent with all the known Cry functions in plants, which are activated only under blue/UVA light (Wang and Lin, 2020; Ponnu and Hoecker, 2022).

If the FADH• is the light-activated redox state, any illumination regimen that depletes FADH• should also reduce biological activity. This prediction was validated by co-illumination experiments using both blue and green light together. Whereas blue light drives the reaction FADox to FADH• to accumulate the biologically active signaling state, green light is only absorbed by the radical redox state and thereby drives the reaction: FADH• to $FADH^{-}$. Therefore co-illumination of green and blue light should deplete FADH• relative to blue light illumination alone. This was indeed observed for both Cry1 and Cry2 (Banerjee et al., 2007; Bouly et al., 2007; see also discussion in Ahmad, 2016). This is indirect evidence that flavin photoreduction to the FADH• redox state activates plant Cry.

Direct evidence for the occurrence of flavin photoreduction *in vivo* involves work by Robert Bittl and Erik Schleicher at FU Berlin. The plant Cry1 and Cry2 photoreceptors were overexpressed in insect cell cultures to achieve a high concentration of receptor protein. Flavin radical formation was subsequently visualized in living cells by whole-cell EPR spectroscopy. This method provided direct evidence of flavin reduction in response to illumination and, using ENDOR analysis, demonstrated the formation of the neutral radical (FADH*) redox state (Banerjee et al., 2007; Bouly et al., 2007; Herbel et al., 2013). Other examples of whole-cell EPR spectroscopy have followed the redox state transition of the Drosophila Cry, which likewise correlates with *in vivo* physiological data (Ahmad, 2016).

Combining physiological methods with *in vivo* spectroscopy has furthermore determined the lifetime of the signaling state of plant Crys *in vivo* (Herbel et al., 2013). This was done by directly measuring the concentration of the signaling state FADH• in living cell cultures over time using EPR spectroscopy. At the same time, the half-life of the biological signaling state *in vivo* was determined by measuring its 'escape time', or time after which the biological response was no longer reversible by green light. In this way, the lifetime of the signaling state as measured by biological activity *in vivo* (escape time) was shown to match the half-life of cryptochrome-bound FADH•, consistent with the deduced photocycle.

An additional approach to define the photocycle has been kinetic modeling of the Cry1 and Cry2 redox interconversion

rates (Figure 4; k1, k2, k1b and k2b), and to compare these rates deduced from *in vitro* studies with purified proteins to those deduced from physiological responses to light in plants. Calculation of the relative quantum yields of the *in vitro* photoreactions showed that flavin photoreduction efficiency and FADH• radical formation was roughly four-fold greater for Cry2 than for Cry1. This calculated greater light sensitivity for Cry2 flavin reduction deduced from '*in vitro*' experiments indeed closely matched the greater light sensitivity of Cry2 *in vivo* (Procopio et al., 2016).

4.2 Genetic evidence: mutants in the Trp triad pathway

The first step in the Cry photocycle is flavin photoreduction from the oxidized (FADox) to the biologically active radical (FADH•) redox state. Studies exploring the Cry photoreduction mechanism *in vitro* have established that the Trp triad pathway is used in plant Crys similarly to that in photolyases (see Section 2.2). Mutation in any of the three conserved Trp residues mediating electron transfer from the protein surface to the flavin effectively abolishes the plant cry ability to undergo flavin photoreduction *in vitro* (Zeugner et al., 2005; Engelhard et al., 2014).

Thus, a prediction of the Cry photocycle is that Trp triad mutants of Cry should lose light sensitivity, as they do not form the FADH• radical state.

This prediction was addressed experimentally using Cry1 Trp triad mutants expressed in transgenic plants. The isolated mutant proteins failed to reduce upon illumination *in vitro*, and did not accumulate the active FADH• radical state. When expressed *in vivo*, the photoreduction-deficient Cry1 mutant constructs showed significantly reduced biological activity in mediating plant growth responses such as anthocyanin accumulation and hypocotyl growth inhibition in responses to blue light (Zeugner et al., 2005). Thus, reduced biological activity correlated with reduced FADH• radical state as predicted. Further indication of the effect of the Trp triad mutants on biological function of Crys came from transient grating experiments of M. Terazima. The diffusion coefficient was determined for isolated plant Cry1, before and after illumination. A significant increase in protein volume was observed upon illumination of wild type Cry as measured by its diffusion rate (Kondoh et al., 2011), which are now known results from the light-dependent oligomerization required for biological signaling (Ponnu and Hoecker, 2022). By contrast, Cry1 proteins with mutations in the Trp triad electron transfer pathway showed no such light-dependent increase in density, indicating an absence of conformational change related to signaling (Ponnu and Hoecker, 2022). Taken together, these results using two different experimental approaches from independent labs, both showed correlation of flavin reduction to the radical (FADH•) in order to reach a biologically relevant signaling state.

There has been subsequent confusion as to the effect of the Trp triad in Cry activation resulting from conflicting (to Zeugner et al., 2005) reports that Trp triad mutants of Cry1 and Cry2 were functional as blue light receptors in plants (Li et al., 2011; Gao et al., 2015). These apparent contradictions are however simply resolved by closer inspection of the actual data. In the first paper (Li et al., 2011) the Cry2 Trp triad mutants assigned as being blue light responsive, were in fact also biologically active in red light and darkness, thereby constitutively active and not light responsive at all. We now know from recent CryoEM studies that these mutants in fact had irreversible conformational changes rendering them constitutively active, and unresponsive to light (Hao et al., 2022). Similarly, Trp triad mutants of Cry1 presented as being blue light responsive, only occurred at blue light intensities far above saturation (Gao et al., 2015). In the supplement of this same paper, a proper dose response curve showed almost 95% reduction in blue light sensitivity of Trp triad mutants as compared to the relevant wild type overexpressing controls (see analysis presented in Hammad et al., 2020 for details). Even the residual biological activity of Trp triad mutants was shown to result from residual *in vivo* (FADH•) formed through alternate (to the Trp triad) electron transfer pathways *in vivo* (Engelhard et al., 2014).

In sum, light responsivity correlates with *in vivo* formation of FADH• and is consistent with analysis of the plant Trp triad mutants. It is also clear that the Trp triad plays a structural role in plant Crys apart from its role in electron transfer, particularly in the case of Cry2.

4.3 The lifetime of the Cry radical state is correlated with Cry biological activity

Another prediction of the Cry photocycle is that mutations that alter the lifetime of the reduced Cry redox states should affect biological function. Indeed, recent reports of mutations affecting the lifetime of FADH• redox state subsequent to illumination in yeast further support its biological signaling role. A two-hybrid genetic screen was performed using the interaction of Cry2 with its relevant plant signaling partner CIB1 as a direct assay for biological activity (Taslimi et al., 2016). In this screen, performed under blue light pulse conditions, mutants were obtained with enhanced biological activity in response to short intermittent pulse conditions. These mutants mapped to the flavin pocket, and were shown to slow down the rate of flavin re-oxidation from the neutral semiquinone to the oxidized FAD redox state in a related algal CRY. The resulting stabilization of the flavin radical, which would prolong its lifetime under pulsed light conditions, explains the corresponding increase in biological activity in yeast.

Consistent with these findings, the effect of mutations that disrupt the ATP binding site in the plant Cry flavin pocket showed diminished Cry biological activity in transgenic plants *in vivo* (Eckel et al., 2018). The ATP binding site was targeted for mutation as ATP binding is known to significantly prolong the lifetime of the Cry radical (FADH•) redox state both *in vitro* and *in vivo* (Kottke et al, 2021). As predicted, there was diminished biological activity in mutants lacking the ATP binding site consistent with a reduced 'lit' state concentration (Eckel et al., 2018). In further support of these experiments, additional mutants slowing the reoxidation rate of plant Cry2 *in vitro* resulted in enhanced biological activity in transgenic plants (Araguirang et al., 2020). These included the L404F substitution mutation in the plant

Cry2 which enabled the purified protein to be maintained in a stable radical redox state in the dark *in vitro*. *In vivo*, this mutation conferred constitutive biological activity in the dark as well as hypersensitivity to light, consistent with FADH• accumulation. Importantly, structural studies confirmed that this particular mutation triggered no misfolding artifacts in the protein, unlike the case with the Trp triad mutants – see above and (Li et al., 2011). Therefore, the increased biological activity can be ascribed to the enhanced stability of the flavin radical in this *in vivo* system.

4.4 Absence of alternative light sensing mechanisms

One alternative hypothesis to a cryptochrome redox photocycle has been proposed to date, namely that the anionic flavin radical (FAD•-) represents the dark-adapted 'resting state' of plant Crys, whose illumination triggers a photocycle involving reversible electron transfer from flavin to ATP (see eg. Liu et al., 2010). This suggestion is possibly inspired by the photolyase DNA repair mechanisms, and the fact that the anionic radical redox state is detected in certain mutants of E.coli photolyase (eg N378D) (Müller et al., 2016) and in the insect type I Cry (see eg. Song et al., 2007). However, this mechanism is not consistent with any of the available evidence for plant Cry. The anionic radical formed in the course of flavin reduction exists only transiently, as it is rapidly and efficiently protonated by the conserved proximal D residue in the flavin pocket (D396 or D393) to form the stable neutral radical FADH• (Burney et al., 2012). There is furthermore no evidence for existence of a stable anionic radical (or indeed for any plant Cry radical flavin state) in dark-adapted cells *in vivo*, either indirectly or by direct whole-cell spectroscopic means (Bouly et al., 2007; Herbel et al., 2013; Goett-Zink et al., 2021). Finally, ATP binding as required by this hypothesis is not essential for Cry biological activity *in vivo* (Eckel et al., 2018).

Nonetheless, it cannot be excluded that additional reactions occurring in the framework of the plant Cry redox photocycle may tune photosensory efficiency and/or respond to different environmental cues. Possible contribution of additional photoreactions to the signaling mechanism of plant Crys therefore awaits experimental evidence.

4.5 How the photocycle triggers structural change linked to biological function

A key 'missing link' in the mechanism for Cry light activation are the intermediate steps between flavin photoreduction and the conformational change that occurs in plant Crys that form the basis of the biologically active 'lit' state. The problem is challenging as flavin does not itself undergo large conformational changes upon reduction.

Recent clues concerning these intermediate steps have come from experiments on the *Drosophila* Cry showing that release of the C-terminal domain from its docking position within the flavin binding pocket can be triggered by photoreduction of flavin. The ensuing conformational change in the protein was initiated through changes in charge and protonation state of a conserved His378 residue in close proximity to the flavin (Lin et al., 2018), which, significantly, did not induce large structural changes within the flavin pocket. Further indirect support for the possibility that subtle electrostatic changes within the flavin pocket could initiate conformational change was obtained using high-resolution serial crystallography of flavin photoreduction in photolyases (Maestre-Reyna et al., 2022). These showed that the formation of the anionic radical induces a strong twist in the isoalloxazine ring which triggers ensuing electrostatic perturbations in the flavin pocket, leading to structural changes at the protein surface.

4.5.1 Light-induced structural changes in plant-like Crys as detected by FTIR spectroscopy

In the case of plant Crys, time-resolved experiments using IR spectroscopy have been used to probe structural changes resulting from flavin photoreduction in the plant-like Cry1 from *Chlamydomonas rheinhardtii*. They show conformational change is initiated within the N-terminal photolyase-like domain of Cry, likely due to electrostatic charge fluctuations in the flavin pocket triggered by photoreduction (Langenbacher et al., 2009). These structural changes occur within microseconds of illumination, in the β sheet region proximal to the flavin. This time scale is consistent with formation of the anionic radical (FAD•-). (Thöing et al., 2015; Goett-Zinc and Kottke, 2021), and the structural changes persist over several minutes subsequent to illumination, consistent with the lifetime of the signaling state (FADH•).

As a consequence, the C terminal domain (CCT), which is folded against this β sheet region in the dark-adapted Cry state, subsequently unfolds and is exposed at the protein surface consistent with its known signaling role (Goett-Zink et al., 2021). In this way, signal propagates from formation of FAD•- to the β-sheet bound to Cry CCT. The resulting reorganization induces the dissociation of the CCT from the PHR – like N-terminal domain, and the activated conformation stabilized in the presence of FADH• (Goett-Zink et al., 2021). All these changes fit with the proposed downstream signaling mechanism of higher plant Crys, which involves unfolding and exposure of the C-terminal domain (CCT) and subsequent oligomerization followed by interaction with cellular signaling intermediates (Wang and Lin, 2020).

4.5.2 Structural changes in Cry as detected by CryoEM

Direct structural information on the biologically active form ('lit state') of plant Crys has been obtained from CryoEM studies on the N-terminal domains of light-activated plant Cry2 (Ma et al., 2020; Palayam et al., 2021). These studies have identified amino acid residues mediating tetramerization of Cry2, as well as residues that interact with the Cry inhibitor BIC2 (Kruusyee et al., 2022), as a result of protein restructuring subsequent to illumination. Flavin reduction to the neutral radical FADH• redox form was thought to cause the weakening of the hydrogen-bonding interactions between amino acids in close proximity to the flavin. The subsequent loosening and enlarging of the FAD-binding cavity was proposed to trigger modifications in the α/β-domain, the connector region, and the α-domain promoting the formation of CRY2 oligomers and subsequent biological activation. These results were in agreement with prior

time-resolved FTIR studies by Goett-Zinc et al., 2021. Similar modifications were observed in CryoEM structures obtained from the dark-adapted form of *Arabidopsis* Cry2 Trp triad mutant (W374A) and its interaction with the partner protein CIB1 (Liu et al., 2020; Hao et al., 2022). This mutant showed light-independent biological activity (Li et al., 2011), caused by structural perturbations that mimic those of the wild type light-activated form. Structures for the rice (Zea Mays) oligomerized Cry1 protein (Shao et al., 2020) also showed expansion of the flavin cavity upon light activation. Thus, evidence from independent groups shows structural change in the flavin cavity, consistent with past work on algal Crys and photolyases (see eg. Maestre-Reyna et al., 2022).

The challenge remains to detect the intermediate steps that occur between light absorption by flavin in the flavin pocket and the ensuing large structural changes in the α/β-domain, the connector region, and the α-domain. These intermediate steps showing initiation and progression of conformational change await higher resolution and time-resolved experimental methods such as serial X-Ray crystallography as performed with photolyases (Maestre-Reyna et al., 2022), or by a combination of single-molecule Förster resonance energy transfer, structural predictions, and solution X-ray scattering methods as recently reported for the light-triggered conformational change of the animal type CraCry from algae (Li et al., 2022). One major hurdle has been that, although the initial structural change predicted for the plant Cry is the dissociation of the C-terminal domain from the flavin pocket (Wang and Lin, 2020), it has not been addressed by CryoEM. The C-terminal tail of Crys adopts a random coil state (Parico and Partch, 2020), and there is as yet no crystal structure of the full-length plant Cry protein. Since the C-terminal domains of CRYs are unstructured, they may adopt different conformations according to their interaction partners. Co-crystallization studies with different interacting proteins, perhaps via cryo-EM, may be a way to study their structural diversity.

5 Special effects related to the Cry photocycle

The Cry photocycle provides an exquisitely tunable, versatile signaling mechanism based on redox chemistry that is sensitive to multiple inputs in addition to light. It is moreover relevant to organisms throughout the biological Kingdom as many fundamental features of Cry photochemistry appear to be conserved. Although a thorough analysis of potential applications and consequences of the cryptochrome photocycle is beyond the scope of this review, a few selected examples of how understanding the photocycle informs scientific advances and novel applications in plants are presented below.

5.1 The cryptochrome photocycle and response to applied magnetic fields

The idea that cryptochromes could function as magnetoreceptors came initially from studies with migratory birds, which use the earth's magnetic field as a compass in seasonal migration. Birds were found to orient preferentially in short-wavelength light (below 600nm) and moreover used an inclination compass which could detect the angle of the earth's magnetic field but was not responsive to the north-south direction. Quantum physical principles suggested that such a 'chemical magnetoreceptor' must undergo a reaction mechanism that generates radical pairs (Ritz and Schulten et al., 2000; Hore and Mouritsen, 2016). As a consequence, cryptochrome, which is localized in the bird's retina, was suggested as a possible avian magnetoreceptor. A series of famous behavioral and histochemical experiments followed in which the light-sensitivity and spin chemical properties of the avian magnetic compass were related to the light-sensitivity and function of cryptochrome 1a in the avian retina (Wiltschko et al., 2021).

There have also been long-standing observations of plant responses to magnetic fields dating back over a hundred years. Concrete evidence implicating plant cryptochromes in sensitivity to magnetic fields was first documented for plant growth experiments, showing that the effect of a static magnetic field of 500 μT (10-fold the earth's magnetic field strength) was to decrease seedling growth in blue light (Ahmad et al., 2007). This response was observed in blue/UV light but not in red light, and moreover failed to occur in cryptochrome mutant seedlings consistent with a role for cryptochromes in this response. Although generating initial controversy (Harris et al., 2009), independent labs subsequently upheld a role for both Cry1 and Cry2 in modulating blue-light dependent plant growth responses to static magnetic fields (see e.g. Xu et al., 2012; Xu et al., 2014; Agliassa et al., 2018). Evidence for cryptochrome mediated physiological responses to applied magnetic fields in plant, Drosophila, as well as in mammalian cell systems has been recently summarized in this Review Series (Kyriacou and Rosato, 2022; Zhang and Malkemper, 2023; Thoradit et al., 2023) although areas of controversy exists in all of these cases (see eg. Bassetto et al., 2023).

5.1.1 The radical pair mechanism and Cry magnetosensing

Magnetic fields of the order of the geomagnetic field (30 – 50 μT) are far too weak to initiate chemical reactions. However, a theoretical explanation for how biochemical reactions could nonetheless be manipulated by magnetic fields plays upon the fact that these weak forces can interact with electron spins of radicals in an oriented manner. This is known as the Radical Pair mechanism of biological magnetosensing (Hore and Mouritsen, 2016), and predicts modest (less than 10%) changes in rate constants of susceptible reactions. It can be simply explained as follows: Free radicals are formed in the course of many biochemical reactions as high-energy intermediate states. The energy comes from making and breaking chemical bonds, not from the magnetic field. However, the magnetic fields can intervene, in certain favorable configurations, by changing the electron spins of the high-energy excited state intermediates. In this way, the efficiency of the biochemical or photochemical reaction and the rate of product formation is altered. In biological terms, the result is an apparent change in the rate constant and therefore light sensitivity of a

photoreceptor. For example, in the case of cryptochromes, if the magnetic field were to induce a change in any one of the rate constants k1, k2, k1b or k2b (Figure 4), then this would be perceived by the organism as a change in light intensity even though the actual number of photons reaching the receptor is exactly the same. The change in efficiency of forming the FADH• signaling state would result in an altered perceived light signal.

The challenge in proving such a mechanism is, first, to demonstrate that it indeed occurs and, second, identifying the magnetically sensitive radical pairs leading to changes in biological activity.

The validity of the Radical Pair mechanism has been directly demonstrated in model compounds and even shown to occur in photoreactions of isolated cryptochromes and photolyases *in vitro* (Hore and Mouritsen, 2016). A further fruitful line of evidence for the Radical Pair mechanism has been the effects of radiofrequency (RF) signals in the MHz range, which were found to disrupt magnetic sensitivity in a number of organisms, consistent with a radical pair mechanism as found in birds (Hore and Mouritsen, 2016; Wiltschko et al., 2021). Such effects are also reported in plants, where exposure to RF was found to disrupt magnetic sensitivity in plant growth phenotypes and to mimic the effect of removing the earth's magnetic field entirely (Albaqami et al., 2020). All of these data are consistent with a radical pair mechanism, but do not resolve the question of whether cryptochrome really function as a magnetoreceptor in a biological system? And if so what radical pairs are involved?

5.1.2 What is the magnetically sensitive step?

There are at least two known steps within the Cry photocycle that can form radical pair intermediates. The first occurs during the initial reaction of light-driven electron transfer to the flavin, whereby Trp/FADH· and Tyr/ FADH· radical pairs are transiently generated as intermediates. These radical pairs can be triggered solely by absorption of short-wavelength blue or UV/A light with a sharp cutoff at 500nm, as oxidized flavin does not absorb in a spectral range above 500nm (Maeda et al., 2012). As we have seen, the primary photoreduction reaction generates the radical redox state and the biologically active form of the receptor (Figure 4, rate constant k1). A further critical characteristic of this reaction step is that it can only occur during illumination; the radical pairs are unstable with half lives in the range of milliseconds (Maeda et al., 2012). Therefore, magnetic field effects could only occur during illumination if this mechanism is involved.

This forward photoreduction reaction was initially favored as a possible target for applied magnetic fields as it showed magnetic field sensitivity *in vitro*. The plant Cry1, for instance, responds to magnetic fields in the mTesla range *in vitro* by altering the quantum yield of radical (FADH•) formation under illumination (k1 rate constant, Figure 4) (Maeda et al., 2012). Subsequently, similar results were shown for other Crys and photolyases, including for one of the crys found in the bird retina (avian Cry4) (Xu et al., 2021). These experiments were supported by numerous theoretical papers 'proving' how variations of Trp/Flavin radical pairs are a suitable target for modulation by magnetic fields (summarized in Hore and Mouritsen, 2016).

However, this reaction mechanism is in contradiction to the available biological evidence. There are literally dozens of behavioral studies on avian magnetosensing, including from independent labs, that have shown that birds can orient under wavelengths (567nm) where the Trp/FADH• radical pair does not exist. Oxidised flavin does not absorb above 500nm, so cannot be activated by 567nm green light of these avian orientation studies. Further experiments with birds were performed under pulsed light conditions. In these experiments, magnetic field exposure was controlled separately from the light pulses. It was demonstrated that birds could orient even if the magnetic fields was given during dark intervals between light pulses – which is a physical impossibility if the Trp/Flavin radical pair was involved (summarized in Wiltschko et al., 2021).

Further evidence that the magnetically sensitive step mediated by the cryptochrome photoreceptor occurs in the absence of light has also been obtained in plants, where magnetic field sensitivity occurs during intervals of darkness between or after illumination periods (Xu et al., 2014; Hammad et al., 2020; Pooam et al., 2021). In some of these experiments there are more than 10 second intervals between the end of the light period and the beginning of the magnetic field exposure. Since the Trp/FADH• radical pair has a lifetime of just a few milliseconds, it cannot mediate magnetic field sensitivity of plant Crys. In other words, as long as an initial step occurs that generates the flavin radical (FADH•), the magnetically sensitive step can be decoupled from illumination.

Further support for these observations came from cryptochrome-dependent magnetic field effects in the *Drosophila* system. These were likewise observed to occur in the absence of the Trp/FADH• radical pair (Kyriacou and Rosato 2022; Bradlaugh et al 2023). In this case, the N-terminal flavin-binding domain of *Drosophila* Cry is not even required for magnetic sensitivity, such that the needed flavin radical is thought to be generated in other flavoproteins that interact with the *Drosophila* Cry C-terminal domain. This provides a new paradigm for Cry-dependent magnetosensing consistent with a redox-related radical pair mechanism.

Intriguingly, a lab without prior publications on *Drosophila* has recently reported being unable to replicate these experiments (Bassetto et al., 2023). However, this same lab likewise failed to replicate other behavioral studies they attempted, claiming to discredit all prior findings on *Drosophila* Cry as a magnetoreceptor (which have been published over the last 15 years). As many of these studies had already been replicated by independent, leading *Drosophila* labs (see eg. Yoshii et al., 2009; Fedele et al., 2014; Bae et al., 2016; Kyriacou and Rosato, 2022), a visit to a *Drosophila* lab with a working behavioral assay system by Basetto and colleagues may be the most effective means to resolve their difficulties (see also Section 5.4 below).

5.1.3 Flavin reoxidation and formation of a radical pair

As stated above, the physiological evidence in plants points to a magnetosensing mechanism in which light is required for Cry activation (reaction step k1 in Figure 4), but in which the actual

magnetically sensitive step occurs during the dark reoxidation reaction subsequent to illumination (Hammad et al., 2020; Pooam et al., 2020; Pooam et al., 2021; Albaqami et al., 2020). Because flavin reoxidation produces ROS, Reactive Oxygen Species intermediates including hydrogen peroxide and superoxide radicals O_2·- together with FADH• have been suggested as possible radical pairs (Müller and Ahmad, 2011).

At present, the precise nature of the radical pair formed by plant Crys in the course of magnetic field exposure has not yet been determined. As they are formed exclusively in the process of flavin reoxidation they likely involve the flavin radical and some species of reactive oxygen (Müller and Ahmad, 2011). There are theoretical constraints suggesting that superoxide, which is formed during the flavin reoxidation step, is an unlikely candidate to participate in magnetoreception, since the spin relaxation time is too rapid (Hore and Mouritsen, 2016). However, alternative mechanisms, such as involving scavenging radicals or other intermediates have been suggested (Kattnig and Hore, 2017; Rishabh et al., 2022) which await experimental verification.

5.2 Magnetosensing mechanisms that may indirectly involve Crys

The evidence shows that plant Crys likely function as direct magnetoreceptors (Pooam et al., 2019; Hammad et al., 2020), as it is possible to visualize effects of magnetic fields directly on receptor phosphorylation and Cry conformational state *in vivo*. These effects occur *in vivo* within minutes of magnetic field exposure and can be most readily resolved under pulsed light conditions.

However, it is becoming increasingly apparent that there are additional mechanisms for plant magnetosensing, which may involve Crys only indirectly, but not as the actual magnetosensors. For instance, plant cryptochrome mutants showed altered gene expression profiles in a magnetic field as compared to wild type plants, even in the complete absence of light (Dhiman and Galland, 2018). This surprising result would implicate plant Cry in magnetic field sensing, but without activating the photocycle. Such indirect mechanisms involving Crys but not the Cry redox photocycle, have also been reported in flies (Kyriakou and Rosato 2022). Some of these may involve mitochondrial redox reactions, that likewise respond to magnetic fields by the radical pair mechanism, and do so independently of light (Usselman et al., 2016; Ikeya and Woodward, 2021).

A further alternate magnetosensing mechanism involving Crys has been the suggestion that ferromagnetite/cryptochrome complexes called MagR may function in plant magnetic field sensing (Xie, 2022). This suggestion is based on the observation that MagR, a conserved iron-sulfur binding protein originally named IscA1, co-purifies with Cry in both animal and plant species. Because ferromagnetite can sense (align with) the polarity of the magnetic field in bacteria and some animals, it was speculated that magnetic field sensitivity of Crys could also be due to bound magnetite as the magnetosensing party (Xie, 2022). However, this MagR mechanism is in contradiction to known Cry-dependent responses to the intensity of the magnetic field but not its direction. Furthermore, RF (radiofrequency fields) have been shown to interfere with Cry-dependent magnetic field perception in plants (Albaqami et al., 2020), which is a diagnostic feature of the Radical Pair mechanism. Such RF interference cannot be mediated through a magnetite-based mechanism. Although it cannot be excluded that plants have some form of magnetite-based magnetic sensitivity mechanism, experimental proof for MagR and magnetite based magnetosensing is currently lacking.

5.3 Plant magnetic field sensitivity by mechanisms that do not involve the Crys

Intriguingly, mutant analysis suggests that other plant photoreceptors distinct from cryptochromes may be implicated in magnetic field effects on plant growth and gene expression, even in the absence of light. These include phytochrome and phototropins (Agliassa et al., 2018; Dhiman and Galland, 2018), neither of which can form radical pairs as reaction intermediates, nor are they known to function in the absence of light. Though unlikely to function as direct magnetosensors, gating by a variety of photoreceptors sets up new paradigms for magnetic field sensitivity as well as identifying new roles for plant photoreceptors that occur in the absence of light. The actual magnetosensing mechanism in these cases remains unknown, however.

5.4 Resolving areas of controversy

The field of magnetobiology has historically been plagued by controversy and contradictory findings and therefore has had a somewhat dubious past (see eg. Galland and Pazur, 2005). As even this brief analysis shows, there appear to be multiple unrelated magnetic field response mechanisms acting simultaneously in plants, and affecting different biological properties to differing degrees. This complexity is compounded by the fact that magnetic field effects are often quite subtle (predicted primary effects of the Radical Pair mechanism are less than 10% change in reaction yield). These kinds of effects require an exceptional mastery of the experimental system to reliably visualize (eg. bird orientation, *Drosophila* behavioral experiments, plant growth experiments). Finally, artifacts due to poor characterization of experimental conditions are compounded with everyone using completely different readouts and exposure conditions, making comparison among past studies virtually impossible in many cases (Galland and Pazur, 2005).

Today, improving methodology has achieved better controlled conditions and resulted in reliable results and greater reproducibility between different labs, particularly for bird, plant (*Arabidopsis*), and *Drosophila* magnetic field exposure experiments. For example, cryptochrome mediated magnetic field effects on plant seedling growth that are dependent on blue light have been extensively replicated (Ahmad et al., 2007; Xu et al., 2012; Xu et al., 2015; Agliassa et al., 2018; Pooam et al., 2019; Hammad et al., 2020;

Albaqami et al., 2020). Current disputes can often be resolved on purely methodological grounds (failure to replicate results due to failure to precisely replicate the experimental conditions under which they were obtained). For example, a reported inability to observe plant blue light-dependent and Cry-dependent magnetic field sensitivity in plants (Dhiman and Galland, 2018; Dhiman and Galland, 2018) can be ascribed to the profoundly different experimental conditions and assays used (as compared to successful experiments presented in Xu et al., 2012; Xu et al., 2015; Agliassa et al., 2018; Albaqami et al., 2020). Thus, strict attention to methodological parameters, possibly including visits to the respective labs should help make methodological issues a thing of the past (and resolve disputes framed by studies such as Bassetto et al., 2023).

A second source of controversy concerns interpretation of findings rather than of the data itself. An ongoing example of such confusion is a continuing stream of publications describing short-lived Trp/flavin radical pairs of Crys as mediators of biological magnetic field sensitivity (for analysis, see Section 5.12 above). This is in contradiction to all of the behavioral evidence: birds are oriented in green light and in darkness, where formation of Trp/flavin radical pairs by Cry is a physical impossibility (see eg. Wiltschko et al., 2021). This impossible mechanism has even recently been proposed as the basis for the avian magnetic compass (Xu et al., 2021), and has been widely disseminated as such in the popular press. Because magnetoreception is a highly interdisciplinary field, such confusion may occur when physicists and chemists do not consider biological data, or vice versa.

However, in order to be valid, a proposed mechanism must first and foremost be consistent with the physiological response. The biological evidence must always take precedence over ever other form of analysis. Truth is a zero-sum game: if something is correct (such as that birds navigate in green light), then a contradictory hypothesis (ie. that Trp/flavin radical pairs of Crys mediate magnetic orientation) must be false, no matter how attractive it may be on theoretical and *in vitro* experimental grounds. To quote the founders and current leaders in the field of bird navigation, Wolfgang and Roswitha Wiltschko: 'the bird is always right'.

6 Emerging technologies involving the cryptochrome photocycle

Cryptochromes impact on practically every aspect of plant growth and development. They can influence seed germination, flowering, plant architecture, fruit metabolic content and resistance to biotic and abiotic stresses (Chaves et al., 2011). Accordingly, cryptochromes have broad potential as targets for adaptive modifications of crop plants (Fantini and Facella, 2020); or through stimulation through electromagnetic fields or other forces to improve crop yield and resistance to biotic and abiotic stress including those related to climate change (Pooam et al., 2020).

Plant cryptochromes have in addition been developed as potent optogenetic tools for regulating gene and protein expression for research and in medicine. For example, Cry2 has been used as a light-induced oligomerization module (see eg. Konermann et al., 2013). These reversible, light-inducible complexes form the basis of light-sensitive tools for gene expression and sequestering heterologous proteins in both mammalian cells and yeast.

Finally, interest in imaging of quantum forces has stimulated the development of new technologies which may eventually permit visualization of magnetic field effects at the single molecule and nanoparticle scale in living tissue (see eg. Winkler et al., 2023; Passian and Siopsis, 2017). This will help resolve the ongoing problem of relating quantum effects to *in vivo* biological responses.

Although a thorough analysis of these topics is beyond the scope of this review, we provide a brief snapshot of two exciting emerging technologies related to applications and visualization of the cryptochrome photocycle; namely optogenetic control of gene expression in yeast for synthetic biology; and development of diamond sensors for eventual visualization of *in vivo* magnetic field sensitivity of cryptochromes in living cells.

6.1 Applications for synthetic biology in yeast

Cryptochromes have significant potential applications in synthetic biology, involving the use of light to control gene expression. Since the yeast *Saccharomyces cerevisiae* (baker's yeast) is commonly used as a chassis for synthetic biology approaches aiming to produce proteins or high-value chemical compounds, here we will review current efforts in the field to develop cryptochrome-based optogenetic tools for yeast.

In yeast cell factories, typically, the genes encoding the heterologous biosynthetic pathway are under inducible control in order to avoid product toxicity or draining of cell resources during the growth phase. Inducible gene expression usually relies on the use of promoters that are activated by the presence of specific chemical compounds (e.g., galactose). This can impose limitations since the inducer is difficult to remove once added while tuning of the promoter activity is frequently difficult to achieve with precision. These challenges can be overcome using light-controlled induction of gene expression, which is readily reversible, dose-dependent, non-toxic, and cost-effective. Yeast is a highly suitable chassis for the construction of optogenetic circuits as it does not contain any native photoreceptors and it can provide endogenous flavins for functional cryptochrome expression.

Thus, fusions between cryptochromes and transcription factor domains have been used to establish synthetic light-inducible transcription factors that control the expression of genes in response to blue light. Most systems take advantage of the *Arabidopsis thaliana* cryptochrome 2 protein (Cry2) and its interacting partner Cib1. By fusing Cry2 with the DNA-binding domain of a transcription factor (DBD) and Cib1 with a transcriptional activation domain (AD), an opto-switch is created that assembles upon blue-light stimulation and drives the expression of the target gene (Liu et al., 2008; Kennedy et al., 2010; Hughes et al., 2012; Melendez et al., 2014; Pathak et al., 2014; Hallett et al., 2016; Taslimi et al., 2016). When blue light stimulation is removed (darkness), the complex dissociates and gene expression ceases (Figure 6). This yeast opto-switch has been optimized by the discovery of a photocycle mutant of Cry2 that exhibits reduced interaction with Cib1 in the absence of blue light (Taslimi et al.,

2016). Further optimization of this system was achieved by employing a range of different constitutive promoters to express its components, allowing fine-tuning of the sensitivity and responsiveness of the system (An-Adirekkun et al., 2020) (Figure 6).

Beyond gene expression, the Cry2 photoreceptor has also been used to control the assembly of enzyme complexes in the context of synthetic compartmentalization and metabolic channeling. This approach takes advantage of the ability of Cry2 to form oligomers upon blue-light stimulation (Yu et al., 2009). This assembly is enhanced by a point mutation in Cry2, giving rise to the variant Cry2olig (Taslimi et al., 2014), and by fusing it to the N-terminal intrinsically disordered region from the protein Fus (Shin et al., 2017). Combination of these two enhancing elements results in two systems with different physical properties. Fusion of the N-terminal region of Fus to wild-type Cry2 forms liquid-like spherical droplets, named optoDroplets, that rapidly exchange monomers in and out of clusters. Fusion of the Fus N-terminal domain to the Cry2olig variant results in rigid clusters that do not exchange subunits with the solution. The latter are called optoClusters (Figure 6). Using the deoxyviolacein biosynthesis pathway as a model system, the optoCluster system was shown to enhance product formation two-fold by decreasing the concentration of intermediate metabolites and reducing flux through competing pathways (Zhao et al., 2019). Thus, Cry2-based inducible compartmentalization of enzymes into synthetic organelles is an efficient strategy to optimize engineered metabolic pathways.

In sum, understanding of the cryptochrome photocycle will enable the design and development of novel optogenetic tools impacting on many different synthetic biology applications in yeast. It may even help in the design of novel Cry variants displaying controlled response to external magnetic fields. In combination with suitable lighting protocols, this new technology of 'magnetogenetics' could exponentially increase the synthetic biology toolkit.

6.2 Novel sensors for measuring Cry activation *in vitro and in vivo*

During the last decade, new bio-sensing schemes based on color centers in diamond crystals have appeared. The most common of these is the nitrogen-vacancy (NV) center which consists of a substitutional nitrogen atom and an adjacent lattice vacancy (Schirhagl et al., 2014; Aslam et al., 2023) (Figure 7). As a key feature, the NV center carries a free electron coupled to the optical transition, allowing for both electron spin polarization and readout. By recording electron spin precession, the system thus provides the ability for optical detection of local magnetic field at room temperature. In addition, the diamond system benefits from its high biocompatibility. With mm-sized diamond crystals hosting surface-layers enriched in NV-centers, or nanodiamonds with few or single NV centers, high sensitivity for detection of radicals has been demonstrated (Tetienne et al., 2013; Nie et al., 2021). In contrast to time-resolved electron paramagnetic resonance spectroscopy or optical methods based on absorption or fluorescence detection, such NV-center in diamond-based detection does not necessarily rely on ensemble averaged information of the spin dynamics. This type of sensor should be suitable for the detection of magnetic field effects in relation with cryptochromes, for which numerical investigations suggest a sensitivity allowing for detection of magnetic field effects in even one or few molecules.

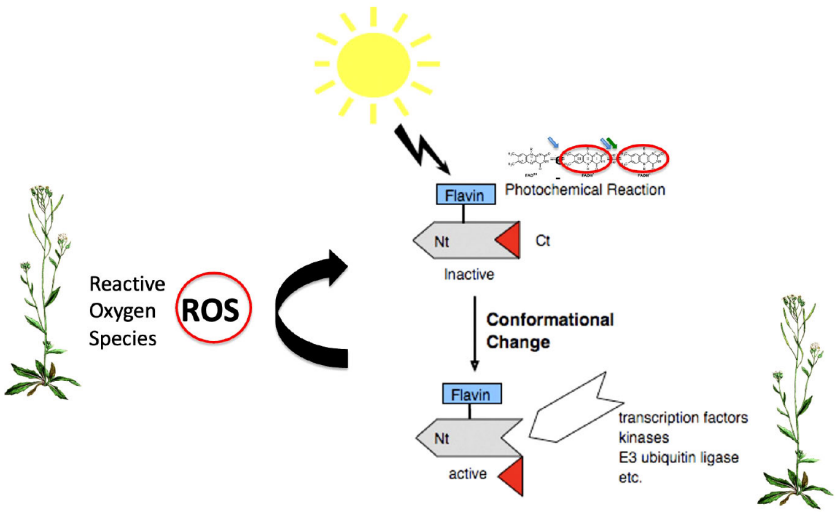

FIGURE 5
Biological signaling mechanisms of Cry. Light is absorbed through the oxidized flavin chromorphore (FADox) inducing the formation of the radical (FADH•) active 'lit' state. Biological activation ensues through conformational change within the Cry protein, leading to unfolding of the C-terminal domain and subsequent access to partner proteins and signaling intermediates (Ponnu and Hoecker, 2022). A second signaling mechanism results from the flavin reoxidation reaction (FADH• to FADox), which occurs in the absence of light and generates ROS (Reactive Oxygen Species) (curved arrow). These in turn can induce ROS-regulated signaling pathways even independently of conformational changes in Crys (El-Esawi et al., 2017).

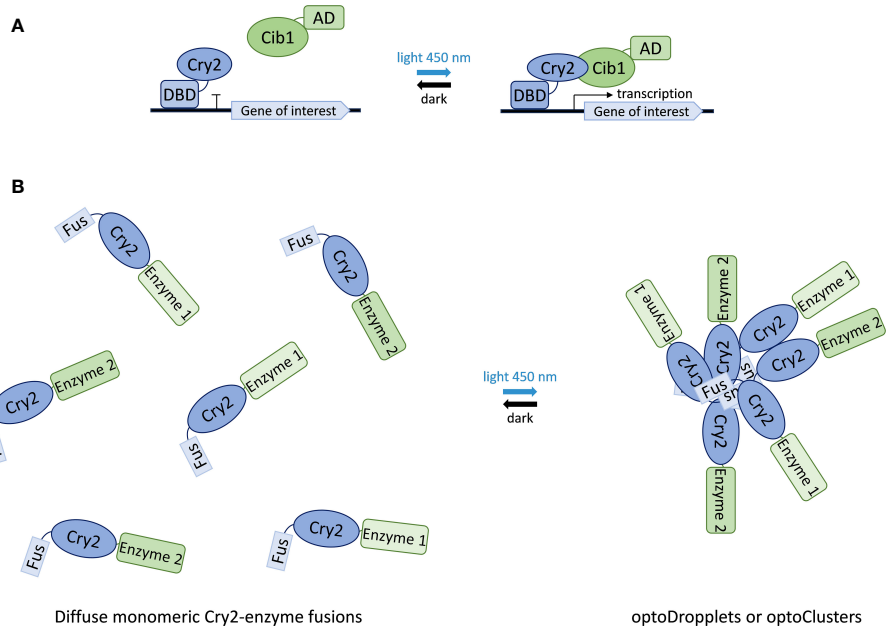

FIGURE 6
Applications of Cry2 in synthetic biology. **(A)** Light-controlled gene expression switch composed of Cry2 fused to a transcription factor DNA binding domain (DBD) and Cib1 fused to a transcription activation domain (AD). Illumination with blue light causes Cry2-Cib1 association leading to the activation of transcription. Reversion to the dissociated state upon light removal (dark state) stops transcription. **(B)** Formation of optoDropplets and optoClusters based on the association of Fus-protein domain fusions of Cry2 results in the sequestration of attached enzymes upon light illumination.

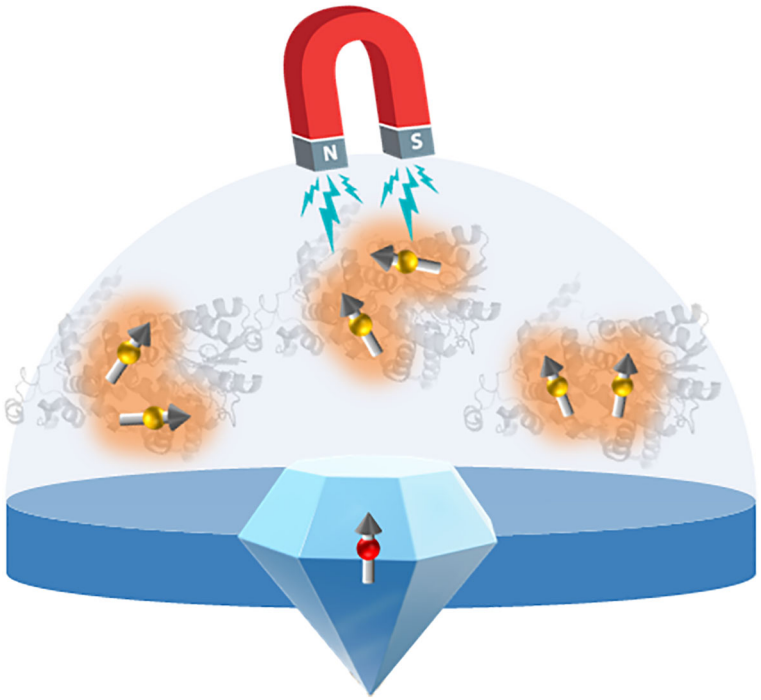

FIGURE 7
Principle of an NV-center in diamond experiment for *in vitro* sensing of the existence of radical pairs as well as their magnetic field effect. The NV-center in diamond provides the electron spin (arrow with red dot within the diamond-shape) which interacts with the electron-spins of the radical pair molecules (shaded orange regions with arrows with yellow dot). An external magnetic field – here symbolized by a U-shaped magnet - may be applied with varying strength, allowing for the investigation of magnetic field effects.

7 Summary and concluding remarks

Cryptochromes are complex and versatile flavoprotein receptors that have evolved many important signaling functions in both plants and animals. Despite an enormous diversity in origin and in their biological roles, the underlying structure and primary photochemical reactions among cryptochromes are remarkably well conserved. This suggests that detailed understanding of key biological mechanisms in one experimental system may have relevance for all.

We accordingly focus on a critical step in the evolution of plant cryptochromes from their photolyase ancestors, which enabled them to mediate physiological responses to light. This has been the development of a redox cycle in which flavin is reduced by light to a biologically activated 'lit' state, followed by spontaneous reoxidation back to a biologically inactive dark-adapted state. This so-called photocycle (Figures 4, 5) provides the basis for plant cryptochrome light sensitivity, and in addition provides a mechanism by which plant cryptochromes can respond to other environmental cues including temperature, applied magnetic fields and even radiofrequency fields.

Furthermore, these Cry signaling mechanisms have been reviewed specifically through the lens of their potential biological effectiveness *in vivo* (Figure 5). For example, many photoreactions of plant cryptochrome that have been extensively studied *in vitro* are actually of little or no physiological significance *in vivo*. These include the exhaustively studied flavin photoreduction reactions showing magnetic field sensitivity *in vitro*, but which are not relevant to magnetosensing by Cry *in vivo*. Conversely, certain key aspects of the Cry photocycle that have been largely overlooked during *in vitro* studies, do in fact play a disproportionate role in physiological responses *in vivo*. These include the flavin reoxidation reaction which contributes both to the magnitude of the biological response and to magnetic field sensitivity. Although cryptochrome photochemistry and physiological response characteristics vary throughout the biological Kingdom, and indeed in some cases cryptochrome signaling appears not even to be sensitive to light, it is hoped that this review will help provide a blueprint on how to link *in vitro* mechanisms and redox properties of Crys with an actual physiological responses *in vivo*.

Future perspectives for this field are continuously opening up and will include new ways to manipulate Crys both *in vivo* and *in vitro*, identification of novel Cry variants, and novel applications for Crys in agriculture and in synthetic biology. All these perspectives benefit from an understanding how light signals are transduced into a meaningful physiological response. Additional prospects include design of targeted strategies for manipulation of endogenous Crys in humans to produce ROS (Sherrard et al., 2018), leading to novel therapies such as in the treatment of inflammation and infectious disease (Ronniger et al., 2022).

In combination with suitable lighting protocols, this new technology of 'magnetogenetics' could increase the optogenetic toolkit as well as providing fundamental insights on how electromagnetic fields impact on living systems.

Author contributions

BA: Writing – original draft, Writing – review & editing. JB: Writing – review & editing. SB: Writing – review & editing. NJ: Writing – review & editing. MP: Writing – review & editing. ME: Writing – review & editing. DE: Writing – review & editing. SM: Writing – review & editing. SW: Writing – original draft, Writing – review & editing. AH: Writing – review & editing. KB: Writing – original draft, Writing – review & editing. SK: Writing – original draft, Writing – review & editing. JL: Funding acquisition, Writing – review & editing. MA: Conceptualization, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Peter Nagy,
University of Debrecen, Hungary

REVIEWED BY
Brian D. Zoltowski,
Southern Methodist University, United States
Chentao Lin,
University of California, Los Angeles,
United States

*CORRESPONDENCE
Brian R. Crane,
bc69@cornell.edu

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A structural decryption of cryptochromes

Cristina C. DeOliveira and Brian R. Crane*

Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY, United States

Cryptochromes (CRYs), which are signaling proteins related to DNA photolyases, play pivotal roles in sensory responses throughout biology, including growth and development, metabolic regulation, circadian rhythm entrainment and geomagnetic field sensing. This review explores the evolutionary relationships and functional diversity of cryptochromes from the perspective of their molecular structures. In general, CRY biological activities derive from their core structural architecture, which is based on a Photolyase Homology Region (PHR) and a more variable and functionally specific Cryptochrome C-terminal Extension (CCE). The α/β and α-helical domains within the PHR bind FAD, modulate redox reactive residues, accommodate antenna cofactors, recognize small molecules and provide conformationally responsive interaction surfaces for a range of partners. CCEs add structural complexity and divergence, and in doing so, influence photoreceptor reactivity and tailor function. Primary and secondary pockets within the PHR bind myriad moieties and collaborate with the CCEs to tune recognition properties and propagate chemical changes to downstream partners. For some CRYs, changes in homo and hetero-oligomerization couple to light-induced conformational changes, for others, changes in posttranslational modifications couple to cascades of protein interactions with partners and effectors. The structural exploration of cryptochromes underscores how a broad family of signaling proteins with close relationship to light-dependent enzymes achieves a wide range of activities through conservation of key structural and chemical properties upon which function-specific features are elaborated.

KEYWORDS

flavoprotein, light-sensing, photosensory receptor, signal transduction, circadian clock, redox chemistry, post-translational modification, protein oligomerization

Introduction

Cryptochromes represent a functionally diverse family of signal transduction proteins that are evolutionarily linked to light-dependent DNA repair enzymes known as photolyases (Sancar, 2003; Chaves, et al., 2011a; Zoltowski, et al., 2011a; Conrad, et al., 2014; Michael, et al., 2017a; Wang and Lin, 2020a; Foley and Emery, 2020; Kiontke, Stephan, et al., 2020). Light provides a ubiquitous environmental cue that shapes many biological processes, from circadian rhythms to developmental pathways (Zoltowski, et al., 2011b; Conrad, et al., 2014; Ahmad, 2016; Wang and Lin, 2020b). Many cryptochromes bind flavin cofactors and in doing so play a key role in light responses for many types of organisms (Figure 1). Other cryptochrome activities are light independent; hence, the utility of their structural framework goes beyond photobiology (Figure 1). Structures of diverse cryptochromes in complex with partner proteins and small molecules have revealed commonalities and differences in their modes of recognition and ability to propagate signals to their targets (Conrad, et al., 2014; Wang and Lin, 2020a; Foley and Emery, 2020).

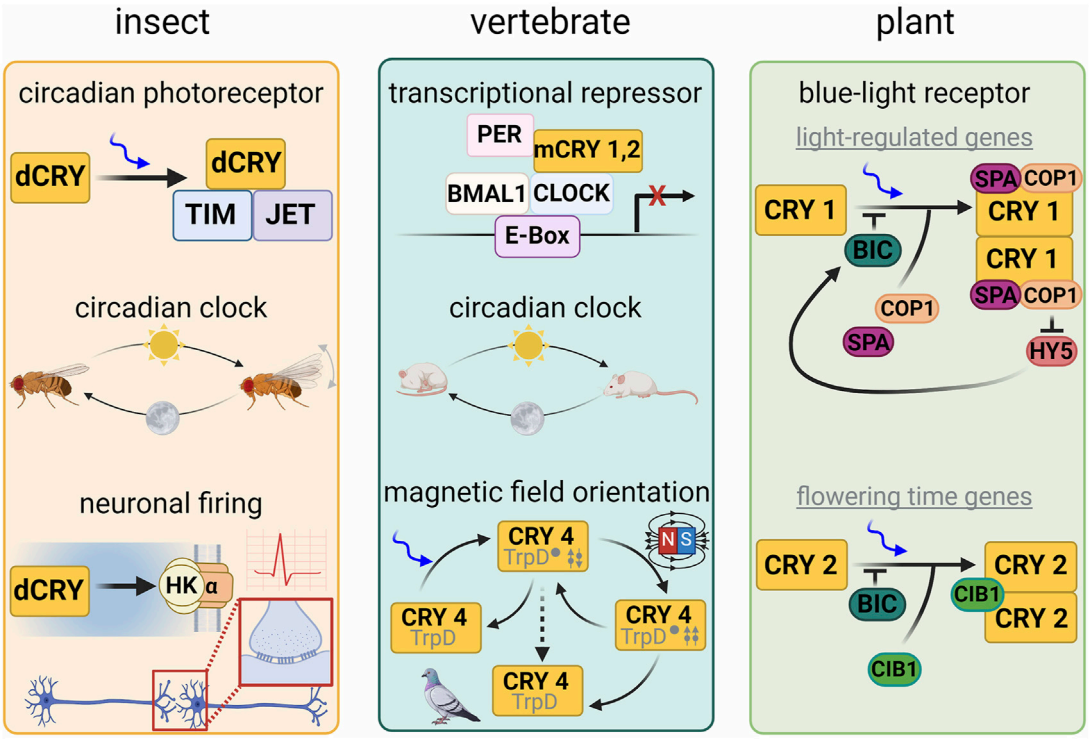

FIGURE 1
Cryptochrome function in different model organisms. In *Drosophila melanogaster*, dCRY acts as a circadian clock photoreceptor by coordinating light-dependent degradation of Timeless (TIM) by the E3 ubiquitin ligase Jetlag. dCRY also interacts with the potassium ion channel β-subunit Hyperkinetic to regulate neuronal firing. In vertebrates, cryptochromes acts as a transcriptional repressor and regulator of the circadian clock. In plants, CRY1 acts as a blue-light receptor in the regulation of light-regulated genes associated with growth and development and couples to circadian clock regulation, whereas CRY2 regulates genes governing flowering time. CRY has also been implicated in sensing earth's magnetic field in invertebrates, plants, birds and certain mammals. Lower schematic of the central panel represents the proposed radical pair (twin arrows) mechanism involved in magnetoreception.

Herein we examine the structural biology of cryptochromes to uncover broad insights into the fundamental mechanisms that underlie the modulation of dynamic protein interactions by reactive cofactors.

Evolutionary relationships of cryptochromes: signaling proteins and photoenzymes

Originally discovered in plants and flies, cryptochromes exhibit considerable sequence similarity with DNA photolyases (PLs) (Aguida, et al., 2024; Ahmad and Cashmore, 1993; Balland, et al., 2009; Emery, et al., 1998; Kanai, et al., 1997; Ozturk, N., et al., 2007; Sancar, 2003; Stanewsky, et al., 1998). Photolyases repair cross-linked pyrimidine dimers in UV-damaged DNA using light-dependent redox reactions mediated by their flavin cofactors (Carell, et al., 2001; Essen and Klar, 2006; Sancar, 2003). Depending on the identity of pyrimidine base lesions that they recognize photolyases fall into two functional types: cyclic pyrimidine photolyases (CPDs) and 6-4 photolyases (Carell, et al., 2001; Sancar, 2003; Essen and Klar, 2006). Although cryptochromes have evolutionarily diversified into a wide array of roles that extend beyond these activities, including circadian regulation and phototaxis, sequence homology to photolyases remains high (Chaves, et al., 2011b; Essen, et al., 2017; Ozturk, N., 2017). Moreover, photolyase and cryptochrome sequences across all kingdoms of life do not segregate into clear functional groups, emphasizing the close relationships within the greater family (Chaves, et al., 2011a; Ozturk, N., 2017) (Figure 2). Sequence clustering of the CRY/PL family reveals distinct groups of both proteins, such as the Class I and Class II CPD photolyases, distinguished by their antenna cofactors, and the so-called Class III photolyases (Figure 2) that are more closely related to plant cryptochromes (Kiontke, S., et al., 2014; Ozturk, N., 2017). DASH-CRYs (**D**rosophila, **A**rabidopsis, **S**ynechocystis, **H**uman-type cryptochromes), which are found in bacteria, algae or associated with organelles of higher plants (Brudler, et al., 2003; Kleine, et al., 2003; Essen, et al., 2017; Kiontke, Stephan, et al., 2020) may possess both signaling and DNA repair activities. DASH-CRYs segregate into two closely related groups (Figure 2): one that includes the plastid CRY3 from *Arabidopsis thalania* (Kleine, et al., 2003; Klar, et al., 2007) and other bacterial CRY-DASH proteins. In the major sequence category of cryptochromes, which also contains the 6-4 photolyases, delineations emerge among the Type I invertebrate CRYs, Type II and Type IV vertebrate CRYs under stricter clustering criteria (Figure 2), whereas land plant CRYs (Wang and Lin, 2020a), as represented by *A. thalania* CRY1 (AtCRY1) and CRY2 (AtCRY2), stand apart, owing in part to their extensive CCEs.

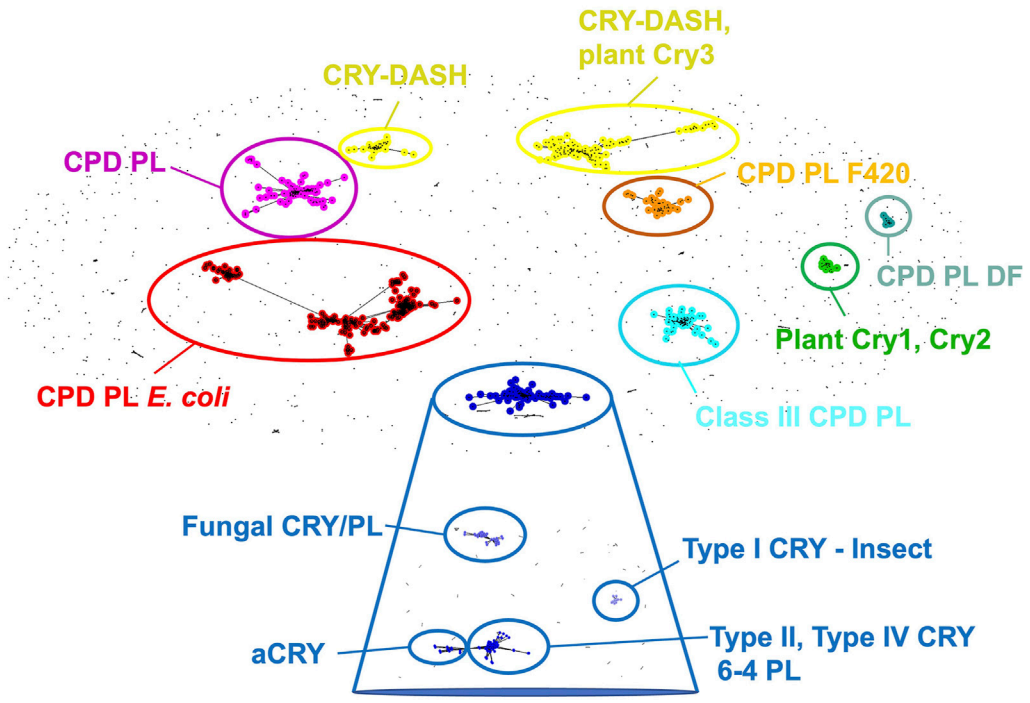

FIGURE 2
Cryptochrome and photolyase sequence clusters. When clustered by pair-wise sequence similarity, cryptochromes are sorted into CPD photolyases, CPD photolyases as found in *E. coli*, CPD photolyases containing the F420 cofactor, CPD photolyases DF, Class III CPD photolyases, CRY-DASH, CRY-DASH and plant CRY3, plant CRY one and 2, and a CRY cluster that upon more stringent differentiation (expanded blue box) segregates into categories represented by fungal CRYs, animal-like algal CRYs (aCRYs), Type 1 insect CRYs (e.g., dCRY), as well as vertebrate Type II, Type IV CRYs and 6-4 photolyases. Sequence clustering performed by clans (Frickey and Lupas, 2004).

Types of cryptochromes: from circadian rhythms to magnetic field sensing

Cryptochromes exhibit a wide range of functions, which is in contrast to relatively modest diversification of their structural and enzymatic features (Figure 1). Type I invertebrate CRYs entrain circadian rhythms to light by interacting with core elements of the circadian oscillator. Type II CRYs, including those from mammals, are unlikely to be light sensors, bind FAD weakly and may not require the cofactor for their functional roles (Sancar, 2004; Kutta, et al., 2017; Calloni and Vabulas, 2023). Recent work however does suggest that mammalian CRYs (mCRYs) may bind flavin as a mechanism to regulate and protect against ubiquitin-mediated degradation (Hirano, et al., 2017) and interestingly human CRYs (hCRYS) can rescue some presumably light-dependent functions of *Drosophila melanogaster* CRY (dCRY) in transgenic fly lines (summarized in (Vanderstraeten, et al., 2020)). Type II CRYs are also key components of the circadian clock, but rather than act in light entrainment, they compose the repressor complexes that feedback to inhibit the positive-acting heterodimeric transcriptional activators of the clock (CLOCK:BMAL1) (Sancar, 2004; Partch, Carrie L., et al., 2014; Michael, et al., 2017a; Michael, et al., 2017b; Takahashi, 2017). (Note that the abbreviation "mCRY" is often used to refer to "mouse" CRY, with the mouse being a key experimental system to study circadian rhythms; here we use the more general definition of mammalian CRY (mCRY), and further designate non-mouse mCRYs where appropriate). Type IV CRYs (Wang, et al., 2018a), found in reptiles, amphibians, fish, and birds, bind FAD and may participate in the sensing of the earth's magnetic field for navigation during migratory behavior (Xu, J. J., et al., 2021), as do Type I CRYs in invertebrates (Gegear, et al., 2008; Maeda, et al., 2008; Hiscock, et al., 2016; Agliassa, et al., 2018; Zoltowski, et al., 2019). Land plant CRY1 and CRY2 (e.g., AtCRY1 and AtCRY2 from *Arabidopsis*) are associated with growth, development, flowering, circadian rhythms and magnetic field sensitivity in plants (for reviews see (Galland and Pazur, 2005; Thoradit, et al., 2023)). Non-circadian-clock-related functions of Type I cryptochromes include the modulation of neuronal firing frequency, UV light avoidance in insects, and sensing of the lunar cycle (Baik, et al., 2019; Baik, et al., 2017; Damulewicz and Mazotta, 2020; Fogle, K. J., et al., 2015; Fogle, K.J., et al., 2011; Poehn, et al., 2022; Zurl, et al., 2022). Type II CRYs also play roles distinct from their repressive functions in circadian rhythms such as regulating the glucocorticoid receptor, cAMP signaling associated with gluconeogenesis (Lamia, et al., 2011; Tan and Scott, 2014; Zhang, E. E., et al., 2010) and the differentiation of brown adipose tissue (Miller, et al., 2020a; Miller, et al., 2021). Many organisms contain both Type I and Type II CRYs, whereas some contain only one or the other (Kotwica-Rolinska, et al., 2022). Notably the loss of Type I CRYs is usually associated with the loss of its target in the clock oscillator: the co-repressor Timeless (TIM) (Kotwica-Rolinska, et al., 2022). Animal-type CRYs (aCRYs) from algal species, act both as circadian clock regulators and 6-4 photolyases (Coesel, et al., 2009; Heijde, et al., 2010;

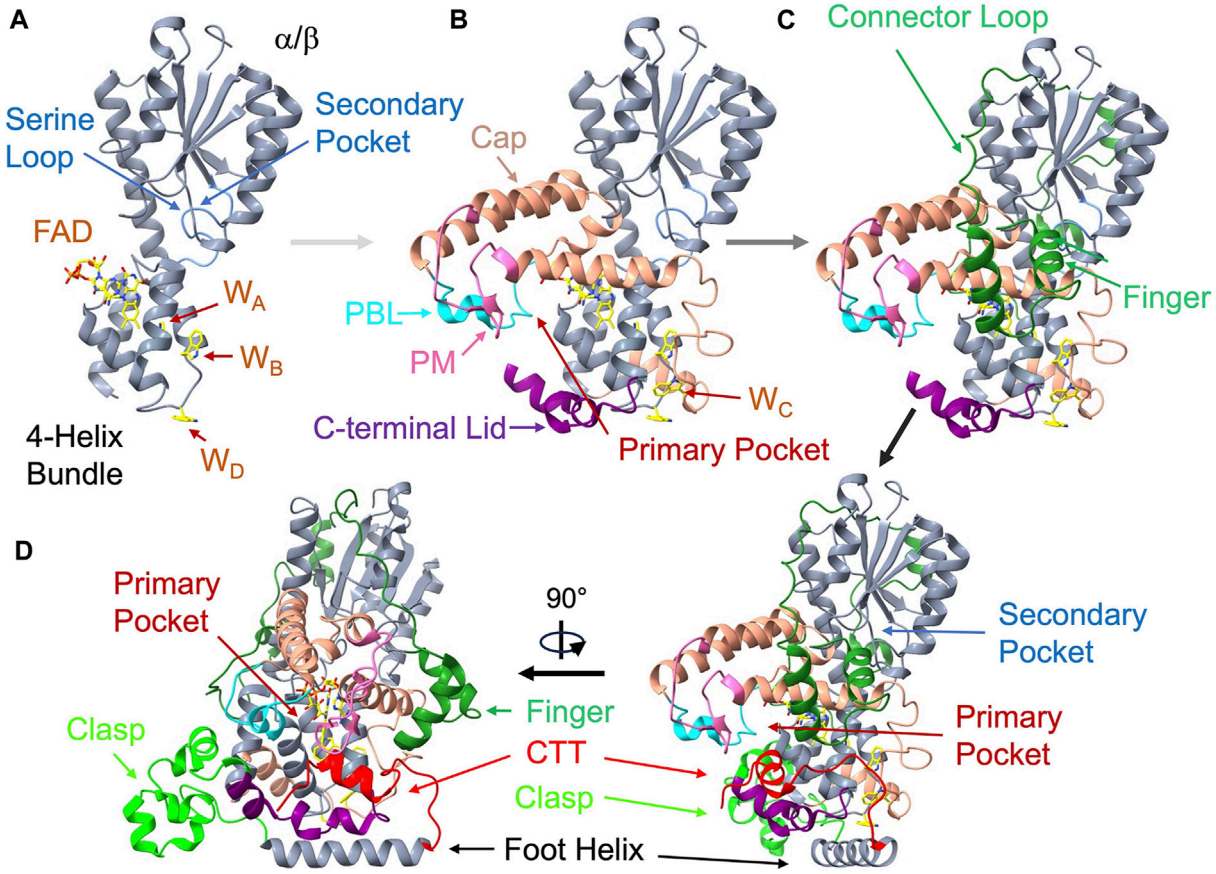

FIGURE 3
Structural anatomy of cryptochrome. The *Drosophila* CRY (dCRY) structure (PDB: 4GU5) assembled from its components. **(A)** On the left top the primase related 4-helix bundle (360-423, grey) binds the isoalloxazine ring below the α/β Rossman fold domain (residues 135-169, grey). The serine loop (light blue, 42-53) borders the secondary pocket. Three of the four redox active Trp residues (W_A, W_B, W_D) locate to the primase bundle. **(B)** Addition of the helical cap (salmon, 226-359) provides interactions to the adenosine moiety and harbors the phosphate-binding loop (PBL, cyan, 288-306) and the protrusion motif (PM, pink, 288-306). W_C is on a loop connecting to the bundle. The C-terminal lid (purple, 420-466) completes the primary pocket. **(C)** On the top right, the connector loop (dark green, 141-225), which contains the finger motif (dark green, 140-170) reaches across the α/β domain to connect the α-helical domain (green). **(D)** To complete the structure (lower right and rotated lower left), the clasp region (lime green, 470-496) follows the C-terminal lid, leads into the foot or CC helix (497-518), and finally the C-terminal tail (CTT, red, 519-542).

Fortunato, et al., 2015; Essen, et al., 2017; Kottke, et al., 2017). The CRY-DASH family (or class 0 PLs) are found throughout archaea, bacteria, plants and even vertebrates, but not mammals, and have quite diverse functions in signaling and transcriptional regulation (Brudler, et al., 2003; Kleine, et al., 2003; Kiontke, Stephan, et al., 2020), maintaining DNA repair activity in some cases, particularly for single-stranded DNA (Selby and Sancar, 2006; Tagua, et al., 2015; von Zadow, et al., 2016).

Underscoring their commonalities, CRYs and PLs often have evolutionarily conserved functions, demonstrated by ectopic and *in vitro* experiments. The *Ostereococcus tauri* (algal) CRY-DASH will inhibit CLOCK:BMAL1-mediated activation of a circadian clock reporter gene (Heijde, et al., 2010). Portorous tridactylus CPD PL interacts with CLOCK and can restore transcriptional oscillations in the liver of clock-deficient *cry1*/*cry2* mice (Chaves, et al., 2011b). At high concentrations, a truncated form of AtCRY1 can repair the CPD lesion (Zwang, et al., 2018).

Clock-associated CRYs from various vertebrate species, link to functions such as nonvisual photoreception, solar compass orientation, and time–place learning, thereby highlighting the adaptability and versatility of cryptochrome function (Emery et al., 1998; Cermakian, et al., 2002; Cermakian et al., 2002; Van der Zee, et al., 2008; Van der Zee et al., 2008; Hitomi et al., 2009). Understanding the interplay between these different phenotypes enriches our comprehension of the broader ecological roles and evolutionary significance of cryptochromes.

Cryptochrome structure: a base to build diverse functionality

CRYs share an architecture with PLs known as the Photolyase Homology Region (PHR) that comprises two domains: the α/β domain and the α-helical domain (Park, et al., 1995; Brudler, et al., 2003; Brautigam, et al., 2004; Hitomi, et al., 2009). Below we describe how distinct elements compose the cryptochrome fold, referencing to the dCRY structure (Zoltowski et al., 2011a; Czarna, et al., 2013; Levy, et al., 2013) that has been decomposed into its

structural elements in Figure 3 (note that the residue and secondary structure numbering may differ slightly for any given CRY, largely due to variability in loop regions). The α/β domain consists of a typical Rossman fold, where the traditional nucleotide diphosphate binding motif sits at the middle of the parallel β-sheet edge. The nucleotide binding region forms a cavity with the α-domain, wherein antenna cofactors, such as 5,10-methenyltetrahydofolate (MTHF), bind in PLs. In CRYs, this cavity forms the so-called secondary pocket that mediates interactions with partners, particularly the CLOCK:BMAL1 transcriptional activators in the circadian oscillators of mammals (Partch et al., 2014; Rosensweig, et al., 2018; Fribourgh, et al., 2020). A flexible loop that connects the second β-strand of the α/β domain to the second α-helix, known as the "serine loop" (residues 38–48 mCRY1, 42–53 dCRY), borders the secondary pocket and its dynamics modulate interactions between the mCRYs and CLOCK:BMAL1 (Fribourgh, et al., 2020).

Following the α/β domain a helix-turn-helix region (α5-α6, residues 135-169 in dCRY) curls like two fingers down against α11 of the α-helical domain before returning as a long connector to loop (170-266) that tracks back over the top of the entire α/β domain and then into the first helix, α8, of the α-helical domain. α8, α10 and α11 partially align to cap the top of the flavin cofactor by interacting with the adenosine moiety and FAD diphosphate groups and forming the primary pocket. Within this cofactor-capping region reside two loop elements that diverge in sequence and conformation among CRY homologs but have important functional and ligand binding properties: the phosphate binding loop (PBL, residues 249-263) following α8 and the protrusion motif (PM, residues 288-306) following α10 (Hitomi, et al., 2009). At the center of the α-helical domain, a skewed 4-helix bundle (α13-α16; residues 360-423) binds the isoalloxazine ring of the flavin adenine dinucleotide (FAD) cofactor at the center of its C-terminal three helices. CRYs and PLs share this core 4-helix bundle with DNA primase enzymes and hence these proteins may be related through an ancient connection involving single-stranded polynucleotide binding of primases and DNA repair activity of PLs and CRY-DASHs (Sauguet, et al., 2010).

Following the last helix of the bundle (α16), the Ser-rich (and in some cases, Cys-rich) extended C-terminal lid (residues 420-446) forms a cavity with the PBL and PM adjacent to the flavin. In dCRY this cavity accommodates the C-terminal tail (CTT) helix. The C-terminal lid, also known as the lid loop in mCRYs (residues 405-412 in mCRY1, residues 423-430 in mCRY2), partly determines specificity of the mCRY flavin pocket for targeted small molecules by influencing the conformations of conserved residues in the binding pocket (Miller, et al., 2020a; Miller, et al., 2020b; Miller, et al., 2021). In crystal structures, the lid-loop conformation is often ill-defined or influenced by crystal contacts. The C-terminal lid also contains key cysteine residues (mCRY1 Cys412 and Cys414) that play a role in regulating potentially redox-dependent interactions with the Period protein (PER) in mCRYs (Nangle, S. N., et al., 2014; Schmalen, et al., 2014). The C-terminal lid then leads into an irregular helical region that includes α18 and α19 (residues 449-497) that we will call the "clasp". The clasp associates adjacent subunits in the higher oligomeric states of land plants. Finally, C-terminal to the clasp is a long foot helix (α20) that is the last conserved region of the PHR. For mCRYs, the foot helix was identified as a potential coiled-coil (CC) motif by sequence analysis (the CC-helix) (Chaves, et al., 2006). Appended to α20, the cryptochrome C-terminal extension (CCE) varies substantially among CRY homologs.

CRYs and PLs typically bind FAD (Lin, C. T., et al., 1995) in an unusual "U" shaped conformation that associates the adenosine with the isoalloxazine ring. FAD acts as the primary blue-light sensitive chromophore (Sancar, 2003; Berndt, et al., 2007; Chaves, et al., 2011a; Zoltowski, et al., 2011b) and the secondary pocket may bind antenna chromophores in some cryptochrome types to improve photosensitivity, likely for DNA repair activity (Kiontke, S., et al., 2014). The electrocatalytic reactions of PLs benefit from the U-shaped conformation of FAD that allows electronic communication between the adenosine and isoalloxazine moiety (Zhang, M., et al., 2017).

Most CRYs and PLs undergo photoreduction of their flavin cofactors when the singlet excited states of the flavin oxidize an adjacent Trp triad or tetrad of residues (Banerjee, et al., 2007; Biskup, et al., 2013; Giovani, et al., 2003; Kottke, et al., 2006; Kutta, et al., 2018; Lin, C. F., et al., 2018; Nohr, et al., 2016; Paulus, et al., 2015; Zeugner, et al., 2005). These residues propagate an electron hole to the surface of the protein by successive oxidation where it eventually reacts with external reductants to stabilize the reduced flavin. Three of these four Trp residues (A, B, C) reside in the primase 4-helix bundle, with A and B at the peripheral end of the last two helices, respectively, the third (C) situated on a connecting loop leading from α11 in the cofactor cap across the base of the bundle. Often CRYs conserve a fourth, even more solvent-exposed Trp residue (D) that acts as the terminal position of oxidation and resides on the loop connecting to the last two bundle helices (Biskup, et al., 2013; Kutta, et al., 2018; Lin, C. F., et al., 2018; Nohr, et al., 2016; Oldemeyer, et al., 2016; Paulus, et al., 2015). Alternations of this terminal Trp can completely abrogate stable flavoreduction and downstream partner engagement in dCRY (Lin, C., et al., 2022; Lin, C. F., et al., 2018; Nohr, et al., 2016). In addition, variations in the Trp tetrad are found across the PL/CRY family, particularly in proteobacterial CRYs (Geisselbrecht, et al., 2012) and Class II photolyases (Kiontke, S., et al., 2011). In some cases, the terminal Trp residues can be further augmented by an additional Tyr residue (Oldemeyer, et al., 2016; Zoltowski, et al., 2019; Vu, et al., 2023) and in others, alternative Trp-triads are operative (Kiontke, S., et al., 2011). In general, efficient photoreduction of the flavin requires at least a Trp triad (Kao, et al., 2008; Kiontke, S., et al., 2011; Lin, C. F., et al., 2018; Nohr, et al., 2016; Oldemeyer, et al., 2016). Notably, the initial charge separation reaction that localizes radical states on both the flavin and a tryptophan residue provides a basis for the radical-pair mechanism of magnetic field sensing proposed for avian migration and other CRY-mediated geomagnetic field responses (Ritz, et al., 2000; Rodgers and Hore, 2009; Hiscock, et al., 2016). Trp radicals in the Trp-tetrad may also have structural effects if they are sufficiently long-lived (Ma, et al., 2020a; Cellini, et al., 2024), although whether such changes have the ability to modulate partner interactions important for signal transduction remains to be determined.

Single-molecule folding studies on dCRY find tight coupling between FAD binding and polypeptide folding, with FAD interacting with largely unfolded intermediates at faster than diffusion-controlled rates and at high affinity (Foroutannejad, et al., 2023). Interactions of the isoalloxazine dominate this

process, thereby indicating that the cofactor plays a role in structuring the primase-related 4-helix bundle, and perhaps capping region. Association of the CCE with the PHR appears to be the final folding step, in keeping with a low barrier to the dissociation of this element (Vaidya, et al., 2013; Berntsson, et al., 2019; Chandrasekaran, et al., 2021), which varies across CRY paralogs and described further below.

CRY C-Terminal extensions (CCEs): structural versatility and functional impact

Positioned at the C-terminal end of the PHR, the CCE, (sometimes abbreviated as CCT) and also called a carboxyl-terminal extension (CTE), distinguishes CRYs from their PL counterparts (Chaves, et al., 2011b). CCEs vary in length (from ~20 residues in invertebrate CRYs to ~200 residues in plant CRYs) and often contain flexible regions that lack a well-defined, known tertiary structure (Partch, C. L., et al., 2005). These extensions exhibit structural plasticity, which allows them to modulate the interactions of the PHR, particularly the flavin pocket and serve themselves as a hub for protein-protein interactions (Chandrasekaran, et al., 2021; Chaves, et al., 2011a; Parico, et al., 2020; Partch, C. L., et al., 2005). Light often modulates conformation of the CCE and interactions of the CCE with the PHR.

In Type I invertebrate CRYs, such dCRY, a 23-residue helical CCE or CTT containing an 11-residue helix inserts into the flavin-binding pocket in the dark state. Interactions of the CTT with the flavin pocket prevent dCRY from engaging signaling partners until light undocks it (see below) (Berntsson, et al., 2019; Chandrasekaran, et al., 2021; Lin, C. F., et al., 2018; Vaidya, et al., 2013). Removal of the CTT generally activates dCRY (Busza, et al., 2004; Ceriani, et al., 1999; Dissel, et al., 2004; Hemsley, et al., 2007; Lin, C., et al., 2022).

The CCEs of mammalian CRYs also play key functional roles, although these mechanisms are not necessarily linked to flavin chemistry. With respect to maintaining circadian rhythms in mouse embryonic fibroblasts (MEFs), the CCEs of neither mCRY1 nor mCRY2 are required, yet they have substantial and differential effects on rhythm period length and amplitude (Khan, et al., 2012). Furthermore, sequencing of human subjects with delayed sleep phase disorder (DSPD) identified a genetic polymorphism that excised exon11 of mCRY1 (Patke, et al., 2017; Parico, et al., 2020). Loss of the exon 11 coding sequence shortens the CCE and increases interactions with and thereby repression of the transcriptional activators CLOCK:BMAL1 (Patke, et al., 2017). Biophysical experiments demonstrate that the segment coded by exon11 indeed interacts with the PHR and thereby blocks interaction with CLOCK (Parico, et al., 2020). Contacts between the PHR and the CCE are conserved in mCRY2 and modulate interactions with the mCRY2 binding partner mPER2 (Ozber, et al., 2010; Parico, et al., 2020). The specificity of small molecule inhibitors that have been targeted to the flavin pocket of mCRYs also depends on the CCE (Miller, et al., 2020a), suggesting interactions between the CCE and the flavin pocket where the molecules bind. Furthermore, phosphorylation of the mCRY1 CCE is linked to period length, likely because, as with plant CRYs (see below), phosphorylation affects its conformation and hence access to the primary pocket, where the E3 ubiquitin ligase FBXL3 targets mCRY (Gao, Peng, et al., 2013; Xing, et al., 2013). In addition to harboring a nuclear import signal (Chaves, et al., 2006), the mCRY1 CCE also makes important contacts in the transcriptional repression complex with CLOCK:BMAL1, competing with the BMAL1 transactivation domain (TAD) for binding coactivators (Chaves, et al., 2006; Xu, Haiyan, et al., 2015).

In another related example, aCRYs from algae (e.g., *Chlamydomonas reinhardtti*) contain a ~100 residue CCE whose conformation gates interaction with the clock component Rhythm of Chloroplast (ROC) (Li, P., et al., 2022). aCRYs may function both in signaling and single-strand DNA repair (like some CRY-DASH proteins) (Essen, et al., 2017; Kottke, et al., 2017). The aCRYs are both blue and red-light sensitive because blue light drives the FAD to the neutral semiquinone, a state that then absorbs red light to be further photoreduced to the hydroquinone (Lacombat, et al., 2019; Li, P., et al., 2022; Oldemeyer, et al., 2016; Spexard, et al., 2014).

The avian CRYs associated with light-dependent magnetic field sensing (Niessner, et al., 2011; Xu, J. J., et al., 2021) also undergo conformational changes at their C-termini in response to light (Watari, et al., 2012; Niessner, et al., 2013; Niessner, et al., 2014; Zoltowski, et al., 2019). Avian CRY4 of chickens and CRY1a of both chickens and European robins localize to the retina (Niessner, et al., 2011; Watari, et al., 2012). A CRY1a epitope-specific antibody that targets the C-terminus only reacts with the protein in the discs of the retinal outer segments after illumination (Niessner, et al., 2013; Niessner, et al., 2014) and a C-terminally-directed chicken CRY4 antibody also reacts in a light-dependent manner (Watari, et al., 2012), thereby suggesting that the C-terminus becomes more accessible upon flavin photoreduction in both cases. However, biochemical studies of Type IV pigeon CRY4 (Zoltowski, et al., 2019) indicate rather that the C-terminus becomes more sequestered in light (see below).

Land plant cryptochromes (e.g., AtCRY1 and AtCRY2) have extensive and notably disordered CCEs (Partch, C. L., et al., 2005; Wang and Lin, 2020b). A wide variety of interacting proteins bind to AtCRY1 and AtCRY in both dark and light (see Table 1 in (Wang and Lin, 2020a; Qu, et al., 2024)). The CCEs themselves bind key targets (Yang, et al., 2000; Wang and Lin, 2020b): for example, the CCEs of AtCRY1 and AtCRY2, which differ considerably in sequence, both interact with the WD repeats of the ubiquitin ligases SPA/COP1, although the PHR domain of AtCRY2 also interacts with the kinase-like domain of COP1 (Lian, et al., 2011; Liu, B., et al., 2011; Liu, B. B., et al., 2016; Yu, et al., 2007; Zuo, et al., 2011). Light is proposed to release the CCEs from interaction with the PHRs so that both components can engage partners (Goett-Zink, et al., 2021; Kondoh, et al., 2011; Partch, C. L., et al., 2005; Yu, et al., 2007). It has long been recognized that the CCEs themselves exhibit signaling properties and when expressed alone confer constitutive activation of signaling pathways (Yang, et al., 2000; Yu, et al., 2007). A particular 80-residue region proximal to the PHR, called NC80, confers CRY2 functionality (Yu, et al., 2007). This motif is sequestered in the dark, but light-induced phosphorylation of the CCE exposes it for signal transduction (Yu, et al., 2007). The intrinsic disorder of the CCEs is thought to contribute to their signaling capabilities, allowing induced folding upon binding either by their targets or the cognate PHR domains (Michael, et al., 2017b;

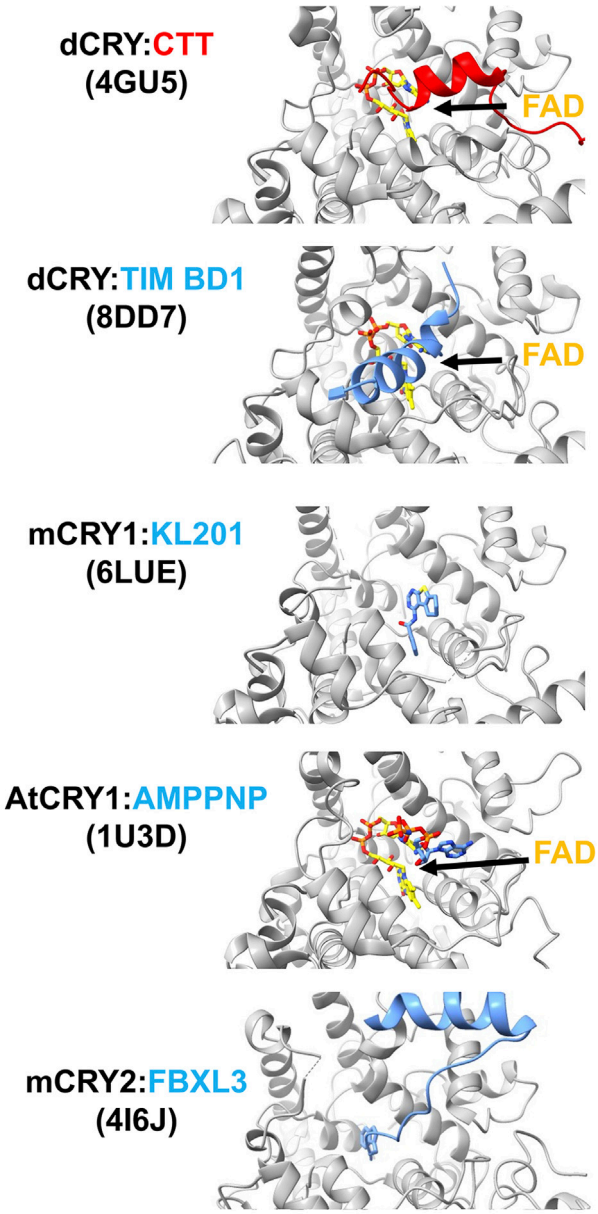

FIGURE 4
Interactions of cryptochromes in their primary pockets. When dCRY is in the dark state, its CTT rests in the FAD pocket. Upon light activation, the N-terminus of TIM binds dCRY by replacing the position of the dCRY CTT. The isoform-selective mCRY1 stabilizer, KL201, binds mCRY1 in the primary pocket. The non-hydrolyzable ATP analog AMPPNP binds in the plant CRY pocket. E3 ubiquitin ligase protein FBXL3 binds mCRY in the pocket to target degradation by the proteosome. FAD is shown in yellow and PDB codes of the structures are given in parentheses.

Partch, C. L., et al., 2005). Furthermore, the CCEs play a critical role in the stability of photobodies, which are a highly aggregated form of plant CRY (Más, et al., 2000; Wang, Q., et al., 2016; Yu, et al., 2009) with properties of liquid-liquid phase separation (LLPS) (Liu, Q. W., et al., 2024; Qu, et al., 2024). LLPS is primarily driven by the PHR domains, but light gating, and phosphorylation-associated stability of the condensates depend on the CCEs (Gao, L., et al., 2022; Liu, S. Y., et al., 2022; Ma, et al., 2023; Wang, et al., 2021a). These phase-separated states recruit and sequester a large number of protein partners (for an extensive summary see (Qu, et al., 2024)) and are considered to mediate an alternative mode of molecular recognition, distinct from those interactions mediated by complimentary 3-dimensional structure (Qu, et al., 2024). For example, AtCRY2 interacts with and regulates different classes of N^6-methyladenosine (m6A) RNA methyltransferases via LLPS to influence m6A modifications and RNA stability (Jiang, et al., 2023; Wang,et al., 2021b).

Primary and secondary pockets: interactions and signaling

The CRY primary pocket binds the FAD cofactor (Lin, C. T., et al., 1995)), small molecule inhibitors (Nangle, S., et al., 2013) and protein partners (Khan, et al., 2012; Gao, Peng, et al., 2013; Xing, et al., 2013) (Figure 4). It largely forms at the interface of the capping helices (primarily α11) with the primase bundle and is perimetrically structured by the PBL, C-terminal lid and PM (Figure 3). In most CRYs (and PLs) this pocket hosts the FAD cofactor in a distinctive conformation, stacking the isoalloxazine ring system close to the adenine ring. In mCRYs, small molecule effectors that were initially discovered in screens for disruption of the transcriptional oscillator bind in the primary pocket (Hirota, et al., 2012; Nangle, S., et al., 2013). A large number of inhibitors have been developed that have selectivity for either of the two mCRY isoforms and exhibit considerable effects on circadian rhythms and other biological activity (Miller, et al., 2020a; Miller, et al., 2020b; Miller, et al., 2021). The small molecules have been shown to compete with E3 ubiquitin ligases, especially FBXL3, for the mCRY primary pocket. The ubiquitination of CRYs by FBXL3 (Busino, et al., 2007; Siepka, et al., 2007) and FBXL21 (Hirano, et al., 2013; Yoo, et al., 2013) leads to their degradation by proteasomes, providing a mechanism to reset the CLOCK:BMAL1 transcriptional cycle. In recognizing mCRY, FBXL3 inserts a C-terminal Trp residue directly into what would be the FAD-binding pocket (Xing, et al., 2013) (Figure 4). Small molecule inhibitors that stabilize mCRY *in vivo* block the site where the C-terminus of FBXL3 binds (Miller, et al., 2020a; Miller, et al., 2020b; Miller, et al., 2021) and correspondingly, knockdown of FBXL3 substantially limits the inhibitor effects (Miller, et al., 2020a). In addition to it's C-terminus targeting the primary pocket, the leucine-rich repeats (LRRs) of FBXL3 encircle the CC-helix (Xing, et al., 2013), competing with interactions between mCRY and mPER2 (see below).

The primase bundle and C-terminal lid fashion both the primary and secondary pockets, which face roughly opposite directions. Switching these elements between mCRY1 and mCRY2 conveys the strong CLOCK:BMAL1-repressor characteristics of mCRY1 onto mCRY2 and visa versa (Khan, et al., 2012). Several specific residue substitutions at the base of these elements that impair repressor activity and reduce rhythm amplitude align more toward the face of the secondary pocket, but are distributed such that interfaces involved in CLOCK:BMAL1 interactions are likely to be extensive (Khan, et al., 2012).

In PLs, the extended pocket formed by the PBL, PM, and C-terminal lid accepts the substrate DNA lesion (Mees, et al., 2004; Glas, et al., 2009). As noted above, in dCRY the CTT binds

next to the flavin in the primary pocket, and the CCEs of other CRYs may also interact here (Figure 4). Upon light activation the dCRY CTT is replaced by the N-terminus of the TIM circadian corepressor, which provides a remarkable structural mimic for the 6-4 lesion repaired by DNA photolyases (Lin, C. F., et al., 2023). In plant CRYs, ATP binds in this position and appears to stabilize the CCE against the PHR (Brautigam, et al., 2004) (Figure 4). Release of ATP upon light activation may be coupled to destabilization and release of the CCE (Cailliez, et al., 2014; Müller, et al., 2014; Eckel, et al., 2018; Iwata, et al., 2020). Notably, ATP and other metabolites also facilitate photoreduction of the plant CRY flavin, by either directly acting as reductants or making the protein more susceptible for photoreduction (Engelhard, et al., 2014). In mCRYs, substitutions of residues in and surrounding the PBL and C-terminal lid affect period length without affecting protein stability, i.e., E3 ligase interactions (Ode, et al., 2017).

The primary pocket is also a main conduit for oxygen to access the flavin cofactor (Mondal and Huix-Rotlant, 2019; Deviers, et al., 2024). Return to the dark-adapted state requires oxidation of the semiquinone, which can be mediated by reduction of molecular oxygen to superoxide and other reactive oxygen species (Müller and Ahmad, 2011; van Wilderen, et al., 2015; Arthaut, et al., 2017), that themselves may have important signaling roles (Jourdan, et al., 2015). Oxygen radicals have also been suggested as a paired spin with the flavin semiquinone in the radical pair mechanism of magnetic field sensing (Hogben, et al., 2009; Müller and Ahmad, 2011); however, stabilizing interactions within the pocket would be required to promote spin-relaxation times long enough for O_2 to be a feasible player in magnetoreception (Hogben, et al., 2009; Player and Hore, 2019; Ramsay and Kattnig, 2022). Simulations find that superoxide localizes close to the flavin, by Trp A, flavin N5 and the N5-interacting residue (Asn391 in CRY IV), and on the other side of isoalloxazine ring by a conserved His residue (353) in the primary pocket (Deviers, et al., 2024). Behavioral data in birds (Wiltschko, et al., 2016) and plants (Pooam, et al., 2019; Hammad, et al., 2020) indicates that the magnetically sensitive reactions take place in the dark, following CRY illumination, and hence the implication of the oxidative reactions. There is also the possibility of O_2 playing the role of a third spin in a modification of the radical pair mechanism, which again would require localization of O_2 within the pocket (Ramsay and Kattnig, 2022). In dCRY, oxidation of the flavin is accompanied by some loss of FAD by the protein (Kutta, et al., 2018), which is unusual for flavoproteins, especially given the high affinity of dCRY for FAD (Foroutannejad, et al., 2023) and thus may have functional implications (Kutta, et al., 2018). With respect to the role of the primary pocket in magnetoreception, surprisingly, the 52 C-terminal residues of dCRY appear sufficient to mediate magnetic field responses in flies (Bradlaugh, et al., 2023). This region of the protein corresponds to largely just the foot helix and the short CCE, which is unlikely to maintain an ordered structure. It is currently unclear how such a mechanism would operate and it is not without controversy (Bassetto, et al., 2023).

In PLs, the secondary pocket, formed by the cleft between the nucleotide-binding site of the α/β domain and the α-helical domain accommodates the antenna cofactors (Figure 3). The variety of these moieties, which harvest light to accentuate excitation of the flavin for redox-mediate DNA repair, continues to grow and includes 5,10-methenyltetrahydofolate (MTHF), 7,8-didemethyl-8-hydroxy-5-deazariboflavin (8-HDF, also known as F0), flavin mononucleotide (FMN), FAD, and 6,7-dimethyl-8-ribityl-lumazin (DLZ) (Geisselbrecht, et al., 2012; Ozturk, N., 2017). CRYs do not generally bind antenna cofactors, except the CRY-DASH family, members of which maintain DNA repair capabilities (Bayram, et al., 2008; Pokorny, et al., 2008; Kiontke, Stephan, et al., 2020). Nonetheless, there were original reports of dCRY binding some MTHF (Berndt, et al., 2007), and it has been noted that plant CRYs conserve two MTHF-binding Trp residues found in class III PLs (related to plant CRYs) (Scheerer, et al., 2015). The secondary pocket

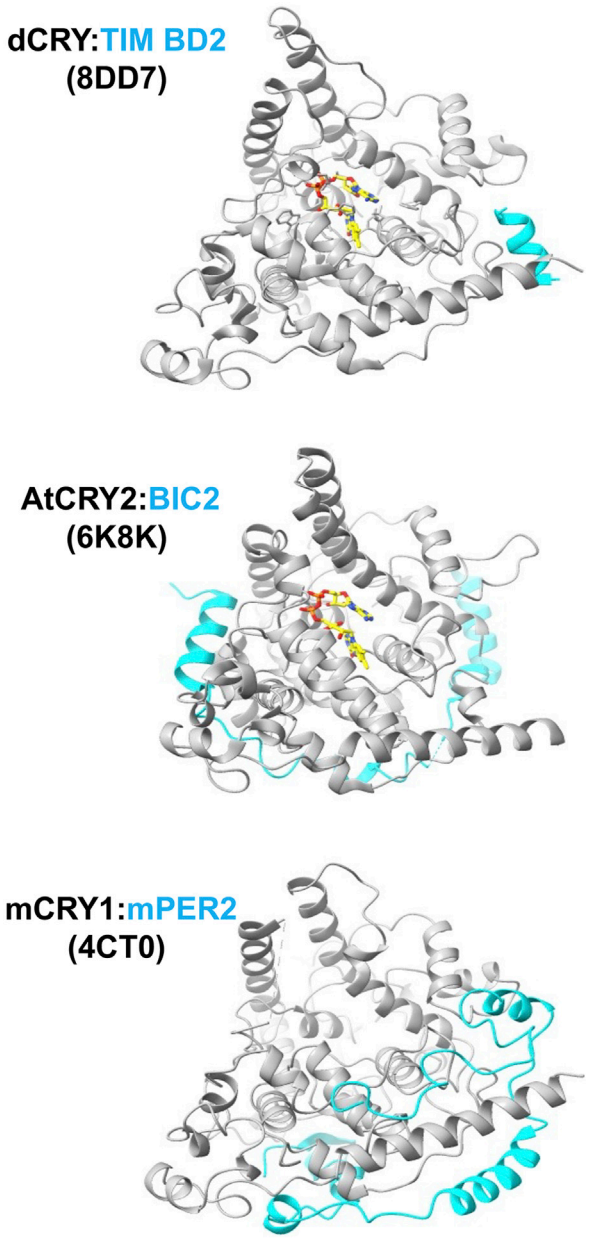

FIGURE 5
Interactions of cryptochromes at their peripheral sites. The C-terminus of TIM binds dCRY by the foot helix, similar to where regions of mPER bind mCRY. BIC2 wraps around the periphery of plant CRY. PDB codes of the structures are given in parentheses.

mediates interactions between mCRY and its transcriptional target CLOCK (Michael, et al., 2017b; Rosensweig, et al., 2018; Fribourgh, et al., 2020). The secondary pocket has been called "an evolutionary hotspot", wherein subtle residue changes alter the ability of Type II CRYs to repress CLOCK:BMAL1 and thereby affect circadian rhythms (Rosensweig, et al., 2018). In particular, minor residue variations in the serine loop at the rim of the secondary pocket affect CRY stability, nuclear import and CLOCK repression (Fribourgh, et al., 2020; Parlak, Gizem Cagla, et al., 2022). Type IV vertebrate CRYs cannot rescue the circadian clock function of Type II CRYs, perhaps because of residue changes that block access to the secondary pocket (Zoltowski, et al., 2019).

Interaction modes of binding partners: beyond the pockets

Key contacts of partners with CRYs involve peripheral regions of the PHR domain and not necessarily the primary and secondary pockets (Figure 5). mCRY1 and mCRY2, pivotal components of the circadian clock negative feedback loop, exert their regulatory influence by inhibiting the transcriptional activity of the CLOCK: BMAL1 complex (Khan, et al., 2012; Michael, et al., 2017a). mCRY1 interacts directly with the CLOCK:BMAL1 PAS-AB core and multivalent interactions between CLOCK PAS-B and the BMAL1 TAD are also required for mCRY1-mediated repression (Xu, Haiyan, et al., 2015; Michael, et al., 2017b).

mCRYs also associate with the mPER proteins to form the central repressor complex (Takahashi, 2017). The mCRY:PER interaction has been characterized crystallographically for the C-terminal regions of mPER2 with mCRY1 (Schmalen, et al., 2014; Michael, et al., 2017a) and mCRY2 (Nangle, S. N., et al., 2014). The mPER2 binding region forms extended polypeptide segments that wrap the protein behind the flavin pocket to the clasp region and particularly engage the C-terminal CC helix in a coiled-coil-like interaction (Figure 5) that is consistent with prior mutagenesis studies probing the mCRY2:PER2 contact (Ozber, et al., 2010). In one structure, a C-terminal β-hairpin of the mPER2 binds alongside of the serine loop of mCRY1 (Schmalen, et al., 2014). The interaction between mCRY1 and mPER2 is further mediated by a zinc ion that tetrahedrally coordinates Cys414 from the C-terminal lid with His473, and two Cys residues on mPER2 (residues 1210 and 1213) (Nangle, S. N., et al., 2014; Schmalen, et al., 2014). This intermolecular zinc site has been designated a "zinc-finger" (Nangle, S. N., et al., 2014), although it has tertiary structure and ligand spacing that differ from nucleic-acid binding CCCH-type zinc fingers. A neighboring mCRY1 disulfide bond involving Cys363 and Cys412 that forms in the unbound structure, breaks to potentially allow the conformational changes and dynamics required for the zinc site to form, thereby providing the means for redox state to mediate mPER binding (Schmalen, et al., 2014). The zinc site contributes to the mCRY:mPER binding affinity *in vivo* (Schmalen, et al., 2014), but substitutions of the ligating residues only have minimal effects on molecular rhythms (Nangle, S. N., et al., 2014). Both mCRY2 and PER2 cysteine residues are sensitive to oxidative conditions that could alter their interactions via the disulfide/zinc ion switch (Baidanoff, et al., 2022), but the full functional impact of such a mechanism remains to be explored. Alternations to mCRY upon mPER binding are particularly evident in the C-terminal lid, the serine loop, and other C-terminal structural elements. Notably, a naturally occurring human CRY2 (hCRY2) variant (Ser420Phe) at the junction of the primase bundle and C-terminal lid curtails repressor activity by reducing hPER2 affinity, but also reduces nuclear import of hCRY2 (Parlak, G. C., et al., 2023).

dCRY also participates in interactions that involve regions distinct from the primary and secondary pockets. For example, in addition to its principle interaction mode with the primary pocket, the *Drosophila* TIM protein supplies a peripheral helix to bind dCRY in regions similar to where the mPERs bind mCRY (Lin, C. F., et al., 2023). In a novel form of association, some evidence suggests that the iron-sulfur-cluster-containing protein designated MagR complexes with dCRY to form a rod-shaped, magnetically sensitive protein complex; however, the resolution of the structural characterization was insufficient to fully define this interaction (Qin, et al., 2016). dCRY also influences neuronal firing in a relatively fast response that involves its functional coupling to the voltage-gated potassium channel β-subunit (Kvβ), known as hyperkinetic (HK) (Baik, et al., 2019; Fogle, K. J., et al., 2015). Molecular dynamics-guided modeling of the dCRY:HK complex associates Trp D of the dCRY Trp tetrad in the α-helical domain close to the presumed binding site of NADPH in HK, thereby providing a mechanism for direct coupling between dCRY photochemistry and redox regulation of HK channel activity (Hong, et al., 2018).

In plants, interactions of the Blue-light Inhibitor of Cryptochromes (BIC) proteins block light activation by also engaging in peripheral interactions outside of the primary and secondary plant CRY pockets (Wang, et al., 2017a; Wang, et al., 2018b; Ma, et al., 2020b) (Figure 4). BICs specifically engage the PHR subdomains, adopting a 'U-shaped lock' conformation that impedes oligomerization and interactions with signaling partners (Ma, et al., 2020a). The BIC interaction involves PHR regions quite different from those of the PER proteins, with key contacts on the opposite side of the PHR (Ma, et al., 2020b). For example, BIC2 interacts with the C-terminal clasp region of plant CRY2 in a manner that prevents this domain from mediating tetramerization (Ma, et al., 2020a).

Type I cryptochrome light activation: flavin reduction and CTT undocking

The mechanisms by which light activation propagates conformational signals in cryptochromes for their signaling functions has been most extensively studied in Type I CRYs and plant CRYs because these systems have both been structurally accessible while also having clearly defined signaling functions in their respective organisms. As noted, there is little evidence that the vertebrate Type II CRYs are light sensors (Kutta, et al., 2017), although they may interact with flavin in modes that allow for responses to redox and metabolic state (Hirano, et al., 2017; Calloni and Vabulas, 2023). In the case of Type 1 invertebrate CRYs, blue-light absorption by the oxidized flavin produces the anionic semiquinone by photoreduction via the Trp triad/tetrad (Berndt, et al., 2007; Kao, et al., 2008; Kutta, et al., 2018; Ozturk, N., et al., 2014; Ozturk, N., Selby, C.P., Annayev, Y., Zhong, D., Sancar, A.,

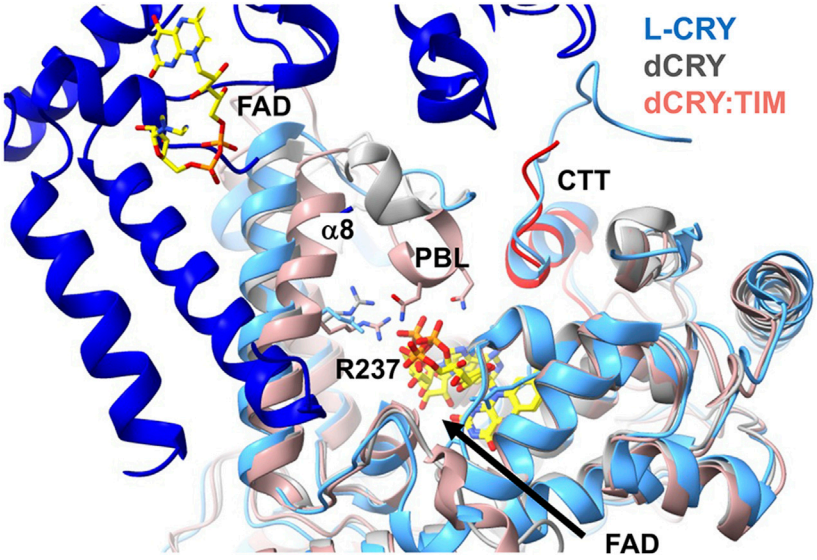

FIGURE 6
Conformational changes in Type I CRYs. Superposition of the structures of dark-state dCRY (grey) and light-state dCRY bound to TIM (red) show that Arg238 and α8 shift toward the rearranged phosphate groups of FAD. The PBL also collapses into the pocket. Also superimposed is the dark-state dimer of L-CRY. L-CRY conserves the analog of Arg238 and its movement toward the flavin may disrupt the dimer contact formed by α8. The longer CCE of L-CRY also contributes to the dimer interface. The CCE of L-CRY contains a helix that aligns with the CTT of dCRY (red) that displaces upon light activation.

2011; Song, et al., 2007; Vaidya, et al., 2013). dCRY mediates the degradation of the transcriptional co-repressor TIM (Koh, et al., 2006; Lin, F. J., et al., 2001) and its own light-driven self-degradation (Busza, et al., 2004; Dissel, et al., 2004; Hemsley, et al., 2007). These processes require light-gated interactions with E3 ubiquitin ligases, of which two have been implicated: Jetlag (Peschel, et al., 2009) and Ramshackle (Brwd3) (Ozturk, N., et al., 2013). For dCRY, variants in the Trp triad with varying light sensitivities correspondingly affect the ability to degrade TIM and undergo self-degradation (Lin, 2019). The CCE of dCRY gates interactions with targets. dCRY has one of the shortest CCEs, and the structure of the full-length protein revealed that the majority of this element forms a C-terminal tail helix that inserts into the flavin pocket (Zoltowski, et al., 2011b; Czarna, et al., 2013; Levy, et al., 2013). Proteolytic sensitivity measurements identified regions in the CTT and surrounding CTT-coupled motif (i.e., the C-terminal Lid, PM and PBL) that become differentially sensitive upon light activation (Vaidya, et al., 2013). Spin-labeling studies in concert with pulse-electron-spin resonance spectroscopy measurements demonstrate that undocking of the CTT depends on forming the FAD anionic semiquinone (ASQ), and this undocking was recapitulated in molecular dynamics (MD) simulations (Berntsson, et al., 2019; Chandrasekaran, et al., 2021; Ganguly, et al., 2016; Lin, C., et al., 2022). Time-resolved SAXS studies of dCRY were also consistent with CTT displacement upon ASQ formation (Berntsson, et al., 2019). The precise mechanism of CTT undocking is not fully determined. Two conserved histidine residues that undergo protonation changes during the enzymatic reactions of photolyases may similarly respond to flavin photoreduction (Ganguly, et al., 2016). These residues have been shown to affect the fidelity of CRY interactions with its target TIM, but their substitution does not block CTT release (Berntsson, et al., 2019; Lin, C., et al., 2022). The cryo-EM structure of light-exposed dCRY bound to TIM defined key interactions of the signaling state (Lin, C. F., et al., 2023). The ~1400 residue TIM proteins inserts its N-terminal helix into the flavin binding pocket, effectively replacing the CTT. The regions that change proteolytic accessibility (e.g., the PBL, PM, C-terminal lid and CTT) (Vaidya, et al., 2013) rearrange conformation upon TIM binding. TIM is a large helical protein made from armadillo (ARM) repeat modules, wherein the first 3 ARM repeats make extensive interactions with dCRY. Moreover, a C-terminal helix of TIM that resides in an otherwise disordered region binds to a separate dCRY element near the C-terminal foot (CC) helix. The dCRY FAD substantially rearranges in the TIM complex, particularly the diphosphate groups which change orientation to interact with an Arg residue (258) on helix α8, which inserts further into the active site to replace the otherwise coordinating Mg^{2+} ion (Figure 6). The PBL refolds from the surface into the flavin pocket, with its TIM-bound conformation stabilized by His377, one of the two conserved His residues that influences TIM recognition and tail release (Figure 6). Peptide binding assays with the N-terminal region of TIM show that Arg237 and the residues in the PBL that undergo the most change are critical not only for TIM binding, but also for CTT undocking, suggesting that the ASQ state of the flavin promotes the movement of Arg237 and the PBL prior to CTT displacement (Schneps, et al., 2024). Indeed, the TIM-bound conformation of the PBL clashes with the CTT when it is bound in the flavin binding pocket (Schneps, et al., 2024). MD simulations of dCRY activation in the absence of TIM suggest that a key salt bridge involving a highly conserved Arg-Asp pair that buttresses the isoalloxazine ring of the flavin disrupts when the flavin is photoreduced (Wang, et al., 2021a), consistent with the cryoEM structure (Lin, C. F., et al., 2023). Similar movements in this salt-bridge have been observed in serial

crystallography experiments of photoreduced PLs (Maestre-Reyna, et al., 2022; Cellini, et al., 2024). Finally, the dCRY PHR domain alone binds the N-terminal peptide, independent of light activation, but the affinity for the peptide increases in the light (Schneps, et al., 2024). Thus, light-dependent CTT release is not the only factor involved in the increase of CRY affinity for TIM in the photoreduced state.

Type IV avian CRY displays some intriguing similarities and differences with respect to their conformational activation mechanism compared to dCRY. Pigeon CRY4 (ClCRY4) binds FAD, forms a neutral semiquinone when photoreduced and has a similar length CCE compared to dCRY (Xu, J. J., et al., 2021; Zoltowski, et al., 2019). Proteolytic protection assays guided by structural analysis indicated that the PBL becomes more protected in the semiquinone form (Zoltowski, et al., 2019). A closed PBL or "gate" in the semiquinone state is also consistent with all-atom (Schuhmann, et al., 2021) and course-grain MD simulations of ClCRY4. This movement of the PBL in the MD simulations produced a state similar to the collapsed PBL conformation of dCRY observed in the TIM complex. However, the CCE of ClCRY behaviors opposite to the CCE of dCRY upon photoreduction, with the former also becoming more protected in the flavin photoreduced state (Zoltowski, et al., 2019; Schuhmann, et al., 2024), whereas the latter undocks to reveal the TIM binding pocket (Chandrasekaran, et al., 2021).

A variant Type I CRY (L-CRY) found in the marine bristle worm *Platynereis dumerilii* paces reproduction to the lunar cycle (Poehn, et al., 2022; Zurl, et al., 2022). L-CRY displays an intriguing modification to the dCRY activation mechanism (Vu, et al., 2023). In this case, the dimeric L-CRY associates in the dark-state through an interface that involves α8 and interactions between the CCE and the clasp region (Figure 6). The protein is proposed to undergo a two-step light activation process. At low light levels only one subunit photoreduces, sending a specific signal within the nucleus (Poehn, et al., 2022). Upon photoreduction of both subunits at higher light intensity and reduced quantum yield, the protein dissociates into subunits, revealing a nuclear export signal. Asymmetric activation at low light and high quantum yield is proposed to produce a signaling state that then distinguishes dim moonlight from higher intensity sunlight. The addition of a terminal Tyr residue to the Trp tetrad may stabilize charge-separated radicals to a greater degree and fine-tune light sensitivity (Vu, et al., 2023). Indeed, manipulation of the Trp -tetrad in dCRY can also increase light-sensitivity (Lin, C. F., et al., 2018). Based on the structure of the dimeric dark state, the dimer interface may be disrupted by CTT undocking and a shift of α8 when conserved Arg (dCRY 237, L-CRY 234) responds to flavin photoreduction analogous to that observed in dCRY (Schneps, et al., 2024) (Figure 6). Notably, family members of other flavoprotein light-sensors such as LOV domains also show either light-dependent associations or dissociations by coupling similar flavin pocket chemistry to perturbations of peripheral structural elements that increasingly diverge from the cofactor pocket to the protein interaction surface (Vaidya, et al., 2011; Conrad, et al., 2013; Conrad, et al., 2014).

Animal-like CRYs (aCRYs) from algae also undergo light-depend rearrangements of their CCEs. Although the sequences of CCEs are very different from Type I CRYs they also appear to associate with the flavin pocket in the dark and become displaced in the light. aCRY accesses the oxidized, semiquinone and hydroquinone flavin states which can be interconverted by blue and red light, respectively (Franz, et al., 2018; Kottke, et al., 2017; Li, P., et al., 2022; Spexard, et al., 2014). HDX studies indicate a modest change in PHR protection upon conversion to the fully-reduced hydroquinone state due to diminished interactions of the CCE with the PHR (Franz-Badur, et al., 2019). However, Fourier transform infrared (FTIR) spectroscopy did not reveal substantial conformational changes between either the FADox and $FADH^{\circ}$ states or the $FADH^{\circ}$ and $FADH^{-}$states (Spexard, et al., 2014; Oldemeyer, et al., 2016). On the other hand, FRET studies indicate a CCE displacement by ~ 15 Å upon blue-light irradiation to the presumed neutral semiquinone (Li, P., et al., 2022).

Plant cryptochrome light activation: CCE undocking and oligomerization

Light activation of plant CRYs has been informed by recent cryoEM and crystal structures of the protein in dark and light-exposed states (Hao, et al., 2023; Liu, B. B., et al., 2016; Ma, et al., 2020a; Ma, et al., 2020b; Palayam, et al., 2021; Shao, et al., 2020; Wang and Lin, 2020a). The first cryptochrome crystal structure was of the AtCRY1 PHR domain (1U3C) (Brautigam, et al., 2004); it displays the characteristic photolyase fold as well as a head-to-head (HH) association in the crystal involving the α/β domain with α10-α11 that has been found to manifest in nearly all of the other structures determined by crystallography or cryoEM (see below). Light photoreduces AtCRY1 and AtCRY2 to primarily the neutral or protonated FAD semiquinone state via a Trp triad (Giovani, et al., 2003; Zeugner, et al., 2005; Kottke, et al., 2006; Banerjee, et al., 2007). Although there has been debate on the identity of the key light-activated state within the cell (Li, X., et al., 2011), there are strong arguments that the NSQ is the functionally relevant light-adapted state (see (Ahmad, 2016) for a thorough discussion of this issue). Furthermore, NSQ formation clearly promotes both changes in interactions between the CCE and the PHR (Goett-Zink, et al., 2021; Kondoh, et al., 2011; Partch, C. L., et al., 2005) as well as the oligomerization of both full-length CRY proteins and their PHR domains *in vitro* (Ma, et al., 2020a; Palayam, et al., 2021). Plant CRY PHRs form both dimers and tetramers, with the tetrameric state favored by the photoreduced flavin as well as other conditions (Shao, et al., 2020). These tetramers are also preferentially formed by variant proteins with single residue substitutions (W368A AtCRY2 or W374A in *Zea mays* (ZmCRY1)) that result in light-independent activation *in vivo* (Shao, et al., 2020). Notably, AtCRY1 has also been found to oligomerize independent of light through chemical cross-linking studies in plant seedlings (Sang, et al., 2005); however, more recent quantitative pull-down experiments show that both AtCRY1 and AtCRY2 oligomerization is enhanced by light *in vivo* (Liu, Q., et al., 2020; Wang, Q., et al., 2016). Diffusion measurements suggest that AtCRY1 does not oligomerize with light, but rather undergoes a large conformational change involving the CCE (Kondoh, et al., 2011), although these measurements are sensitive to both conformation and oligomeric state.

The tetrameric state of plant CRY, which has been observed for both CRY1 and CRY2 of several plant species (Figure 7), are composed of two distinct interfaces (Ma, et al., 2020b; Shao, et al., 2020; Palayam, et al., 2021). The plant CRY tetramer has

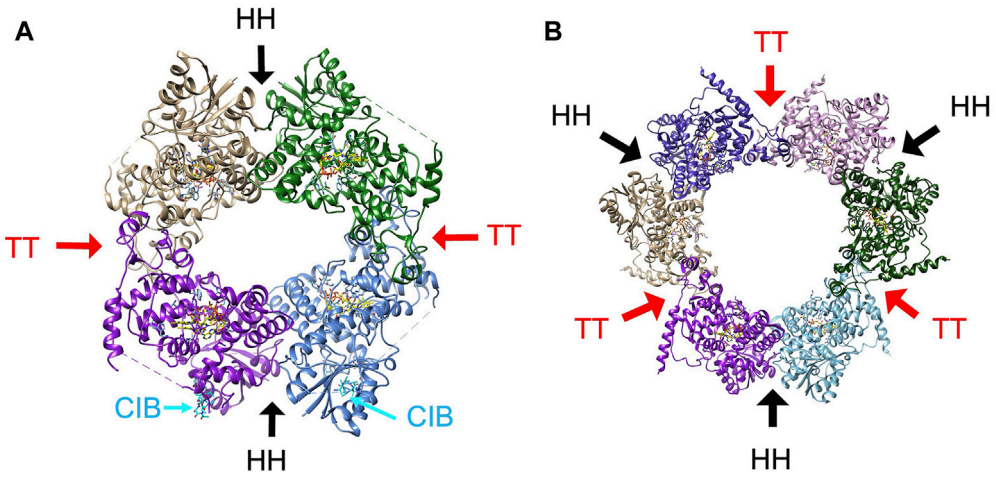

FIGURE 7
The oligomeric states of plant CRY. **(A)** The tetramer characteristic of activated plant CRY1 and CRY2 has two subunit interfaces, one involving the α/β domain, connector and Cap (HH, black arrows) and the other involving the Clasp region (TT, red arrows). Cryo-EM electron density indicative of CIB1 helix binding (cyan) to AtCRY2 was found near the HH interface (PDB:7x0Y). **(B)** The hexamer that composes the crystal lattice of AtCRY1 PHR domain (PDB; 1u3c) maintains the HH interface and a similar TT interface as found in the tetrameric structures of full-length plant CRY1 and CRY2.

222 symmetry, which produces a head-to-head (HH) contact and a tail-to-tail contact (TT) (Figure 7). The TT interface has been called a head-to-tail contact (HT), but such a description is usually reserved for oligomers in which the twofold symmetry axis does not reside within the interface itself and hence pairs one region of the protein with another, which is not the case for the AtCRY tetramer. Furthermore, the two interfaces have been interchangeably described as interface one and interface 2. Here we will refer to HH and TT interfaces which respectively correspond to the HH and HT of Palayam et al. (Palayam, et al., 2021), interface two and interface one of CRY1 in Shao et al. (Shao, et al., 2020)) and interface one and interface two of Ma et al. (Ma, et al., 2020a). The HH interface primarily involves the α/β domain and associated connector region preceding the α5-α6 finger motif and the α10 capping helix of the α-helical domain (Figure 7). The TT interface is formed by a symmetric interaction of the irregular clasp helices (α18 and α19) that extend from the C-terminal lid and includes contacts from a region of the connector that precedes α8 and the C-terminus of α13 (Figure 7).

There is good evidence that light affects association about the TT interface. Single residue substitutions in this region (AtCRY R439L, W349A on α13) prevent light-induced tetramerization of AtCRY2, reduce interactions with a CIB1-derived peptide and only partially rescue the long hypocotyl phenotype by AtCRY2 in a *cry1* mutant (Shao, et al., 2020). In addition, genetically selected mutants that map to the TT interface of AtCRY1 (G347R, A462V) and CRY2 (G254R, P339L) disrupt light-independent oligomerization *in vivo* (Liu, Q., et al., 2020; Sang, et al., 2005) and Q333A and E462A at the TT interface reduce the formation of photobodies (Ma, et al., 2020a). Mutations at both the HH and TT interfaces reduce AtCRY2 photo-oligomerization *in vitro* (Ma, et al., 2020b). As noted above, BIC2 wraps the PHR domain as an extended polypeptide of disconnected helices to partially block the TT association (Ma, et al., 2020a). BIC2 binds to AtCRY2 in the dark and inhibits flavin photoreduction by perhaps slightly altering the position of the Trp-triad and flavin-protonating Asp residue (Ma, et al., 2020b). When illuminated by blue light, AtCRY2 targets the transcriptional regulator CIB1 (cryptochrome-interacting basic helix-loop-helix 1) through the PHR domain to regulate flowering (Liu, H. T., et al., 2008). CIB1 appears to bind near the HH interface (Figure 7), although the detailed structure of the protein could not be discerned in cryo-EM maps, which only revealed modest density for a helix-like structure (Hao, et al., 2023). The HH interface forms in the absence of light and thus may be available to bind CIB1 in the dark (Shao, et al., 2020). However, light-dependent TT oligomerization would recruit four CIB1 proteins together, which may be required for their transcriptional activation activity.

Of considerable interest are the conformational changes that propagate from the flavin to transform the plant CRY structure into the signaling state. Spectroscopic studies have determined the primary reactions of light activation in plant CRYs (reviewed in (Goett-Zink, et al., 2021; Aguida, et al., 2024)). In brief, flavin photoreduction to the ASQ precedes flavin protonation from a conserved Asp residue (Thoing, et al., 2013; Hense, et al., 2015; Thöing, et al., 2015), which ionizes in the dark-to-light transition. Infrared difference spectroscopy identified secondary structural regions affected by the electron and proton transfers at the AtCRY1 active center; early structural intermediates involve helical and turn rearrangements followed by later stage remodeling of the β-sheet within the α/β domain (Goett-Zink, et al., 2021). Like Type I CRYs, light releases the CCE from the PHR to gate downstream effects (Partch, C. L., et al., 2005; Sang, et al., 2005; Wang, et al., 2017b), and the spectral perturbations in the β-sheet likely reflect loss of interaction with the CCE. Phosphorylation or modification of the CCE also diminishes its interaction with the PHR domain, modulates its photosensitivity and activates downstream signaling (Yu, et al., 2007; Yu, et al., 2009; Wang, et al., 2017a). Notably the light-dependent TT interface is far

removed from the α/β domain where spectroscopic changes reside, but the CCEs are large. One then might expect that CCE release impacts association about the TT interface. However, the PHR domains alone undergo light-activated tetramerization (Ma, et al., 2020a; Palayam, et al., 2021) without the need for CCE gating. In all, it is not yet entirely clear how flavin photoreduction generates a functional signaling state. Under physiological conditions, tetramerization may be a necessary, but insufficient condition to activate plant CRY. Flavin photoreduction could regulate both CCE release and other structural changes that encourage association about the TT interface. Oligomerization may also be important for the CCE itself to send signals because artificially induced CCE dimerization in the absence of the PHR alters gene expression in plants (Rosenfeldt, et al., 2008).

To better understand the molecular mechanism of plant CRY light activation, light-state crystal structures (Palayam, et al., 2021), cryoEM structures of light-activated CRY2 alone (Ma, et al., 2020b) and with CIB1 (Hao, et al., 2023), as well as cryoEM structures of activating mutants (Shao, et al., 2020) have been compared to dark-state structures, primarily determined in crystals (6K8I, 1U3C) with and without BIC2 (Brautigam, et al., 2004; Ma, et al., 2020a). Comparisons between the CRY2 light-state tetramer to the CRY2 crystal structure in darkness (PDB: 6K8I) identified several residues that appear to change position slightly between the clasp and the flavin pocket (Ma, et al., 2020b). These residues were then substituted, most with little effect; however, two substitutions Y232A and W353A promoted tetramer formation in the dark. The challenge with these analyses is that the dark state structures typically form the same or similar oligomeric states in crystals as the light state structures. Hence, crystallization may favor conformations and subunit assemblies found in the light-state. For example, 6K8I, although referred to as a monomer structure, forms a similar tetramer in the crystal lattice as the light-activated structure (Ma, et al., 2020a; Ma, et al., 2020b). This recapitulation of light-state-like oligomers in crystals is also the case for the original dark state of CRY1 PHR, which does not form a tetramer in the crystal, but does form a hexamer with very similar HH and TT interfaces (Brautigam, et al., 2004) (Figure 7). The lower resolution of the cryoEM structures also makes it challenging to compare more subtle conformational changes in the dark and light state. If one superimposes the individual subunits of the various structures there does not appear to be a consistent pattern that distinguishes light (or mutationally activated) subunit conformations from the dark-state ones. The lower resolutions of the cryo-EM structures also limits evaluation of changes in flavin conformation.

The Trp triad may also participate in propagating conformational changes from the initial photochemistry. The activating mutations of W374A (CRY1) and W368A (CRY2) involve one of the Trp-triad residues. Photoactivation is blocked in these variants, but conformational changes induced by the residue substitutions appear to activate oligomerization (Engelhard, et al., 2014). The structural perturbations are removed from the HH and TT interfaces, but they do interact with the connector region and thereby could affect the clasp. Whether a chemical change in the Trp triad (e.g., a cation or neutral radical) is stable long enough to drive a change in oligomerization that must act on the timescale of physiological signaling is an open question. Going forward, the structure of a dark-state plant CRY monomer with an intact CCE would provide an important reference for further understanding plant CRY photoactivation.

Summary and Outlook

Despite their diversity of function, CRYs share a remarkably conserved structure with two domains whose general folds are largely invariant. This architecture provides two pockets to accommodate cofactors, small molecules and partner proteins. The CRY conformation, particularly involving the CCE and the PBL, responds to moieties that bind in the pockets, thereby gating pocket access or revealing interaction motifs at the periphery or on the CCE itself. Photoreduction of FAD bound in the primary pocket to a semiquinone state (anionic or neutral) alters the protein properties in complex ways that share some commonalities among CRYs close to the cofactor but then diverge as more peripheral structural elements differ. Photoreduction utilizes an internal chain of Trp and Tyr residues that enables stabilization of charge separation over a relatively long distance and time. It remains to be fully understood how changes in flavin and Trp redox states propagate to other regions of the protein such as the CCE, clasp and α/β domain to regulate new interactions or facilitate changes in oligomeric state, although considerable details are becoming apparent. Non-photoresponsive CRYs, such as the mammalian Type II proteins, couple pocket reactivity to conformation in distinct ways whose details continue to be revealed, whereas the CRY-DASH family couple signal transduction with DNA repair activities. Thus, the understanding of the structure and mechanism of CRYs informs on many biological processes. This review has not discussed the application of cryptochromes to bioengineering applications, such as in the area of optogenetics (de Mena, et al., 2018; Lu, et al., 2020; Yamada, et al., 2020; Iwata and Masuda, 2021; Aguida, et al., 2024), but as new mechanisms reveal themselves, there is increasing potential to utilize cryptochromes as versatile sensors and control elements in entirely new processes that may reach beyond biology and into the realms of material science, device design and information processing.

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Conflict of interest

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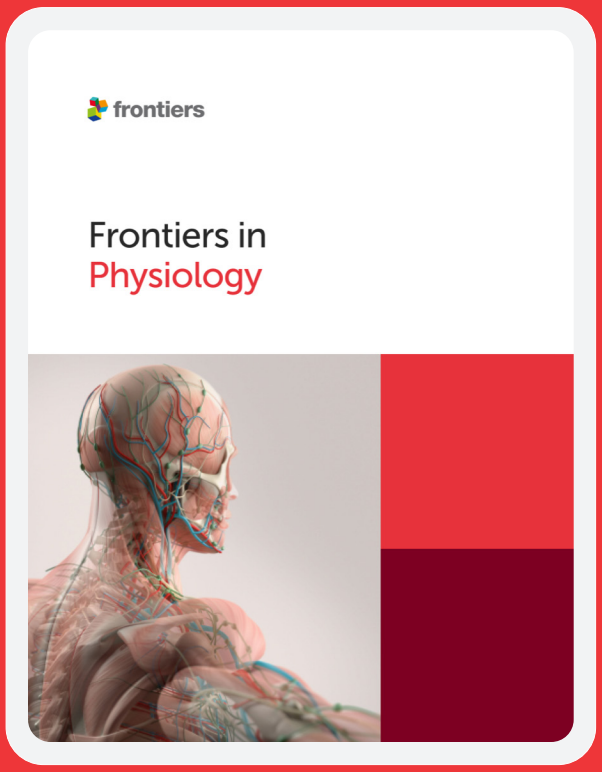

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