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Review

The Red Edge: Bilin-Binding Photoreceptors as Optogenetic Tools and Fluorescence Reporters

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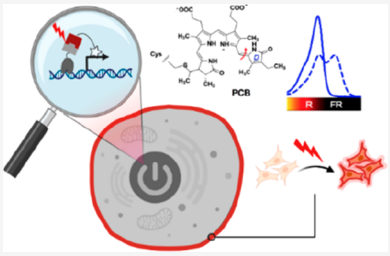
ABSTRACT: This review adds the bilin-binding phytochromes to the *Chemical Reviews* thematic issue "Optogenetics and Photopharmacology". The work is structured into two parts. We first outline the photochemistry of the covalently bound tetrapyrrole chromophore and summarize relevant spectroscopic, kinetic, biochemical, and physiological properties of the different families of phytochromes. Based on this knowledge, we then describe the engineering of phytochromes to further improve these chromoproteins as photoswitches and review their employment in an ever-growing number of different optogenetic applications. Most applications rely on the light-controlled complex formation between the plant photoreceptor PhyB and phytochrome-interacting factors (PIFs) or C-terminal light-regulated domains with enzymatic functions present in many bacterial and algal phytochromes. Phytochrome-based optogenetic tools are currently implemented in bacteria, yeast, plants, and animals to achieve light control of a wide range of biological activities. These cover the regulation of gene expression, protein transport into cell organelles, and the recruitment of phytochrome- or PIF-tagged proteins to membranes and other cellular compartments. This compilation illustrates the intrinsic advantages of phytochromes compared to other photoreceptor classes, e.g., their bidirectional dual-wavelength control enabling instant ON and OFF regulation. In particular, the long wavelength range of absorption and fluorescence within the "transparent window" makes phytochromes attractive for complex applications requiring deep tissue penetration or dual-wavelength control in combination with blue and UV light-sensing photoreceptors. In addition to the wide variability of applications employing natural and engineered phytochromes, we also discuss recent progress in the development of bilin-based fluorescent proteins.



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1. INTRODUCTION

Biological photoreceptors are the essence of optogenetics, a biomolecular method that enables remote-controlling a wide range of biological processes with light. Since its establishment, the field has strongly influenced nearly all imaginable applications in biomedicine, cell-physiology, bioengineering, and modern superresolution microscopy techniques. "Optogenetics" in all its variations is the topic of a series of articles in *Chemical Reviews*,[1−4] and the pioneers of this revolutionary concept have provided many historical surveys detailing the birth and potential of the idea and also how broad the applications are and will be in the future.[5,6] The unique property of biological photoreceptors, being regulated in their activity by light in a noninvasive fashion, has stimulated their introduction into "blind" orthogonal organisms that allowed rewiring cellular functions with light perception.

The term "optogenetics" is in many aspects synonymous with "channelrhodopsin" (ChR) that in fact was the first and still is one of the most frequently employed tools in biotechnological and biomedical applications.[7] The number of published articles detailing ChR-regulated optogenetic applications easily witnesses their popularity. ChRs carrying the vitamin-A aldehyde (retinal) as chromophore have been developed with a wide range of absorption maxima using extensive mutagenesis screenings. However, ChRs are membrane-intrinsic proteins serving as light-gated ion channels with moderate selectivity for a particular ion including protons, although recently ChR-controlled enzyme activities have been described.[8] Thus, conditions requiring a selective light-regulated function call for alternative methods. Soluble biological photoreceptors with unique specificity and activity have now overcome these bottlenecks and expanded the range of possible applications.

In fact, nearly all classes of biological photoreceptors have been engineered and employed in optogenetics, deploying a wide range of molecular tools responding to wavelengths across the light spectrum for precisely controlling cellular functions. Besides the overwhelming number of applications performed with the retinal-based microbial and animal rhodopsins, flavin-based blue light-sensing photoreceptors, LOV- or BLUF-domain proteins, or the cryptochromes and also green light-absorbing cobalamin-containing receptors have been employed. In particular the flavin-based photoreceptors have made their way into optogenetic applications, due to their dual activity serving as light-regulated enzymes and as fluorescent tag. These features even allowed their implementation as fluorescence-switching components in modern microscopy applications.[1] These blue light-sensing chromoproteins exist as soluble but also as membrane-attached proteins and therefore provide a much wider range of light-activated functions than ChRs. However, their absorption maxima exclusively reside in the blue light region, despite many attempts to shift the activation light color by mutagenesis.

This review on the spectroscopic and physiological functions of phytochromes (Phys) and related bilin-binding proteins and their applications in optogenetics highlights their inherent properties that distinguish them from most other photoreceptors. The information presented here definitely cannot cover all details of structure, functions, and physiological aspects of phytochromes. For selected areas of phytochrome research, e.g., phylogeny,[9−11] domain composition, and development of phytochromes and phytochrome chromophore biosynthesis,[10] and for the ever-expanding field of CBCRs providing new mechanisms of chromophore modification and color-tuning,[12] the reader is referred to more detailed and expert literature.[13,14]

We provide a comprehensive overview over those specialized applications that rely on bilin-binding photoreceptors, excellently complemented by the recent review of Losi, Möglich, and Gardner on blue light-sensing photoreceptors,[1] completing the description of background and applications of soluble, red and blue light-sensitive photoreceptors.

There is common agreement in the community to abbreviate "phytochrome(s)" as "Phy(s)". In contrast, later discovered Phys spread across various organisms generated a variety of different abbreviations (and classifications of the individual members of Phy subfamilies). We here abstract Phys of bacterial origin as "bPhys", which shall include "bacteriochromes", "bacteriophytochromes", or "bacterial phytochromes" (all three names refer to the same type of phytochromes carrying biliverdin IXα-BV as chromophore) and also "bathychromes". An exception to this group constitutes cases where selected bacterial phytochromes were given individual names, such as BphP1.

BV as chromophore is also employed by classes of eukaryotic organisms; here we mention fungi and diatoms (section 2.1.4), and we also add here various groups of (micro)algae to this section.

A particular challenge remains for the classification of phytochromes with unique domain composition, or definitions taking chromophore identities as classification criterium. For Phys from some species, both ordering systems do not concur and request exceptions from the classification rules. In this review, we define a classification system based on the domain

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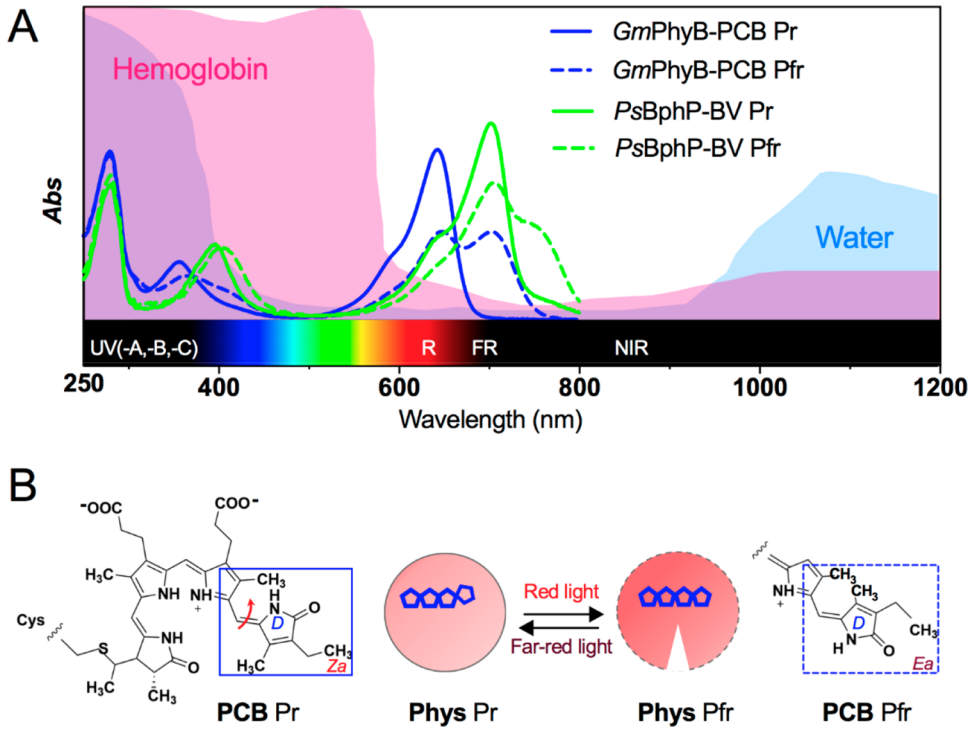

Figure 1. (A) Transparent window in the long wavelength range, relevant for optogenetics applications in animal cells. The strong absorption of hemoglobin (pink, short wavelength edge) and the ingrowing absorption of water (light blue, long wavelength edge) determine the boundaries of the transparent window. The overlay of both absorptions is shown in purple (generation of this figure by combining six absorption spectra was inspired by a figure by A. Fuchtenbush, V. Kriesel, and P. Rosen, (Eds.) "Laser Field Therapy"). Note that the high absorption of the heme group in the short wavelength range (around 400 nm) is due to the high extinction coefficient of the Soret band. The absorption spectra of a PCB-binding plant- (*Gm, Glycine max*) and a BV-binding bacterial phytochrome (*Ps, Pseudomonas syringae pv. tomato*) are shown in blue and green, respectively. The spectra of *Gm* were recorded using a recombinant, PCB-carrying variant, composed of only the PAS-GAF-PHY domains. The native *Gm* protein likely carries phytochromobilin as chromophore. Solid and dashed lines indicate Pr and Pfr states, respectively. (B) Photoisomerization of a protein-bound chromophore (here: PCB) leads to conformational changes of the protein and further affects downstream functions. In canonical Phys, the holoprotein is formed in Pr, where the chromophore adopts a *Z,Z,Z,s,s,a* geometry. The photoproduct is a far-red-absorbing Pfr state with only one double bond photoisomerized (*Z,Z,E,s,s,a*); the isomerization is indicated by a red arrow. A general chemical structure is shown here to highlight the photochemical changes not indicating the stereochemistry at positions 2, 3, and 3′. The stereochemistry at these three positions, as it is determined through the covalent binding of the chromophore, is detailed in Figure 2C.

composition of the photosensory unit that yields three major groups, some of which divided into subgroups.

Our definition follows the history in phytochrome research and takes (land) plant Phys as anchor group. For these proteins, e.g., the phytochromes from *A. sativa*, the CBD (chromophore binding domain) was defined as being composed of PAS-GAF-PHY (PAS, Per-Arnt-Sim; GAF, cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA; PHY, a protein domain characteristic of phytochromes). This three-domain architecture was found as essential to maintain the spectroscopic and kinetic properties of phytochromes, and it serves here as the criterion to classify all Phys posssessing that CBD composition as canonical (group 1). This then calls for several subclasses to separate the *land plant* Phys from their *cyanobacterial* "orthologs" (Cph1, CphA), and to separate these from the "other" *bacterial* Phys = bPhys (e.g., *Deinococcus radiodurans*), from the *fungal* Phys, and from the *algal* families, and also from the cryptophytes (this latter classification follows the phylogeny in Duanmu et al).[11] Also to be mentioned as a separate group are Phys originating from diatoms. The bPhys (wherever identified) fall into two subclasses, the "normal" (= Pr → Pfr) and the bathy-bPhys (= Pfr → Pr). Recently, a new group of cyanobacterial PAS-GAF-PHY-composed proteins from, e.g., *Nostoc punctiforme* ATCC29133 (the first one in this group) were reported to carry a second cysteine capable of modifying the PCB chromophore by attachment to position 10 (see Figure 2).[15] This enzymatic bilin conversion resembles the reactivity found in CBCRs and generates remarkably blue-shifted absorption bands. We thus split the subgroup of canonical, cyanobacterial phytochromes into one represented by Cph1 (and orthologs) and a second one named TCCP (tandem cysteine cyanobacterial Phys).

To be distinguished from the canonical Phys are the PAS-less Phys such as Cph2, All2699 from *Anabaena* PCC7120, or the thermostable Phys from *Synechococcus* (group 2).[16] The third group would then be the CBCRs, again with several subgroups, e.g., the red/green switching ones (in either direction) or those proteins carrying the "second" cysteine modifying the chemical structure of the chromophore. The many examples of novel phytochromes with unexpected variations in their domain structure and employment of chromophores that do not concur with the commonly found representatives in that subclass are exceptions from the rule and will be indicated if necessary.

Phytochromes are involved in regulating a variety of biological functions.[10] Their photobiological properties make them attractive candidates for developing optogenetic applications: phytochromes are soluble proteins, they absorb in the red and far-red regions of the visible light spectrum, and the absorption

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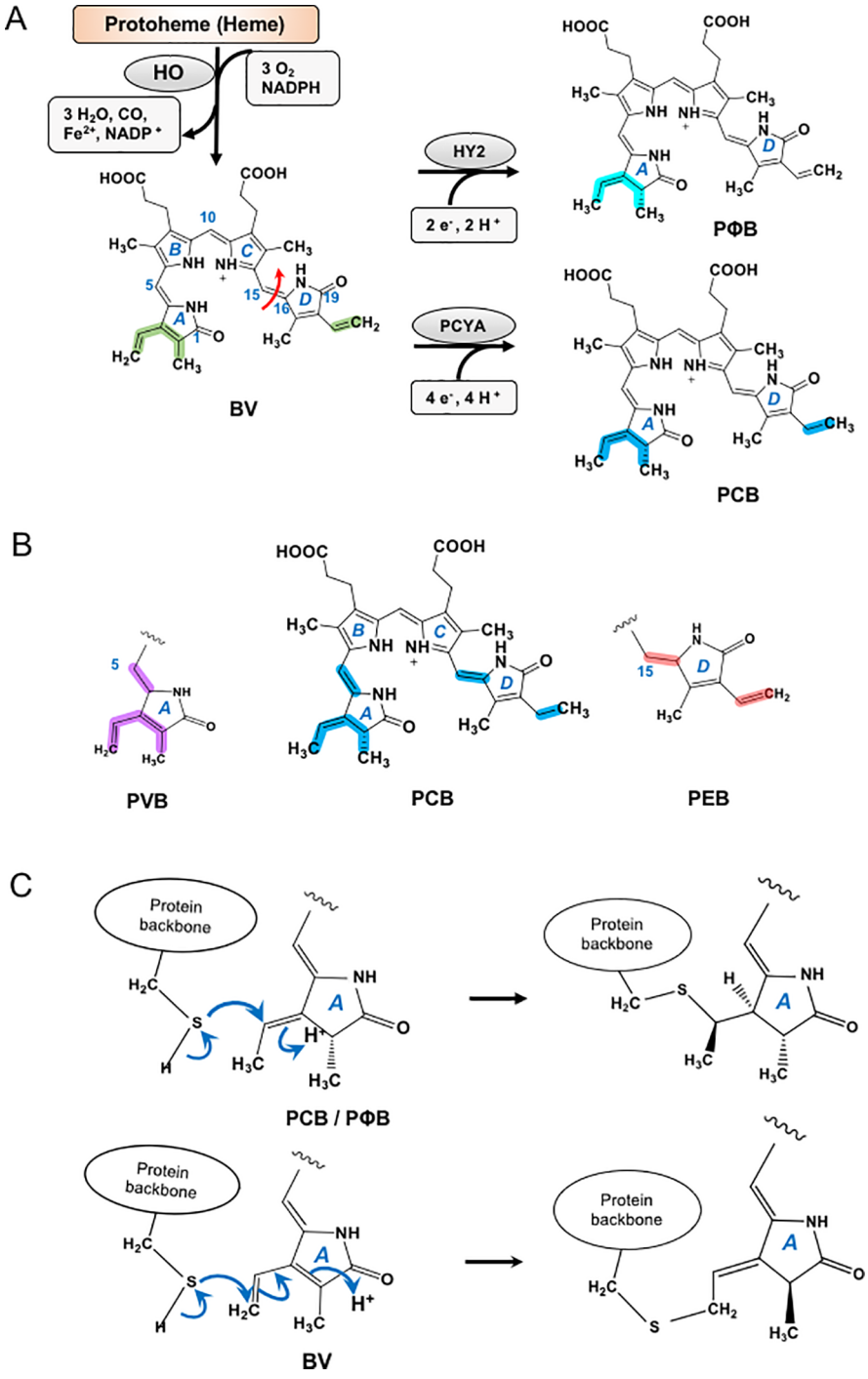

Figure 2. Generation, electronic rearrangement, and binding mechanism of bilin chromophores to apoproteins. (A) Left: Generation of biliverdin IXα (BV) from protoheme by a heme oxygenase (HO) and NADPH (converted into $NADP^+$); reaction products are water (H_2O), Fe^{2+}, and CO. Right: Generation of phytochromobilin (PΦB) and phycocyanobilin (PCB) from BV. PΦB is generated in plants by HY2, a ferredoxin-dependent bilin reductase (FDBR) by addition of $2e^-$, $2H^+$. PCB is generated directly (in cyanobacteria) in a concerted $4e^-$, $4H^+$ step by the ferredoxin-dependent enzyme PcyA. Generation of PCB from PΦB (a concerted $2e^-$, $2H^+$ step) in plants might exist but has not yet been identified; however, in some streptophyte algae the HY2 enzyme generates PCB that is then incorporated as chromophore into the apo-Phy. The photoisomerization of the C15−C16 double bond (interconversion Pr ⇌ *P*fr) is indicated by a red arrow in the biliverdin structure. In all panels, the bilins are presented in a positively charged state (charge on the tetrapyrrole moiety), and the propionate side chains are shown undissociated. Upon assembly into the apoprotein, the propionate side chains dissociate, as shown in Figure 1. (B) Electronic rearrangement in PCB yields either phycoviolobilin (PVB, left panel, conversion of the double bond between C4 and C5 into a single bond) or phycoerythrobilin (PEB, right panel, double bond between C15 and C16 is converted into a single bond). PEB is formed in cyanobacteria directly from BV by the enzyme couple PebA/B or by PebS in a concerted $4e^-$, $4H^+$ step similar to that described in panel A for PCB formation in bacteria. If both C4−C5 and C15−C16 double bonds are converted into single bonds, the product is called phycourobilin (PUB, structure not shown). (C) Top, covalent attachment of PCB or PΦB to a cysteine of the apo-phytochrome. This reaction yields, besides the originally present chiral center at C2, two new chiral centers at the bilin A-ring (*3R, 3′R*). For clarity, we show only the backward directed hydrogen atom as indication of the chirality. Bottom, covalent attachment of BV to a cysteine residue of the apo-bacterial phytochrome. The resulting electronic arrangement resembles a 3′′-attached PΦB. In this chemical form, the same nucleophilic attack of a *second* cysteine (as present in

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Figure 2. continued

some BV-binding Phys) can occur, shifting the π-electrons of the 3−3′ double bond to position 3 to capture again a proton. These double-Cys substituted chromophores then show absorption spectra akin to covalently bound PΦB. If in some algal Phys and CBCRs the bound chromophore is 18′,18″-dihydrobiliverdin, the structure of the chromophore from double Cys attachment would then be a covalently bound, doubly-Cys (3′-, 3″-) substituted PCB. The finding of a double-Cys substitution is discussed in the text. Note that in some CBCR-GAF domains and in modified proteins, bond formation between position 3′ of BV and the cysteine residue has been reported.[18] Alternative electronic rearrangements in bound BV chromophores have also been proposed, such that nucleophilic attack of the sulfur atom to position 3′′ shifts the electron density to carbon 3′, thereby allowing binding of a proton.[20] This possibility simply removes the vinyl double bond and leaves the electronic structure of the BV molecule in the four rings unchanged.

maxima of the biosynthetically formed state, here furthermore called the parental state, and photoproduct differ significantly (Figure 1). The early identified Phys with two interchangeable forms were considered prototypal, and the two forms were named Pr (r, red-absorbing) and Pfr (fr, far-red-absorbing). This "historical" definition would exclude eukaryotic algal phytochromes, tandem cysteine cyanobacterial phytochromes, and phytochromes from other eukaryotes such as fungi and diatoms. As we explain in the following, Phy- and Phy-related proteins showing large variations in the absorption maxima of their two forms were found, requesting this dogma to be revised.

Phytochromes inherently respond to dual-wavelength exposure allowing rapid and repetitive ON- and OFF-switching or fine regulation of the activation strength by light. Especially this reversible, light-driven photochemistry, discussed below (section 2.3.1), is unique within the ensemble of biological photoreceptors employed in optogenetic applications. Of note, the absorption maxima and their (moderate) fluorescence fall into the "transparent" window, a wavelength range of the visual spectrum between ca. 700 and 900 nm that is relatively free of cell-inherent absorbers. This spectral range thus allows better focusing and penetration into tissues as compared to shorter wavelengths. In this aspect, these absorption and emission ranges raise particular interest for optogenetic applications in animals, located in between the strong absorbances of heme groups on the short wavelength side, and the growing absorption of water on the long wavelength side. This spectral region is also called the "therapeutic window" in, e.g., medical applications such as photodynamic therapy (Figure 1).

As said above, phytochromes, Phy-related proteins, and other bilin-binding receptors have been considered only for a relatively short time as potentially useful tools for optogenetics because they were long seen associated with several restricting properties:

(i) Canonical Phys, for which most of the information for this photoreceptor class has been collected, require a relatively large portion of their protein moiety to maintain their spectral properties (phytochromes are named as "canonical" members, if their photosensory unit comprises three domains, a PAS-, a GAF-, and a PHY-domain; for details see text directly preceding Figure 1 and section 2.1).

(ii) In relation to flavin-based tools that function through a light-regulated enzyme activity, no comparable signaling element could be identified in canonical plant-derived Phys.

(iii) The chromophores of all phytochromes are bilins that, in contrast to retinal and flavins, are not present in every cell and hence require additional engineering efforts to make them available.

(iv) The absorbance maxima of the canonical and bacterial Phy-subfamily (BphP, "bacterio-phytochrome photoreceptor", here subsumized as bPhy) are found invariably in the red/far-red range of the visible spectrum—in principle a wavelength range well suited for applications. However, mutagenesis approaches mainly failed in shifting the activation range for generating orthogonal receptors. Only recently, cyanobacteriochromes (CBCRs, see section 2.1.3) were found as a large subgroup of the phytochrome photoreceptor family showing a large variability in the absorption maxima of their parental and photoproduct states, making CBCRs useful tools for optogenetic applications.

These apparent disadvantages likely caused an unpopularity of phytochromes in the optogenetics field compared to retinal- or flavin-binding photoreceptors. We therefore focus in detail on the functions of these photoreceptors, the relationship between the various phytochrome-related subfamilies, the generation of their bilin chromophore, and their photochemical properties. We will not dwell on the physiological role of phytochromes except for examples where complexes to other proteins are formed as potential lead structures for optogenetic tool development.

1.1. Light-Key Player in Photoreceptor Function: Light-Activated Chromophore

Common to all photoreceptors is the "photo switch", i.e., the protein-bound chromophore. Ideally, it should also be available or generated in the target cell, in a bacterium, plant, or animal, as any means of external application (injection, infusion, etc.) would impede the advantageous property of photoreceptors: their noninvasive activation. In addition, the chromophore incorporation into a photoreceptor apoprotein—preferentially concurrent with protein biosynthesis—should be autocatalytic, not necessitating further "helper" or chaperone proteins.

Unexpectedly, retinal serving as chromophore in the ChRs, halorhodopsin, and bacteriorhodopsin, has been found in animal cells making an external injection obsolete. The same holds true for the flavin-based photoreceptors, as flavins (as FAD, flavin-adenin-dinucleotide; FMN, flavin-mono-nucleotide; or riboflavin, i.e., Vitamin B2) are essential and ubiquitous components in redox-driven physiological processes redox couple FAD/$FADH_2$, exploited in the Krebs cycle.

Most applications based on bilin-binding proteins, however, require engineering of the chromophore synthesis in the target cell or organism from other metabolic pathways. Phycocyanobilin (PCB), phytochromobilin (PΦB), or biliverdin (BV) (Figure 2), the chromophores in the bilin-based photoreceptors, are present in plants and photosynthetic bacteria, but not necessarily in sufficient amounts in living animal cells, archaea, fungi, or eubacteria. Fortunately, chromophore biosynthesis for applications of biliproteins relies on a biosynthetic pathway that is well preserved across organisms for the generation (and degradation) of porphyrinic compounds, e.g., the heme group, a prominent ligand in myoglobin, hemoglobin, cytochromes, or related proteins yielding biliverdin as the first degradation

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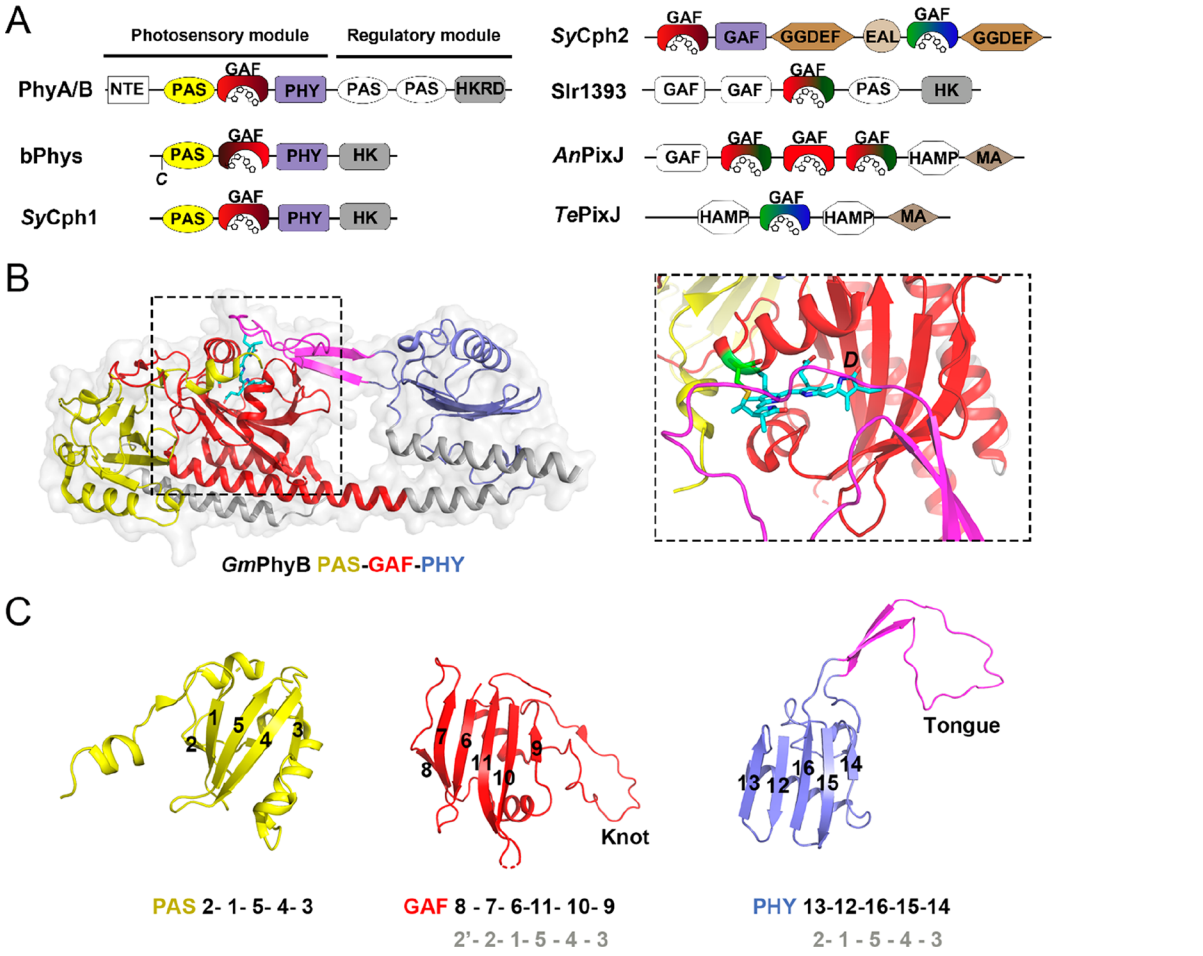

Figure 3. Phytochrome structure. (A) Domain organization of phytochromes. Left, canonical plant phytochromes and bacterial phytochromes (bPhys) comprise an N-terminal photosensory core module (PAS-GAF-PHY) and a C-terminal regulatory module. In *Sy*Cph1 and in many BV-binding bPhys, the regulatory module is a histidine kinase. The domain alignment shows the cyanobacterial phytochrome Cph1 from *Synechocystis* PCC6803. Right, *Sy*Cph2, equally identified in *Synechocystis* is called a PAS-less (also knotless) phytochrome. *Sy*Cph2 has no homology to histidine kinases but instead carries two chromophores and c-diguanylyl cyclases (GGDEF) and a phosphodiesterase (EAL). Cyanobacteriochromes (CBCRs) are phytochrome-related photoreceptor proteins found only in cyanobacteria: the chromophore binding domains in these CBCRs are GAF3 from Slr1393 of *Synechocystis sp*. PCC6803; GAF2, -3, and -4 from *Nostocaceae An*PixJ; *Te*PixJ from *Thermosynechococcus elongatus*. In most proteins, the chromophore binding site is a cysteine residue residing in the GAF domain. Some CBCRs such as *Te*PixJ and the third domain of *Sy*Cph2 have a second cysteine residue in the DXCF motif of the GAF domain (see section 2.1.3 for more details). Only in bPhys, the cysteine is in the N terminal extension preceding the PAS domain (highlighted by a capital 'C' in black). (B) Three-dimensional structure of a plant phytochrome. Left, illustration of PhyB from *Glycine max* (Pr state, PDB: 6TL4), a plant phytochrome comprising a PAS domain (yellow), a bilin-binding GAF domain (red), and a PHY domain (slate). Note that the N-terminal extension (NTE) is missing in the structure. The PAS-GAF region of *G. max* shows high similarity to that of the first PAS-GAF structure of *Arabidopsis thaliana* PhyB (PDB: 4OUR, with RMS Cα differences of 1.3 and 2.0 Å for the A and B molecules, respectively). Right, a detailed view of the PCB-binding pocket in *Gm*PhyB. The D-ring of the PCB chromophore is indicated. In both panels, PCB is shown in cyan. (C) Comparison of the core regions of the PAS-, GAF-, and PHY domains identifying the structural relationship between these three domains. The numbering of the β-sheet elements (1−16) is preserved in all three domains; they all show an antiparallel β-sheet arrangement with strands in the spatial order of 2-1-5-4-3 and a variable connector between strands 2 and 3 that contains an α-helix. The GAF and PHY domains contain functional insertions in their core regions but at different topologic locations. The "knot" and "tongue" insertions are indicated.

product and then the other bilin chromophores (see section 2.2).

Common to all three phytochrome chromophores is the type of covalent attachment. They are bound to the protein via a nucleophilic attack of a cysteine residue to the ethylidene- (3′-position of PΦB or PCB) or vinyl substituent (3″-position of BV) of ring A (see Figures 2 and 3 and section 2.2). Of note, some BV-binding phytochromes (bPhys and CBCRs) engage a second cysteine residue for covalent attachment.[17] It should be mentioned, however, that the chemical structure of the resulting chromophore presented in this publication seems to contradict the chemical reactivity of protein bound bilins. From our understanding, the chemical modification that the 3″-bound BV experiences in a first step is a concerted process consisting of a rearrangement of the A-ring double bond (between C-atoms 2 and 3) to bind a proton to position 2, thereby changing C2 hybridization from sp^2 into sp^3 and rendering position 3 (partially) positively charged and allowing a shift of the vinyl double bond to positions 3 and 3′. At this state, the 3″-bound BV chromophore resembles a 3″-bound PCB. This chemical structure can then be attacked by a second, close-by cysteine present in some bPhy and CBCRs, resulting in a second covalent cysteine attachment to position 3′, concomitant with a shift of the double bond electrons to position 3, to which then a proton

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is attached. As a consequence, these BV-binding bPhys and CBCRs exhibit blue-shifted absorption maxima resembling those of PCB-binding proteins.

Attempts to find novel chromoproteins with further red-shifted absorption maxima led to search BV-binding CBCRs (in analogy to the situation for PCB-binding plant Phys compared to BV-binding bPhys).[18] Interestingly, a GAF domain from the CBCR JSC1 (g4) could be identified that carries the intermediate in the BV-to-PCB conversion process, 18′,18′′-dihydrobiliverdin.[18] The formation of this BV-intermediate was explained by the coexistence of two reducing enzmyes, generating the dihydro-BV derivative and PCB, potentially as a way to shift the absorption to longer wavelengths.[19] It could be shown that also BV can serve as chromophore in this CBCR-GAF domain. The preference for BV or its dihydro-derivative is explained by the architecture of the chromophore binding site that prohibits stable incorporation of PΦB or PCB. Crystal structures of these BV- or dihydro-BV-loaded proteins revealed that the chromophores are bound equally as PΦB or PCB via their 3′ position.

2. BILIN-BINDING, RED-LIGHT-SENSITIVE PHOTORECEPTORS

2.1. Classes of Bilin-Binding Photoreceptors

Considering the functional principle of proteins utilizing covalently bound bilins as chromophores, two major classes can be distinguished: on the one hand, the light-harvesting antenna pigments of cyanobacteria, in which thousands of bilin-binding proteins are organized in phycobilisomes that gather the incident light and funnel it to the reaction center for photosynthetic purposes.[21] These proteins function as gateways and transmitters of incident light, and as such they do not exhibit a distinct photochemical reactivity except for an outstandingly high fluorescence under certain experimental conditions, e.g., when the energy transfer pathway is blocked. On the other hand, one finds the photochromic phytochromes and phytochrome-related proteins that serve as light sensors to generate and mediate physiological signals to the organism. It will be these latter ones that present the main subject of this review with focus on their potential as optogenetic tools, whereas the former ones will only be touched as potential novel fluorescence tags due to their long wavelength absorption and emission and their outstanding brightness (see section 4.2).[22,23] For a long time, phytochromes were not considered useful fluorescence tags. Pioneering studies determined an extremely low fluorescence quantum yield for the plant-derived Phys. In fact, emission could be measured only for the Pr forms, already with a nearly negligible quantum yield ($\Phi_{fl} < 3-4.5 \times 10^{-4}$),[24] whereas no fluorescence at all could be detected for the Pfr forms. Still moderate but clearly larger quantum yields were determined for the prokaryotic phytochromes, as well the cyanobacterial Cph1-type members and other bacterial BV-binding Phys (Φ_{fl} about 0.07−0.1) that already allowed employment as fluorescent tags. Still few applications as fluorescence tags were reported so far for CBCRs. For a recent survey on applications exploiting the fluorescence of various phytochromes, see Chernov et al.;[25] here we only add an update to this excellent compendium.

With only a few exceptions, all phytochromes and related photoreceptors are distinguished from other photoreceptors by their long wavelength absorption maxima in the region from 600 to 800 nm and their photochromicity, i.e., the absorption shift between the parental and the signaling state. This shift of the absorption maxima in some proteins may account for more than 100 nm. Both states show photochemical activity, and the wavelength difference and the extent of conversion, i.e., the quantum yield (*vide infra*, section 2.2.2) may vary, but for most Phys appropriate illumination conditions can provoke a significant conversion between both states.

The most frequently found structural arrangement of the phytochrome photosensory unit (Figure 3) comprises a PAS, a GAF, and a PHY domain. The PHY domain shares high structural similarity to a GAF domain except for the major difference of the antiparallel β-sheet extension—the "tongue"—that unfolds and refolds into a short helical motif in the Pfr state. Comparing GAF or PHY domains to PAS domains, it is apparent that all three domains share the overall three-dimensional fold; that is, all three domains exhibit antiparallel β-sheets architecture that is connected by α-helices (Figure 3C). This similarity in number and arrangement of secondary structural elements seems to point to a common structural ancestor for the three domains and brings the PAS-GAF-PHY arrangement in "canonical" phytochromes close to an oligo-GAF arrangement found in CBCRs.[26] It should be kept in mind, however, that for canonical phytochromes only a complete ensemble of these three domains (PAS-GAF-PHY, often named PGP; in some other applications this fragment is called CBD—chromophore binding domain) maintains the full photochemical reactivity contrasting the mono-GAF functionality of CBCRs or Cph2-type proteins.

Variations in the domain composition exist across phytochrome-related proteins, sometimes lacking the N-terminal PAS domain or containing multiple GAF domains (see following sections, e.g., section 2.1.3 and Figure 3). Here, one or more of these GAF domains, even if expressed as an individual protein, may have the capability to bind a bilin chromophore. The chromophore is always located in the GAF domain, even if covalent binding takes place to an amino acid in the PAS domain, as is the case when biliverdin-IXα (BV) is used as chromophore (section 2.1.2). From the historical background, it was considered that all phytochromes and their variants bind their bilin chromophores covalently. Here, one has to discriminate between phytochromes (and −variants) extracted from their original organism and recombinant proteins produced in *E. coli* or yeast. Such recombinant proteins, modified at their chromophore-binding cysteine residue by site-directed mutagenesis, were shown to simply embed the chromophore, to undergo a modified photochemistry, and to exhibit altered absorption maxima.[27] Oka et al. even succeeded in reinserting the gene of a double-mutated PhyB into *A. thaliana* (impeding cysteine-mediated chromophore binding and generating Y276H for constitutive signaling). These transgenic plants showed responses to light treatments which resembled those of the wild type; however, the responses were weaker.[28] To the best of our knowledge, no phytochromes extracted from their genuine hosts could be found carrying their chromophore noncovalently. This includes endogenous and overexpression studies.[29] Chromophore biosynthesis in plants takes place in the plastids, whereas protein biosynthesis occurs through ribosomes in the cytosol requiring a so far unidentified transport process of the bilin. The combination of chromophore and apoprotein on the other hand is an autocatalytic process, forming a covalent bond between the 3′ or 3′′ position of the bilin unit and a cysteine side chain (see Figures 2 and 3). This bilin lyase activity is intrinsic to all phytochromes and related proteins and currently exploited in applications by a "two

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plasmid approach" independently encoding the phytochrome moiety and the enzymes that break down heme groups and convert them into the required bilin chromophore. This strategy enables chromophore assembly already during protein production in the expression host (see also section 3).

Although in general all phytochrome chromophores originate from heme groups, the extent of structural variation identified several classes of chromophores, concomitant with variations in their absorption maxima that are characteristic to the various phytochrome subfamilies. The biosynthesis of the phytochrome chromophore follows the general pathway of heme degradation, based on the function of a heme oxygenase (HO) under employment of oxygen and NADPH as redox component (for details see Figure 2 and section 2.2). Irrespective of the chromophore variations, the conformation/configuration of the protein-bound chromophore is equal in all phytochromes: the holoprotein is formed in Pr, where the chromophore adopts a *Z,Z,Z,s,s,a* geometry. The photoproduct, Pfr, shows only one configurational change: isomerization of one double bond (between rings C and D; for details see section 2.3 and Figures 1 and 2) (*Z,Z,E,s,s,a*). A subfamily of bPhys, named bathy-phytochromes, was found in which the parental state configuration and the photochemistry are opposed to most other members.[30] Accordingly, as bathy-phytochromes adopt the Pfr state as parental form, their chromophore is found as the *Z,Z,E,s,s,a* isomer. Interestingly, these "bathy"-bPhys assemble in their Pr state and convert in the dark into the thermally stable Pfr form (this is consistent with our chosen definition of the parental state).[30] Their long wavelength "dark" state (in OFF-mode) is even more attractive compared to other Phys, as the activation light is still further away from any other photoreceptors allowing a clean activation.

2.1.1. Canonical Phytochromes. Ever since their initial description in 1959, phytochromes were understood as the red/far-red-sensitive photoreceptors in plants.[31,32] Plant phytochromes are essential at virtually all stages of plant development. They govern a plethora of physiological functions, and an estimation yields that about 20% of the entire plant genome responds to light signals mediated through phytochromes.[33,34] Phytochromes from flowering plants represented the blueprint for the definition of "canonical" Phys, and for a long time they have been the only identified representatives of this protein family with their best studied protagonists from *Avena sativa* (oat), *Pisum sativum* (pea), and *Solanum tuberosum* (potato). Initial studies relied on extracted plant material. Following work on phytochromes increasingly employed heterologously expressed recombinant protein.[35,36] As such, phytochromes of flowering plants represent a small protein family. Within the *A. sativa* genome, three phytochrome-encoding genes (PHYA–PHYC) were identified, equally as in rice (another monocot). Most frequent studies use *Arabidopsis thaliana* (a dicot) that encompasses five PHY-encoding genes (PHYA – PHYE). Some reports mention six Phy-encoding genes in some plants (see Table S1 in Zhang et al.[37]), as found e.g. in *Zea mays*. There are also gene variants denoted as A1, A2, B1, B2, C1, and C2, clearly identified as closely related gene duplications. Common to all these chromoproteins from angiosperms and gymnosperms is the size of their photosensory unit (comprising approximately 600 amino acids), often also called the chromophore binding domain (CBD). The C-terminal sequence part included, these phytochromes have a total length of more than 1,100 amino acids. Phytochromes from cryptogams, for example, those from mosses, ferns, and algae, may vary in their number of amino acids but still exhibit the PAS-GAF-PHY architecture of their CBD.[38] Exclusively the phytochromes from land plants and Archaeplastida lineages (including Streptophytes, Charophyte, and Prasinophyte) and one Cryptophyte carry two PAS domains in a tandem arrangement between the PHY domain and the C-terminal portion of the protein. This additional structural element is not found in bacterial Phys.[9]

The identification of a phytochrome(-like) protein-encoding gene in the genome of the cyanobacterium *Synechocystis* PCC6803 and the demonstration of phytochrome-like spectral properties of the gene product after heterologous expression (cyanobacterial phytochrome #1, Cph1) opened the gate to a much greater variety of phytochromes.[39,40] Cph1 still exhibits the common three-domain PAS-GAF-PHY arrangement in its N-terminal part with a clearly identifiable cysteine residue in a highly conserved peptide sequence of its GAF domain. This residue is prone to bind the chromophore. Cph1 and other cyanobacterial phytochromes (see section 2.1.2) can be distinguished from their canonical plant relatives. Three features make the major differences between plant- and cyanobacteria-derived "canonical" phytochromes: (i) the chromophore remains a bilin derivative, but with higher degree of reduction (PCB, phycocyanobilin) (Figures 2 and 3); (ii) plant phytochromes carry a tandem repeat of two additional PAS domains between the light-sensing PAS-GAF-PHY domain and the C-terminal part; this motif is absent in all prokaryotic phytochromes; and (iii) the C-terminal part of many cyanobacterial phytochromes shows signatures of a histidine kinase (HK) enzyme function[39,41] that is absent in the plant-derived phytochromes.[42]

Of note, signaling in several microalgae could be ascribed to the function of a C-terminally located histidine kinase (HK). The above cited publication (Duanmu et al.)[11] identified light-induced, HK-mediated gene expression control in the picoprasinophyte *Micromonas pusilla* as a representative for several prasinophyte lineages. The identification of HK-mediated signaling in the prasinophytes is of importance, as these microalgae are considered the ancestor of green algae and land plants. A comparison with several members of the streptophyte lineage points to a loss of this signaling pathway.

Into the group of eukaryotic Phys fall also those from the diatoms. These proteins so far have not been given much attention besides their identification in the genomes of various diatoms, the type of chromophore bound, and their spectral characterization, which are similar to bPhys.[43]

2.1.2. Bacterial Phytochromes. Driven by the identification of the Cph1-encoding gene in the genome of *Synechocystis* PCC6803, "genome mining" became the most important tool for the identification of novel phytochromes or phytochrome-related proteins. Robust sequence motifs, identification of amino acids instrumental for phytochrome function, and the PAS-GAF-PHY-HK domain arrangement identified a still growing number of phytochromes in prokaryotes. Genes encoding phytochrome-related sequences could be found neither in an archaeal nor in the genome of the eukaryotic alga *Chlamydomonas reinhardtii*;[38] in this context please see a short description on the existence of phytochromes in other algae (section 2.1.4). Empirically, the sequence motif encoding the residues around the chromophore binding cysteine (Cys323 in *Gm*PhyA, Figure S1) served for probing genomes for the presence of genes encoding phytochromes. However, considering the historical development of phytochrome research, this approach failed to identify Phy-encoding genes in the genomes

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of nonphotosynthetic bacteria and later-on in those from fungi. These Phys bind biliverdin IXα (BV) to a cysteine located in a short sequence preceding the N-terminal PAS domain, and the chromophore-binding ("plant-typical") cysteine in the GAF domain is replaced by another amino acid (Figure S1).[44,45] Yet, many of the other sequence motifs and in particular the domain arrangement of the photosensory module could be mapped, allowing identification of an ever-growing number of bacterial phytochromes.

Phylogenetic studies revealed that BV likely represents the earliest bilin chromophore utilized by photoreceptive chromoproteins of this class. The bilin-binding cysteine apparently evolved several times independently in Phys from various clades and lineages. A recent, excellently composed review provides detailed discussions around the topic of phytochrome development and phylogeny.[10]

As there is no rule without exception, a discrimination between photosynthetic and nonphotosynthetic bacteria failed. Nature always shows the limits of any classification, just considering the case of *Rhodobacter sphaeroides*. This bacterium commonly grows in a vegetative manner but carries genes allowing it to switch to a photosynthetic lifestyle. This conversion is controlled by a protein named AppA, composed of a blue light- (LOV-based) and a heme-based oxygen sensor domain called SCHIC (sensor containing heme instead of cobalamin).[46] Thus, under conditions of low oxygen pressure, *R. sphaeroides* initiates expression of photosynthetic genes to use light as energy source.

A curiosity emerged among BV-binding bPhys. Several bPhys were identified that form biosynthetically in the Pr form and immediately convert into the Pfr form to adapt this form as the thermally stable parental state with λ_{max} around 750 nm. The photoproduct of these "bathy-phytochromes" is the Pr form with absorbance maxima around 700 nm that slowly reconverts in a thermally driven process into the Pfr state. The best studied example is *Pa*BphP, the bacterial phytochrome from *Pseudomonas aeruginosa*.[30] Phytochromes with hypsochromically shifted photoproducts were considered initially as curiosities, as they apparently violated the historical dogma of phytochromes, the physiological Pr-OFF, and Pfr-ON response. The finding of an increasing number of Phys with hypsochromic photoproduct maxima, also identified in CBCRs (section 2.1.3), then became simply a variation in phytochrome photochemistry. It has to be noted, however, that the mechanisms and intermolecular interactions between bilin and protein that dictate the position of the absorption band and the direction of the photochemistry are only marginally understood.

These long-to-short wavelengths switching phytochromes and CBCRs offer an additional advantage in optogenetic applications (see Section 4.3), as their thermally stable parental ("dark") state is extremely red-shifted and far away from the absorption bands of any other photoreceptors.

All Phy proteins that carry the discussed PAS-GAF-PHY domain exhibit another feature that only became apparent from the first crystal structure of a phytochrome PAS-GAF fragment. Crystals of the PAS-GAF fragment from the bacterium *D. radiodurans* identified a figure-eight-knot formed within the N-terminal extension slipping through a loop of the GAF domain.[47]

In contrast to plant phytochromes, the C-terminal regions of the bacterial phytochromes often harbor an enzymatic activity that accomplishes the signaling function. Most common are histidine kinase (HK) domains interacting with their genuine response regulator (RR) in a mode of a classical two-component signaling system. Frequently found are one or both enzymatic functions that regulate the intracellular concentration of c-di-GMP, a classical prokaryotic second messenger.[48] For several bPhys it could be demonstrated that the C-terminally located enzyme activity is strongly involved in the regulation of the bacterial lifestsyle. Activation of individual genes or a gene cluster that completely changes the lifestyle of some bacteria has been demonstrated by applying a Phy-specific illumination protocol or, alternatively, by disrupting the Phy-encoding genes. To mention only few selected examples, carotene biosynthesis was found to be light-controlled by a bPhy from *D. radiodurans*. In *Agrobacterium fabrum*, infectivity is controlled by the two identified phytochromes. In the well-studied protagonist *Pseudomonas syringae*, Phys regulate changes in motility and infectivity upon illumination; however, disruption of the Phy-genes yields a phenotype already in the dark.[49] Attention is given in this context to the Myxococcales, a group of bacteria that can convert from a unicellular into a multicellular lifestyle resulting in the formation of fruiting bodies as a more complex form of living. For one bacterium from this order, *Stigmatella aurantiaca*, phytochromes were shown to be master regulators of their lifestyle. Detailed structural characterization and spectroscopy of the two phytochromes from this bacterium disclosed the role of phytochromes in this dramatic change of physiological behavior.[50] More examples of the relation between red/far-red illumination in nonphotosynthetic bacteria can be found in a review on the structure and function of bPhys.[51]

2.1.3. Cyanobacteriochromes. A survey for multidomain proteins in the cyanobacteria *Anabaena* sp. PCC7120 yielded a multitude of proteins that carry a GAF domain. Their sequence similarity and their deduced function allowed grouping them into subfamilies.[52] Among these, one particular set of GAF domains showed sequences akin to the GAF domains of canonical Phys. More detailed analysis identified these GAF domains indeed as capable of binding a bilin and yielding spectral features known for phytochromes and related proteins. These proteins were termed cyanobacteriochromes (CBCRs). They show a remarkable chemical reactivity with respect to the modification of their covalently bound chromophore. Though most work on their spectral, kinetic, and signaling functions was performed with recombinant proteins, it seems that in their native form all CBCRs bind PCB as chromophore, but in some cases the bound PCB is subjected to structural modifications yielding phycoviolobilin (PVB) (Figure 2).[53] Common to all CBCRs is also their photochromicity. Yet, the overall domain structure and the direction of the absorption shift upon generating the photoproduct are highly variable. CBCRs are composed of arrangements of several GAF domains, out of which one or sometimes several show the capability to bind PCB as chromophore. Their C-terminal parts often harbor enzymatic functions such as histidine kinases or nucleotidyl- (adenylyl-, guanylyl-, or cyclic-diguanylyl-) kinases and phosphodiesterases.[38,54]

In fact, the unexpected variability in GAF domain arrangements (instead of the canonical PAS-GAF-PHY motif) obscured the identification of the first bacterial "phytochrome". A gene *rcaE* was identified in the cyanobacterium *Fremyella diplosiphon*, and a knockout experiment pointed to a potential correlation with the chromatic acclimation. From similarity analysis, it had been proposed that *rcaE* encodes a prokaryotic phytochrome (like)-protein.[55] Despite the finding that an *rcaE* deletion mutant lost the capability to adapt its antenna composition,

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initial attempts failed to demonstrate chromophore binding in the heterologously expressed protein,[55] and also the domain architecture prediction did not indicate a PAS-GAF-PHY motif. This was apparently caused by the fact that the RcaE-encoding gene does not have an ATG start codon. Thus, when the recombinant gene was designed, the first ATG was used as a starting point leading to the loss of a larger part of the N-terminal PAS domain and consequently to a misfolded protein. At that time, it was not considered a "phytochrome" (and CBCRs had not been discovered). Reinvestigation of this protein identified it as the "first" CBCR protein.[56] Thereby, although published ahead of Cph1 from *Synechocystis* PCC6803, this latter one was then considered the first bacterial phytochrome.[39,40] Despite sequence similarities to PhyE, resemblance of this protein to ethylene sensors was suggested.

There seem to exist two large groups of CBCRs that can be distinguished by the functional chromophore. CBCRs with a red-absorbing parental state (λ_{max} around 650 nm) and a photoproduct maximum around 540−550 nm are frequently found; thus, they were termed RGS (red-green-switching).[57] Like in the bPhys, CBCRs with both parental states have been identified, i.e., converting red-to-green and green-to-red; it seems, however, that the majority of the RGS-switching CBCRs generate a hypsochromically shifted photoproduct. In contrast, the BV-binding bathy-proteins seem to form the smaller fraction in the bPhys. Though simply exchanging the parental (or dark) state as a green- or a red-absorbing form, both groups of CBCRs seem to be not much related to each other, as phylogenetic studies and structural investigation show.[58−60] There is evidence that these RGS-switching CBCRs might have evolved from the dual-Cys group (discussed in the following paragraph).[60]

The second group of CBCRs, more diverse, but probably equally comprehensive as the RGS-type one, comprises those proteins that sport a second reactive cysteine for chemical modification of the chromophore. The various absorbance ranges caused by the reactivity of this second cysteine allowed clustering of CBCRs into several subfamilies.[61] This differentiation arises from the finding that this second cysteine is located in loops inserted at different positions in the protein and also from the finding that, in some proteins, the cysteine reactivity causes an isomerization of the bound PCB into PVB. In this case, short wavelength absorptions are accomplished via a chemical modification of the chromophore π-electron system. A double bond rearrangement upon cysteine-induced formation of PVB (see section 2.2) reduces the conjugation to expand only over the B−C−D rings of the chromophore. This modification results in shorter wavelength absorption.[53,62] In other dual-Cys proteins, a nucleophilic cysteine attacks the central position C10 of the bilin, causing two electronically separated pyrromethine units. This further modification of the conjugated system yields absorbance maxima in the blue/near UV range of the light spectrum (see Figure 2 and section 2.2).[63] Both conjugation length modifications occur through chemical activity of a second cysteine (besides the canonical chromophore-binding one). As exemplified for these two cases, two "second-cysteine" protein subfamilies could be identified within the CBCR community; one of them carries the reactive cysteine in a highly conserved sequence motif "DXCF", whereas in the other group of proteins (so-called "insert cysteines") the second reactive cysteine is in an additional loop.[64] These cysteines, which reside in loops, usually attack the chromophore at position 10; however, some do not keep the capability to bind to C10 in the 15-*E* isomers of the bilin.[65] The situation with the "second cysteine" is much more complex. Phylogenetic investigation points to a gain-and-lose situation for these extra cysteines.[66] Considering the remarkable variability of CBCRs, reports of additional lineages with surprising properties will likely appear in the future.

Probably triggered by the finding that some cyanobacteria have found an ecological niche and "harvest" far red light through a modified chlorophyll with bathochromic absorption within the "transparent window" (Figure 1),[67] a survey was started aiming to identify CBCRs with absorption in the long wavelength range.[68] Besides screening WT-cyanobacteria, also mutagenic efforts were performed to modify GAF domains of CBCRs to bind BV. In fact, a phylogenetic analysis revealed lineages with long wavelength absorption. Not only BV as chromophore provided a red shift in absorption, but also PEB in an unusual configuration (all-*Z*,*syn*) in CBCRs from *Anabaena cylindrical*. Anacy_2551g3 from *Anabaena cylindrica* PCC7122 is so far the protein with the longest absorption among CBCRs that also shows a most red-shifted fluorescence with λ_{max} = 728 nm and λ_{em} = 740 nm;[68] its fluorescence quantum yield Φ_{fl} = 1.2% is moderate, but this protein might serve as a "lead structure" initiating further improvements reminiscent to bPhys.[69] Other potentially relevant candidates for applications might be proteins such as the dual-Cys CBCR Am1186g2 from *Acaryochloris marina* that has been converted to bind BV as chromophore.[70] A detailed analysis on this representative for an entire lineage revealed that proteins from this group bind BV or its 18′,18″-derivative, keeping an eye on the possibility that these chromophores might allow an extension of the absorption further into the red range of the spectrum.[18] It is important to note that in all cases of chemical modification of those bilins that serve as chromophores, the double bond prone to light-induced isomerization (between rings C and D) remains intact (Figures 1 and 5).

By combining the expression of apo-CBCR-GAF domains with various heme oxygenases (HOs) and ferredoxin-dependent bilin reductases (FDBRs), nearly all imaginable bilins have been incorporated into GAF domains. A very extensive study has been performed with GAF domains from *N. punctiforme* and from *Thermosynechococcus elongates*.[63] Depending on the individual BV-converting enzymes employed, PΦB, PCB, or PEB were formed and covalently incorporated, and as the selected GAF domains have the capability to convert the bound bilins, also "phytoviolobilin" (ΦVB), PVB, and PUB were found as bound chromophores. These latter three derivatives were not present in pure form but together with their origin bilins. It should be mentioned that, similar as in plant Phys, PEB most probably is not present in CBCRs as natural chromophore due to the lack of photoisomerization capability.

Similar work has been reported by Sun et al.,[71] who incorporated PEB and PUB into GAF domains from several CBCRs. Here, the PEB-generating enzyme PebS was used as the BV-modifying enzyme. The aim of these scholars was the generation of fluorescent proteins with a strong orange emission.

The wide variation of absorption maxima of CBCRs spans virtually the entire visual spectrum and beyond. The finding that the chromophore binding capacity and the spectral properties remain intact, even for an isolated chromophore-binding GAF domain expressed as an autonomous protein, makes CBCR-GAF domains most interesting tools for future optogenetic applications. Their properties would eventually allow orthogonal applications combining more than one CBCR GAF domain (see below). In addition, CBCRs exhibit a noticeable

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fluorescence (clearly higher than that of BV-based bPhy), an aspect that adds to their potential.

2.1.4. Phys in Other Eukaryotic Organisms. Recent surveys on classes of organisms, for long not considered to harbor phytochromes, have widened the presence of these red/far-red sensitive proteins and even brought information on a much wider range of absorption maxima.

BV as chomophore is also present in a number of phytochromes from eukaryotic organisms such as fungi and diatoms. The role of Phys in fungi is well determined by a series of detailed studies, including gene knockout experiments with an impact on sexual/asexual reproduction.[72] Their phytochromes, studied in a well-characterized protagonist, *Aspergillus (A.) nidulans*, undergo a relatively simple photoconversion with short-lived intermediates akin to the photoreactions of bPhys.[73] Similar to bPhys is also the signal function, in most cases accomplished by a classical TCS (HK and RR). So far, none of these fungal proteins has made its way into optogenetic applications.

Another group of eukaryotic organisms was shown to carry phytochromes, the diatoms.[43,74] Several of these microorganism species, highly abundant in aquatic environments, were recently studied in detail with emphasis on a potential red light sensitivity. The authors were driven by the fact that practically only blue light penetrates a water column to a significant depth, whereas all other wavelength ranges are absorbed at the surface or slightly below. Preliminary photochemical experiments were performed, and behavioral (action spectra) studies and knockout experiments of Phy-encoding genes clearly identified an important role of Phys for the lifestyle of diatoms. A phylogenetic survey was also reported. However, so far, no optogentic applications came into view.

Much more promising results emerged from a recent survey that identified phytochromes in many eukaryotic (micro)-algae.[11,75,76] Equally abundant in aquatic environments, the phylogenetic analysis found several lineages of microprasinophytes carrying Phy-encoding genes, exemplified in the detailed studied *Micromonas prusilla* as the common progenitor of green algae and land plants. These eukaryotic Phys carry PCB as chromophore that, like in the CBCRs, is subjected to enzymatic changes of its chemical structure. Sequence alignments in this group of small eukaryotes point to a prevalence of histidine kinase-mediated signaling. However, the domain composition as also the signaling components, located in the C-terminal part of the protein, show a large variation: some Phys of these organisms carry—alike, e.g., land plants—two PAS domains between the PHY domain and the signaling components; some others carry only one or no intermittent PAS domain at all. Similarly, complete TCS (two component systems) are present, in some cases together with their fused response regulators; in some other algae these domains were lost. Besides the important phylogenetic findings, an even more surprising feature has been identified in these algae: their phytochromes show a broad distribution of their absorbances. This aspect, very similar to the spectral variation seen in the CBCR proteins, is apparently not restricted to the variation of chromophores in prokaryotes but finds its equivalent also in these microalgae. While not reported so far, one might expect optogenetic applications exploiting the algal "rainbow of colors".

2.2. The Tetrapyrrole Chromophore

2.2.1. Biosynthesis. Linear tetrapyrroles represent the chromophores in all phytochromes and related proteins. These fully oxidized compounds were given the IUPAC-defined name "bile pigments" or "bilins" because they are the breakdown products of the blood pigment hemoglobin and are excreted in bile. Porphyrines are ubiquitous compounds serving, for example, as cofactors in cytochromes or heme proteins across most eukaryotic and prokaryotic organisms (just consider anaerobically growing microorganisms as an exception). Following the biosynthetic pathway, first pyrrolic rings (porphobilinogen) form through condensation of two molecules of 5-(δ-)aminolevulinic acid, which themselves derive from a condensation/decarboxylation of succinyl CoA and glycine (as one of two biosynthetic pathways, the alternative process starts from L-glutamate). Stepwise condensation of four pyrrole units generates a helical conformation that allows cyclization into the porphyrinic structure. The entire biosynthetic pathway is more complex than presented here, as e.g., the enzyme catalyzing the condensation of the porphobilinogen units, hydroxymethylbilane synthase, itself carries a dipyrromethane unit as cofactor making the condensation a six-pyrrole-unit intermediate. Also the final ring closure step includes several rearrangement/reorientation processes that we will not outline in detail here, as it is well covered in textbooks. These cyclic tetrapyrroles then undergo a series of modifications depending on their final fate, among others complexation of central ions such as Mg (chlorophyll pathway), Fe, or Cu during the synthesis of heme or cytochrome components. Formation of the bilin chromophores utilized by phytochromes follows the reverse pathway, through ring-opening of heme groups concurrent with demetalation through the enzymatic activity of heme oxygenases (HO).[77] Of note, the degradation of chlorophyll follows an entirely different pathway.[78] The ring-opening reaction of a heme group requires NADPH as a redox-active compound and three molecules of oxygen to extract the iron ion and to yield one molecule of carbon monoxide (CO) and BV (Figure 2). Most HOs generate the IXα isotopomer originating from cleavage of the methine bridge opposite of both propionate substituents; however, HOs with other topological specificity have been reported.[79]

It is neither fully understood how the transfer of the heme-split BV to a docked apo-bPhy is accomplished, nor is the selectivity characterized in detail with which HOs identify their "cognate" apo-bPhy. Only few experiments address this high specificity between the two binding partners that in one case (*P. syringae*) leads to a selection of only one of two present apo-bPys as the binding partner for BV transfer.[80]

2.2.2. Naturally Occurring Modifications of Phytochrome Chromophores. Bacteriophytochromes (bPhys) employ BV-IXα that derives from the HO-catalyzed porphyrin ring-opening reaction of heme, concomitant with the loss of the central iron ion and a CO molecule.

Further modification into the surprisingly large number of BV-derivatives depends on individual members of highly specific ferredoxin-dependent bilin reductases (FDBRs). The enzymes that follow HOs in generating chromophores for Phys might serve as a classification of the organism, but as a second aspect, knowledge on their specificity, i.e., the type of modified bilin that they generate, is of importance also for optogenetic applications. A particular enzyme involved in the generation of the phytochrome derivative generates holo-Phys with quite different absorption maxima. An instructive overview on the various bilins and their chemical relation is found in comprehensive reviews: Scheer and Zhao[81] (specific for phycobiliproteins) and Dammeyer and Frankenberg-Dinkel.[82] Seven different FDBRs

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are known, varying in substrate specificity and regiospecificity of reduction: PcyA, PebA, PebB, PebS, PcyX, land plant HY2, and algal PUBS.

Most surprisingly, two of these FDBRs, PebS and PcyX, have only been found in cyanophages to date. PebS can itself catalyze a four-electron reduction to produce PEB. PcyX is related to PcyA but converts BV to PEB instead of PCB. Another isomerization activity that converts PEB to PCB has already been reported for the red algae *Cyanidium caldarium*.[83]

Chemically, the generation of the chromophore for plant phytochromes (PΦB) and for related proteins from cyanobacteria (PCB) requires further reduction. The reactive enzymes in these reducing reactions are usually members of the ferredoxin-dependent oxidoreductases.[84] The PΦB chromophore of plant phytochromes is produced by a two-electron reduction of BV. The chromophore of cyanobacterial phytochromes, PCB, instead, is generated by one synchronized ($+4e^- + 4H^+$) step. So far, reduction reactions to yield PCB from PΦB could not be identified in plants, and the generation of PCB seems to be restricted to cyanobacteria. Though being discussed here for land plants and cyanobacteria, reduced bilins (originating from BV) are not as rare as sometimes assumed. We still find cases where genes encoding HOs and FDBRs, the enzymes generating reduced bilins from BV, are found in the genomes of several organisms, though to the best of our knowledge no genes for common receptors could be identified. Enigmatic is the lack of a known bilin-binding protein in the eukaryotic alga *C. reinhardtii* (see section 2.1.2), as it requires PCB for phototrophic growth, although no "classical" phytochrome- or phycobiliprotein-encoding genes could be identified.[75]

There are again exceptions to be mentioned: as identified so far, two streptophyte algae, *Mesotaenium caldariorum* and *Mougeotia scalaris*, carry PCB instead of PΦB as chromophore, as a comparison of the extracted algal material with recombinant PΦB-binding proteins revealed.[85,86] These algae lack the PCYA gene, but additional research identified that, e.g., HY2 from *Klebsormidium flaccidum* produces PCB instead of PΦB.[87] Even though HY2 is the enzyme of chromophore biosynthesis in land plants, in this case it serves for PCB generation. In the case of *M. caldariorum*, Wu et al. proposed that PΦB is an intermediate from BV to PCB. It appears as if a simple classification of enzymes to bilin chromophores does not hold, and more "exceptions of the rule" are to be expected.

Phycoerythrobilin (PEB, Figure 2) is chemically at the same reduction level as PCB, just showing a different double bond arrangement. From a chemical point of view, PEB is related to PCB through addition of a proton to position 15 of the bridge between rings C and D that would then cause a shuffle of the π-electron system and loss of a proton from position 18, resulting in a vinyl substituent at position 18 (instead of the ethyl group present in PCB). Targeting the double bond connecting the rings A and B of PCB, a proton can be added to position 5, similarly as formulated for position 15 in PEB formation, thus converting the A-B ring-connecting double bond into a single bond and generating a double bond in ring A. This compound has been named phycoviolobilin (PVB, Figure 2). This same π-electron rearrangement of both connecting double bonds (between rings A and B and simultaneously between rings C and D) into single bonds then results in phycourobilin (PUB).

However, the biosynthetic pathways do not follow a "simple" chemical explanation. Starting from BV to generate PEB, cyanobacteria employ two FDBRs: PebA and PebB. This reaction pathway has been shown to run through formation of the intermediate 15-16 dihydrobiliverdin. As said, the saturated bond between positions 15 and 16 does not qualify PEB as a Phy-chromophore. Instead, it is an abundant chromophore in the phycoerythrin in the phycobilisomes.

PUB formation from PEB is formally similar to the generation of PVB from PCB. PUB was first found in cyanobacteria and isomerized by a PEB lyase RpcG.[88] Later, a phycourobilin synthase (PUBS) was identified in the moss *Physcomitrella patens*, able to catalyze a four-electron reaction, reducing the C15−C16 and the C4−C5 double bonds to yield PUB.[89]

The formation of PVB is equally complex. The direct formation from PCB in cyanobacteria, without the involvement of a lyase, has been discussed in an overview given by Ikeuchi and Ishizuka.[90] They report this reaction for TePixJ; a mixture of chromophores was found in a time-dependent manner when PCB was added to the apoprotein. The adduct formation preceded the PVB formation. Other isomerization reactions require the covalently bound PCB and are directed by a specific lyase, probably involving a rubinoid intermediate (transient addition of a cysteine to position 10 of PCB).[91] Information on PVB-specific lyases, PecE/F, has been found from *Mastigocladus laminosus*.[92,93] These authors have identified the lyase-catalyzed, ligation-coupled isomerization of PCB to PVB in the process of chromophorylation of alpha-phycoerythrocyanin.

As alluded to briefly in section 2.1.3, some CBCR-GAF domains modify the PCB chromophore even into a rubinoid-type compound without any further modification/isomerization of the chromophore. This happens via a nucleophilic attack of a second cysteine (in addition to the Cys required for covalent binding) to the central position C10, thereby forming a second covalent bond and converting the C10−C11 double bond into a cysteine-substituted single bond.[54,64] PVB, PUB, and also the rubinoid compounds have been synthesized chemically[94] but were never employed in phytochrome assembly experiments.

PEB and PUB are found, besides PCB and PVB, as chromophores in the light harvesting antenna of cyanobacteria (phycobilisomes).[88,95] However, both PEB and PUB are not naturally occurring chromophores in phytochromes and most probably also not in CBCRs. The reaction principle of phytochromes, namely the light-induced isomerization of the double bond between rings C and D, cannot be accomplished by these compounds, as the required double bond is converted into a single bond (Figure 2). *In vitro* incorporation of PEB as chromophore into oat PhyA apo-phytochrome generates a nonisomerizing but strongly fluorescent chromoprotein.[96] Among those two nonisomerizing chromophores, PEB was recently bound to a CBCR-GAF domain *in vivo*.[71] Interestingly, more than half of the PEB chromophores were converted into PUB during the assembly process.

Considering the topic of this article, optogenetic applications of phytochromes require provision of the chromophore. In this aspect, one may favor bPhys as they utilize biliverdin, the first and direct degradation product of cyclic tetrapyrroles readily generated by a HO-encoding gene. In fact, BV formation is a ubiquitous reaction pathway in nearly all organisms essential to excrete excess of potentially harmful porphyrinic compounds, whereas PΦB or PCB is present only in plants and cyanobacteria (the need to provide these chromophores is still a major challenge in optogenetic applications, see section 3). A further advantage of BV-bearing phytochromes is given by the π-electron system that is further extended than that of PΦB or PCB; as such, bPhys show absorbances in the red/far-red spectral region (λ_{max} Pr: ca. 700 nm, λ_{max} Pfr: ca. 750 nm). Red/

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far-red absorption and emission properties are clearly advantageous over blue light-based applications (discussed elsewhere[1]), as longer wavelength light better matches the "transparent window" (Figure 1). Of interest in this aspect is the moderate but detectable fluorescence of bPhys in their Pr state with emission maxima reaching nearly up to 730 nm. Their fluorescence quantum yield reaches Φ_{fl} = 6−10% (significantly larger than that of plant phytochromes of $\Phi_{fl} = 10^{-4}$)[24] after improvement by site-directed mutagenesis.[69] Notably, also Cph1 (and probably closely related Phys, e.g., CphA) shows some fluorescence, but again with fairly low quantum yield (Φ_{fl} = 2.4%) that discourages employment of these proteins in fluorescence-based applications.[97] Still at a very low level of approximately 10%, the bPhy fluorescence has been used in a number of relevant applications in living animals.[25] Similar quantum yields have been reported for GAF domains of CBCRs,[57,98] and long wavelength-absorbing and -emitting representatives of this protein family have been reported,[68] making these small GAF domains interesting candidates for fluorescence and even nanoscopy applications (see section 4.2 for further details).[98] For these red light-absorbing proteins, absorption and emission appear within the "transparent window" (Figure 1), an aspect of importance similar as discussed above for the bacterial phytochromes.

The essential need of the relatively large PAS-GAF-PHY domain arrangement with a molecular weight of more than 65 kDa to maintain spectral integrity, and the tendency to form homodimers, represent a clear drawback for many applications. In principle, CBCRs are better suited for applications due to their wide variation in absorption maxima, significant fluorescence, and smaller size (ca. 18 kDa). Yet, like most bilin-binding proteins, also CBCRs utilize PCB as chromophore. Improved protocols for the generation of PCB in animal cells have already been presented,[99−101] and work has been reported that aimed to convert PCB- into BV-binding proteins (see also section 3).[22,23,102]

2.3. Mechanistic Details

Common features of phytochromes and related proteins are the photochromicity of the two stable states and the large difference in absorption maxima of both photochromic states (in some CBCRs the difference between the two states extends to more than 100 nm). The large photochromicity allows selective irradiation of the parental state or the photoproduct state, thus providing the basis for optogenetic applications of this class of chromoproteins. Light absorption causes an immediate population of the excited state surface from which the chromophore returns to the ground state surface through a conical intersection, combined with the C15−C16 double bond photoisomerization (Figure 4).

The chromophore reactions at the excited state surface are currently under debate with respect to a proposed heterogeneity of conformations with consequences for the quantum yield of product formation. Recent experiments have identified a subpopulation of excited molecules that immediately return to the ground state without performing any light-driven activity due to the small energy barrier on the excited state surface. Thereby, the proportion of molecules that move toward the conical intersection, where the ratio of forward and backward reactions determines the quantum yield, has been addressed by several groups.[103,104]

Early studies determined the chromophore photoisomerization taking place at the double bond connecting rings C and D of

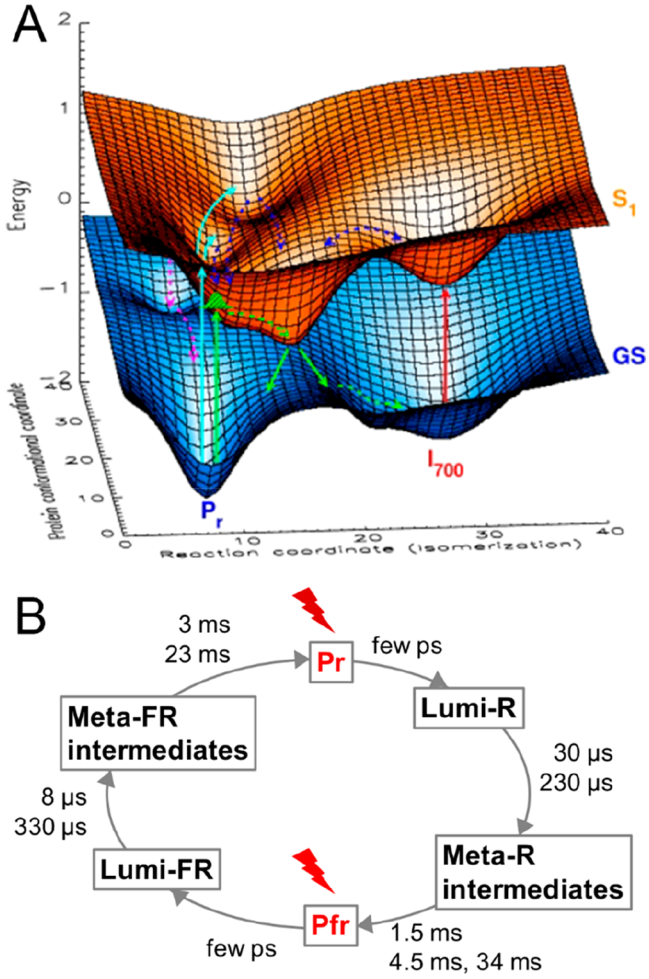

Figure 4. Fast phytochrome photochemistry. (A) Excited state dynamics of phytochrome A from *Avena sativa*. Upon absorption of a photon, the chromophore is promoted to the excited state hypersurface. There, it travels through several local minima, eventually arriving at the conical intersection between both energy levels, where it falls down to the ground state surface, either going back to the S_0 ground state or proceeding forward to generate the Lumi-R intermediate. Back-irradiation of the lumi-intermediate occurring as a side effect of the experiment is shown (red arrow, adapted from Müller et al.[105] with permission, copyright Elsevier 2008). (B) Photocycle of plant phytochrome A from *Avena sativa*. Kinetics were determined at 20 °C. The individual conversion processes either are biphasic (Lumi → Meta) or are determined over several, spectrally very similar, but ill-determined intermediates (Meta-I, -II, and -III).[106]

the bilin moiety ("C15−C16 double bond isomerization").[107,108] Although both "ends" of the chromophore undergo rotations around the A−B- and C−D ring connecting single bonds, this led to the erroneous assumption that the double bond isomerization would occur at the bond between rings A and B (C4−C5).[109] The protein environment, however, restricts conformational freedom sufficient for a *cis*−*trans* isomerization only to ring D (Figure 5). A number of spectroscopic studies added to and confirmed this identification.[110,111] Crystal structures of various phytochromes revealed so far remarkably large distortion angles for the A and D rings with respect to the central B−C plane. These distortions seem to contribute significantly to the absorption maxima.[112] In particular, the recent development of powerful theoretical programs allowed precise quantum mechanical calculations to

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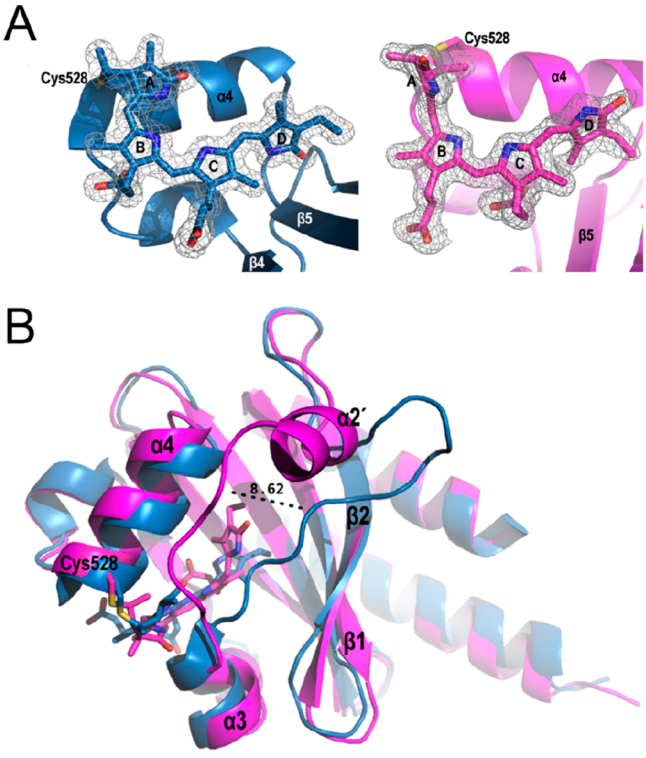

Figure 5. Pr and Pg structures of the red/green switching GAF3 domain of CBCR Slr1393. (A) Left, the red-absorbing parental state Pr (blue, PDB: 5DFX, λ_{max} around 650 nm); the chromophore (with electron density map) is in the *Z,Z,Z,s,s,a* configuration. Right, the green-absorbing photoproduct Pg (magenta, PDB: 5M82, λ_{max} around 540 nm); the chromophore (with electron density map) in the *Z,Z,E,s,s,a* configuration. The D ring is oriented differently in the Pr and Pg states. The dihedral angles between the rings A−B, B−C, and C−D are 24°, −1°, and −150° in Pr and −77°, 8°, and −120° in Pg, respectively; they were determined from the crystal structure coordinates using Pymol. (B) Superposition of the Slr1393g3 Pr and Pg structures, showing the conformational changes associated with the photoconversion.

clearly pinpoint the absorbance-regulating element being the distortional angle between rings C and D over the conformational changes between rings A and B (although, as said, in some proteins the A ring shows a remarkable conformational distortion).[112]

2.3.1. Time Domains, Intermediates, and Efficiencies for Phytochrome Photoconversions. Decay of the vibrationally "hot" chromophore from the excited state surface to the first detectable intermediate during the "forward" reaction (Pr → Pfr) occurs within several picoseconds, whereas the reverse reaction (Pfr → Pr) takes place much more rapidly, requiring only very few picoseconds.[105,113] The efficiency of the photoreaction (quantum yield) has been determined as between 0.15 and 0.2 depending on the protein under study[24] and initially was understood as the amount of Pfr state formed (see above, section 2.1, the discussion on structural heterogeneity with impact on the quantum yield). Studies on the reverse reactions (from the photoproduct back into the parental state) revealed a higher quantum yield than the respective forward reaction. Interestingly, the first detectable intermediate for both directions always shows a bathochromically shifted absorption maximum. Whereas the formation of the first intermediate is light-induced, all following conversions are thermally driven and expand into the time range of several hundred milliseconds (Figure 4).[106,114] The photocycles of canonical phytochromes carrying either PΦB (plant Phys) or PCB (cyanobacterial Phys) are very similar to each other with respect to kinetics, quantum yields, and intermediates. Intermediates that form transiently are named lumi-R (R for Pr), meta-RI, and meta-RII, and for the reverse reaction lumi-F (F for Pfr) and meta-F.[115] The reaction pathways from both Pr and Pfr likely occur in a consecutive manner with each intermediate turnover exhibiting lifetimes longer than the precedent ones. So far, only for one phytochrome has a thermal back-reaction from a before-formed conversion intermediate been reported.[116] However, also for the canonical phytochromes (plant-derived proteins and Cph1 from *Synechocystis*), compelling evidence indicates that the parental Pr state is heterogeneous in its composition, suggested by slightly different conformations of the chromophore.[117] It has even been proposed that at least two Pr states exist (in thermal equilibrium) that travel during parallel reaction pathways (with slightly different lifetimes for the involved intermediates) and eventually form the Pfr state.[115,118] The Pfr state, instead, has been found strictly homogeneous in its conformation. During the Pr → Pfr conversion, a transient deprotonation of the chromophore has been detected, whereas both starting and final states (Pr and Pfr) carry a protonated chromophore, not considering the dissociated propionate substituents.[119] It seems that the reverse reaction (Pfr → Pr) does not include a transient deprotonation of the chromophore.[110] Contrawise, bacterial phytochromes carrying BV as chromophore follow much simpler conversion pathways with only one or two intermediates during the formation of the Pr or Pfr state.[73]

Still relatively little information is available on the photochemical reactivity of the GAF domains of CBCRs. A number of studies describe reactions at the ultrashort time domains.[56,120−124] For conversions in the micro-to-millisecond range, the best characterized ones belong to the red-/green-absorbing subfamily. Neglecting some specimen of CBCRs in which the chromophore chemical structure is changed during photoconversion (PVB, bilirubin type) thereby complicating the photoconversion,[125,126] CBCRs with an unchanged PCB chromophore undergo a relatively simple photoconversion. Following the instantaneously formed first ("lumi"-like) intermediate that decays within the picosecond time range, only one, sometimes two further intermediates with lifetimes below 100 ms can be observed that eventually form the reaction product.[127,128]

2.3.2. Aspects of Protein Structure. Since the first crystal structure of a phytochrome fragment was presented in 2005,[47] a considerable amount of structural information has been gathered, including results for full-length proteins. The pioneering work describes the PAS-GAF fragment of the bPhy from *Deinococcus radiodurans* showing an eight-knot at the N-terminus that forms cotranslationally. Structures of a full photosensory module composed of the PAS-GAF-PHY domain were solved for Cph1 from *Synechocystis*,[129] followed by structures of various phytochromes also including full-length proteins.[130] Here, the group of Winkler reported a full-length structure of a bPhy from *Idiomarina sp.*, A28L, including the enzymatic domain at its C-terminal end.[131] The physiological activity resides in a cyclic-diguanylyl cyclase (DGC) activity (also coined as the GGDEF motif following the conserved functional sequence motif). This finding is of even greater importance, as most so far crystallized light-regulated enzymatic activities are histidine kinases and c-di-GMPases and the counteracting phosphodiesterases (PDEs) that are excellently

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suited for optogenetic applications. The publication from Gourinchas et al. adds further important information: whereas the PAS-GAF-PHY domains of bPHY arrange into parallel homodimers—and the same holds true for histidine kinases—the DGC domains adopt an antiparallel arrangement. Both HKs (known from other 3D structures) and DGCs are linked to the light-regulated units by long α-helical segments.

The authors even elucidated—in part—light-induced conformational changes in the bPHY dimers, for which they employed a sophisticated technique called HDX-MS. This method compares exchanges of protons by deuterons (HDX) over the incubation time, identified by high resolution mass spectrometry (MS). The comparison of the dark- and the light-activated forms identified regions of the proteins that become more or less exposed to H-D exchange.

Out of the many phytochromes identified so far, most optogenetics applications are performed with PhyB from *Arabidopsis thaliana*, due to the light-controlled tight and precise binding of phytochrome-interacting-factors (PIFs). Structural information for *At*PhyB has been obtained in 2014 for the Pr-state of the light-sensitive PAS-GAF-PHY unit. The work revealed a high structural similarity to the Cph1 and the *D. radiodurans* (*Dr*) structures. The authors reported an eight-knot structure just as that found for the *Dr* protein. Further, the antiparallel β-sheet element in the PHY domain protruding toward the GAF-embedded chromophore and long helical elements that pervade the entire lengths of the structure were identified—even longer than the equivalent helices in the Cph1 structure. The N-terminal extension, which is characteristic for flowering plant Phys, appeared longer than in the bacterial Phys and folds back into the vicinity of the chromophore.[132] Further structural information for PhyB-type Phys had been obtained: for PhyB from *Sorghum bicolor* (PG- and PGP-domains) and for PhyB from *Glycine max* (PGP-domain, Figure 3).[133] The same publication (Nagano et al.) reported also structures of truncated PhyA proteins (PG- and PGP-domains of *Gm*PhyA).

The crystallization-based structural information obtained from various phytochromes was recently complemented by a full-length cryo-EM-based structure of *At*PhyA.[134] The electron microscopy experiments yielded an electron density with a resolution of 17 Å and combined with docking of domains from crystallization studies allowed to propose the positions of the two plant-Phy specific PAS domains (named PAS-A and PAS-B) located between the PHY domain and the C-terminal part of the protein.

Still, for many phytochrome(-related) proteins, the crystalline structure of only one state has been resolved at high resolution. The photoproduct structures often have been resolved at lower resolution or have been revealed with alternative methods, e.g., X-ray scattering[135] or solid state NMR spectroscopy.[59,136,137] Structures of both states, parental and photoproduct, have been obtained only for the photosensory PAS-GAF-PHY portion of a few bPhys from, e.g., *D. radiodurans*,[138,139] *P. aeruginosa*, *P. palustris*, or *X. campestris* (a recent compilation of mostly bacterial phytochromes was reported by Gourinchas, Etzl, and Winkler[140]). The very recent application of extremely bright, ultrashort pulses from XFELs (X-ray free electron lasers) yielded three-dimensional structural information on the initiation of the photoprocess and the earliest intermediates for phytochromes from *D. radiodurans* and *Stigmatella aurantiaca*.[141,142] In the most recent paper, the observable time domain was in the range of 33 ms.[143] The future will show that applications of XFEL can be considered a method of choice to follow light-induced structural changes, even more so, as the authors have succeeded to extend the time frame for observation into the milliseconds range. While still pioneering in the field of phytochrome, these publications yield outstandingly detailed and novel insights into conformational changes. A comprehensive discussion of these observed data is beyond the frame of this review, and we refer the interested reader to the original publications. Structural information was obtained also for a small number of CBCR-GAF domains.[136,144,145] As only a few structures have been obtained for full-length phytochromes, structural information on the light-induced conformational changes of the protein that yield the biological signal is still sparse.

The pivotal relevance of phytochromes for plant development has initiated a large number of investigations aiming at understanding how they communicate with other proteins in order to mediate the light-generated signal.[146] Their regulatory function in the cytoplasm,[147] and after photoactivation and transport into the nucleus, has been discussed in recent reviews.[34] The interaction of phytochromes with nuclear proteins has been identified to regulate gene expression. Especially PIF proteins have been utilized in optogenetic applications, e.g., for direct control of gene expression or targeted protein recruitment (see section 4.4 of this review).[148,149] Moreover, binding of PIF3 or PIF6 to PhyB appears fully light-dependent (Pr vs Pfr) and extremely stable, and engineered variants allow utilization also in the cytoplasm. Moreover, PIF proteins could be shortened to sequences of 20 to 40 amino acids in length while still retaining their binding to PhyB (see section 4.1.3).[150−152]

Being a light-regulated kinase on its own,[153] several target sequences for phosphorylation have been identified in plant phytochromes. These preferentially reside in the N-terminal region (which may span various lengths in the canonical phytochromes)[154] but also in the "hinge region" that connects the photosensory PAS-GAF-PHY unit with the C-terminal portion of the proteins.[155] Initiated by the finding that Cph1 relays light-generated information downstream via a C-terminally located histidine kinase (HK),[39,41] the C-terminal domain of canonical plant phytochromes has been inspected for a potential HK activity.[156] However, substitution of the histidines in this part of the protein did not impede their function, indicating that the C-terminal part may carry a HK-related Ser-/Thr-kinase activity.[42]

Three structural features of phytochromes shall be highlighted due to their relevance for potential optogenetic applications: (i) structural analysis in combination with vibrational spectroscopy revealed a stabilizing element of the PHY domain that extends "backward" into the GAF domain. An antiparallel β-sheet structure (called the "tongue") comes close to the D-ring of the chromophore when the protein is in the Pr state, likely providing a stabilizing function (see Figure 3). Light-induced conversion into the Pfr state causes a disintegration of the β-sheet element and formation of a short α-helix.[157,158] This conformational change is considered important for transmittance of information into the C-terminal signaling domain. (ii) Long α-helical elements stabilize the photosensory PAS-GAF-PHY unit and arrange the three domains to dangle like fruits on a tree branch. The helical structures even extend further into the C-terminal portion of the phytochrome proteins and likely contribute to signal transmission from the photosensory part to the C-terminally located enzyme activities. This structural element is conserved throughout canonical plant phytochromes and is also present in their bacterial counterparts.

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The helical extension becomes relevant considering that in many phytochromes, preferentially those of bacterial origin, the signaling function is accomplished by an enzymatic activity located in the C-terminal part of the protein (see sections 2.1.2 and 2.4). (iii) Not present in canonical plant-derived phytochromes, but identified in many canonical cyanobacterial phytochromes as well as in other bacterial phytochromes and CBCRs, are enzymatic activities in the C-terminal part. Most prominent are histidine kinases,[39,41] some of them fused to their genuine response regulators, but also diguanylyl cyclases and phosphodiesterases[48] are abundant.[38,54] Understanding the "communication" between the light-sensing and the enzymatic part of phytochromes will open the door to designing hybrid proteins with light-guided enzymatic activities that do not exist in nature (or so far have not been discovered).

2.3.3. Synchronized Chromophore and Protein Motion: Biological Signal Generation and Propagation. After absorption of a photon, the bilin chromophore of phytochromes instantly undergoes conformational changes on the excited state surface (Figure 4). Discussion among the ultrafast-measuring community indicates a conformationally heterogeneous population of the chromophore with strong dihedral angle distortions, preferentially along the bridges between rings A and B and between rings C and D (Figure 5). However, the final reaction affects the C15−C16 double bond isomerization.[117] The central unit composed of rings B and C is fully conjugated with a nearly identical π-electron arrangement leading to fairly planar conformations throughout the entire photoreaction. The light-excited chromophore undergoes strong vibrational motions that become evident in an oscillation of the absorption monitored by picosecond-time-resolved absorption spectroscopy.[105] Yet, this oscillatory motion is rapidly damped on a time scale of a few picoseconds, indicating that the immediate protein environment around the chromophore couples to these motions and absorbs energy for subsequent thermally driven protein motions.[105] In the respective parental state, Pr, or Pfr for bathyphytochromes, the chromophore is protonated and strongly hydrogen-bonded. This stabilizing network breaks and reforms when the chromophore adopts the photoproduct configuration. It not only isomerizes the D-ring of the bilin with formation of a new hydrogen-bonding network, but the entire chromophore slightly rotates by a few degrees around an axis orthogonal to the plane of the chromophore. This ultimately leads to a change of salt bridges between the propionic substituents at rings B and C and arginine and histidine side chains, respectively. Further structural changes have been detected in the N-terminal extension. Unstructured in Pr, the sequence becomes more tightly fixed to the A-ring in the Pfr state of the chromophore.[158] The most important aspect for signal transmittance and optogenetic applications alike is the unfolding/refolding of the "tongue" (antiparallel β-sheet into α-helix), alluded to above (see also Figure 3), as it releases the PHY domain and thus mediates the light-generated signal into the C-terminal part of the protein. A comparison of structures of the parental state and the photoproduct, obtained for a CBCR-GAF domain (PDB: 5DFX, 5M82, Figure 5) highlights the changed interactions of chromophore and protein and the changes in the conformational states of both components.[144]

2.4. Physiological Functions Controlled by Canonical Phytochromes and Cyanobacteriochromes

Phytochromes, especially those of higher plants, are global players in light-driven plant development (photomorphogenesis), and they have been assigned to control about 20% of all plant genes. However, a detailed presentation of their variegated functions remains highly challenging to assess. In plants, phytochromes can interact with their paralogs in some of their regulatory functions; that is, some physiological processes require joint activity of more than one phytochrome, and in other cases each phytochrome type (PhyA−PhyE) may perform selectively very specific functions not to be taken over by another Phy. In addition, individual phytochromes communicate for their activity with other photoreceptors such as phototropins, cryptochromes, and most probably also with the ultraviolet-sensing receptor UVR8. Many of these light-driven physiological processes contribute to the function of plant hormones. Additional interactions with other actuators can have an attenuating or intensifying effect. Their ubiquitous appearance, their phylogenetic relation and origin, and many details of their physiological functions have been outlined recently in several comprehensive reviews.[33,34,147]

Our knowledge on phytochrome function in prokaryotes is even more sparse. Beyond the finding that many bacterial phytochromes relay signals via C-terminal enzymatic activities using histidine kinases (HKs) and their cognate response regulators (RRs), phosphodiesterases, or nucleotidyl cyclases, no further detailed information on the signaling pathway can be given. The physiological effects can often be described only in a phenomenological manner. Several physiological functions in cyanobacteria are clearly linked to Phys, e.g., chromatic acclimation and virulence. CBCRs were reported to control many aspects of cyanobacterial physiology and metabolism, in many cases through the C-terminally located HK-motifs in interaction with their cognate RRs, just to mention far-red light photoacclimation (FaRLiP), complementary chromatic acclimation (CCA), low-light photoacclimation (LoLiP), photosystem composition and stoichiometry, short-term acclimation (state transitions), circadian rhythm, phototaxis, photomorphogenesis/development, and cellular aggregation.

The relation of RcaE to chromatic acclimation has briefly been discussed further above (section 2.1.3). Here, physiological functions are highlighted a bit more precisely. A detailed analysis revealed that RcaE induces phycocyanin formation under red light, but phycoerythrin is controlled by DpxA (by green, but not by blue light), as demonstrated for *Fremiella diplosiphon*.[159,160] The signaling components of both regulatory elements are HKs and their cognate response regulators (RRs). The CcaR/CcaS sytem has already entered the stage as an optogenetic tool. Far-red light acclimation was demonstrated through the FaRLiP system, to which the knotless RfpA takes responsibility.[161,162] Also, sessile or free-swimming lifestyles of some plant pathogenic bacteria are under control of bPhys.[163] However, besides the HK/RR system also c-di-GMP as second messenger constitutes an important optogenetic target. Only several selected examples should be outlined here in detail: the c-diguanylyl cyclase SesA regulating cell aggregation in *Thermosynechococcus*. It requires the concerted action of three CBCR-proteins to induce the generation of this second messenger. Tlr1612 has been identified as a light-controlled repressor of cell aggregation in *Thermosynechococcus volcanus*.[164] Further, pigment biosynthesis in *Deinococcus radiodurans* was reported to be phytochrome-regulated,[44] but in nearly all these

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examples the signal transduction chain, which finally leads to the observed effect, has not been identified. Thus, out of the many regulatory functions that phytochromes accomplish, we will discuss only those with direct relevance to the topic of this article. In particular, naturally occurring systems with direct application potential came into focus for optogenetic applications.

Early visions of Quail and co-workers put focus on the strictly light-regulated, very specific and strong interaction between phytochromes (preferentially PhyB) and individual members of the PIF family for controlling gene expression.[149] Their seminal study of phytochrome signal transduction shown by a random mutagenesis approach using PhyB (and also PhyA) identified mutations in the tandem PAS domain arrays following the PHY domain, with clearly observable plant phenotypes.[165] This tandem PAS region is defined as positions 621−873 in PhyA and 655−905 in PhyB. For *Arabidopsis* PhyA, the authors found a cluster of effective mutations between the amino acid positions 680 and 840, and similarly for PhyB between 770 and 840. A hot spot for both proteins was identified between positions 775 and 795. No direct PhyA- or PhyB-interacting partner proteins were identified in this study, but signaling proteins from the COP-DET-FUS group were proposed to bind to the identified region of PhyA or PhyB. This reactivity was not only used for its natural function for regulation of gene expression but was also exploited in optogenetics for recruiting proteins to cellular compartments or to specific intracellular membranes. In fact, the couple PhyB/PIF represents the majority of phytochrome-based applications. Interestingly, PhyB-based applications discriminate between fragments that span either amino acids 1−651 (not comprising the tandem PAS array) or others extending up to position 908 (sections 4.1.3 and 4.4). After the first structure of a PhyB photosensory core module (PAS-GAF-PHY) was presented,[132,133] further information on residues contributing to the PhyB/PIF complex formation remain to be identified. At least, some more quantitative studies emerge. The association/dissociation reactions between PhyB and PIF were recently studied and modeled kinetically using a truncated *A. thaliana* phytochrome, with a short piece of PIF6 (PIF6.A variant) that remains preferentially monomeric in solution.[150] PIF6.A comprises only 40 amino acids of the entire protein (denoted as APB.A) yet retains binding properties (280 nM for the Pfr state and 2 μM for the Pr state). To follow the binding experiments, PIF6.A was fused with the homotetrameric fluorescent protein (FP) *Ds*Red in a manner that retains the monomeric conformation of the PIF fragment, whereas the tetrameric FP enhances the detection of fluorescence to allow quantitative measurements. These experiments indicated rapid binding of PIF6.A to the Pfr state (even close to the diffusion limit) and nearly no binding capacity of the Pr state for PIF, in contrast to former experiments. However, the latter experiments were performed by alternative detection methods.[151] The study also included longer PIF6 variants (APB.A plus APB.B), which revealed stronger binding due to a slower dissociation process.

3. PHOTORECEPTOR ENGINEERING

Phytochromes were initially utilized in optogenetics due to their modular domain architecture carrying a photosensory unit in their N-terminal and a signaling activity in their C-terminal half. Their natural function as photoreceptors comprises a process as follows: light absorption generates a biological signal through chromophore-triggered conformational changes of their protein moiety. This releases a given enzymatic activity or formation of complexes with other proteins that become activated, thereby allowing the plant or bacterium to respond to the environmental conditions accordingly. Phytochromes bear another property that makes them promising candidates for optogenetic applications: already the wild type proteins—without further sequence variation—absorb and fluoresce in a spectral region much further red-shifted than any other photoreceptor class.

Bilin-binding bPhys and CBCRs further broaden the options for optogenetic applications. They benefit from their far-red light absorption using BV as chromophore, the first heme degradation product that is ubiquitous in plants, animals, and bacteria. The other chromophores of canonical phytochromes (PΦB or PCB) are characteristic for plants and cyanobacteria. In addition, BV shifts the absorption maxima of the photoreceptor parental state and photoproduct by ca. 50 nm (in some proteins even further red-shifted to 710/760 nm, always referring to the λ_{max} values) toward longer wavelengths relative to canonical phytochromes (λ_{max} values: 700 and 750 nm for the Pr and the Pfr states of bPhys), moving them into the applications-relevant "transparent window" (Figure 1). Together with their noticeable fluorescence of the Pr state (λ_{em} > 720 nm), bPhys have already been applied as fluorescent markers, excellently compiled recently by Chernov et al.,[25] and are therefore here only discussed marginally (section 4.5). Fluorescence-based applications have also been performed employing CBCR-GAF domains and proteins originating from the phycobilisome antenna complexes (section 4.6.2).

Complex formation between plant phytochromes and cytosolic or nuclear proteins, some of which turned out to be transcription factors, led to the earliest proposals for light-regulated biological functions such as gene expression.[148] Interactions of phytochromes with other cytosolic photoreceptors, e.g., phototropins, can be used to direct phytochromes to specific cell compartments or to the membrane, as was already demonstrated in 2012 in the moss *Physcomitrella patens*[147,166] and in applications combining multiple photoreceptors.[167−169] Complex formation between PhyB from *A. thaliana* and PIF proteins (most frequently PIF3 or PIF6) has been tuned to a minimal size of both proteins. PhyB could be truncated to 600 amino acids, still accomplishing reversible complex formation with PIF, and also PIFs were down-sized to only 20 to 40 amino acids (depending on the addressed research topic).[151] These truncated proteins might be the preferred choices when used as tags in protein-recruitment experiments (section 4.1.3). As mentioned above, these short peptides are usually fused to larger proteins in order to prevent proteolysis.

There were more direct attempts to employ canonical phytochromes from cyanobacteria and bPhys based on the finding that these proteins accomplish signaling through enzyme activities located in their C-terminal portion, e.g., histidine kinases[39,41] or diguanylyl cyclases coupled with phosphodiesterases.[48] Early approaches still required the addition of the genuine chromophore into cell cultures, which poses a major experimental issue when developing *in vivo* applications, and in fact, several more recently presented optogenetic applications still depend on exogenous addition of the bilin chromophore.

An important aspect regarding the chromophore availability during protein biosynthesis is often overlooked: although apophytochromes show the capability to autocatalytically bind an added chromophore covalently (even in *in vitro* experiments and also in cases when single GAF domains were expressed), the extent of incorporated chromophore (A_{665}/A_{280}) is lower in recombinant phytochromes than in plant-extracted phyto-

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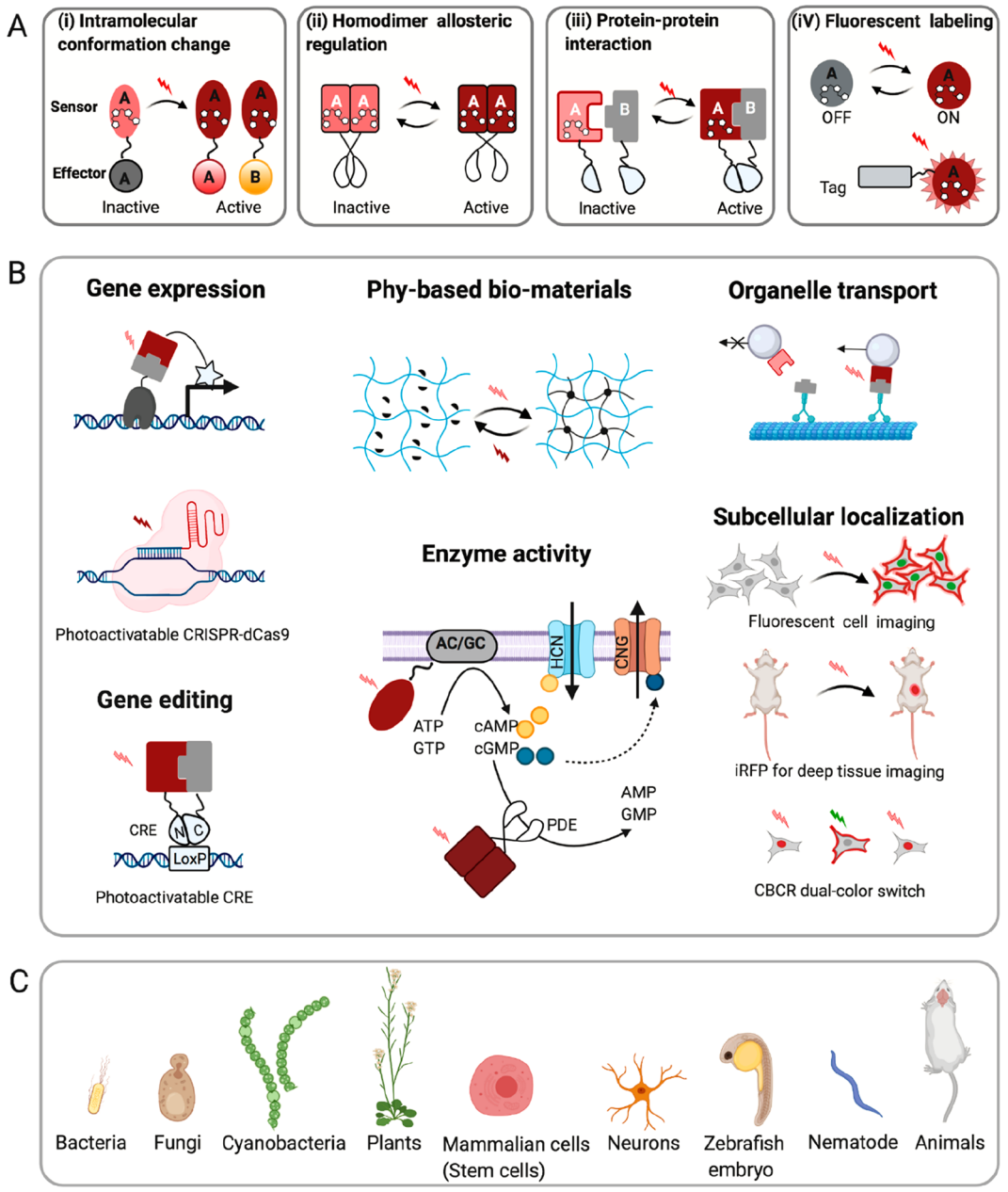

Figure 6. Phytochrome-mediated optogenetic and imaging applications. We restrict the figure to the most frequently applied techniques, e.g., gene expression control, protein recruiting, or organelle-directed transport. We do not include less frequently applied methods, e.g., bioprinting, photoacoustics, protein splicing, or biosynthetic process control. (A) General engineering strategies of photoreceptors. (i) Covalent linkage to an output effector, i.e., utilizing natural architecture ("A"-"A") or engineered hybrid proteins ("A"-"B") not existing in nature. In these engineered variants, the design of the linker between both domains is usually important for the functionality. (ii) Light-induced homodimerization. The light-dependent conformational changes in regulatory mechanisms of dimeric enzymatic functionalities. (iii) Light-induced heterodimerization enables interactions of phytochromes or photoswitchable FPs with their binding partners. The couples PhyA/B and PIFs represent the majority of phytochrome-based applications. Another example is the *Rp*BphP1 and *Rp*PpsR2 couple, where red light (660 nm) interrupts the interaction. (iv) Engineered bPhys, CBCRs, and APC (allophycocyanin) as a source of near-infrared fluorescent probes. (B) Selected cellular applications include the regulation of gene-expression, enzyme activity, organelle transport, gene editing, and subcellular localization with red light. See main text for detailed discussion and further applications. (C) Phytochrome-based optogenetic tools find applications ranging across different organisms including prokaryotes, fungi, animal systems, and plants.

chromes. Also, in bacterial, BV-binding Phys and in CBCRs, the yield of chromophore binding is moderate, even if PCB and apoprotein are coexpressed. Apparently, the apophytochromes, when expressed under their genuine cellular conditions, experience better conditions to incorporate the chromophore, and the present chromophore might also help folding of the polypeptide chain into its three-dimensional structure and protect the holoprotein from intracellular digestion.

The dependence of phytochromes on chromophores (BV or PΦB/PCB for plant phytochromes or the canonical phytochromes from cyanobacteria) called for strategies that prevent the need for their exogenous addition. Consequently, biosynthetic strategies have been developed early on. In bacteria,

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a two-plasmid approach[99] or, alternatively, a fused gene construct turned out to be efficient.[57] The two-plasmid approach requires transforming (or transfecting) the phytochrome-encoding gene (or a Phy-related protein) and simultaneously inserting genes encoding enzymes that break down heme groups (HO, heme oxygenases) into BV along with enzymes that further reduce BV either to PΦB or PCB. Alternatively, the fused-gene approach simply combines the DNA of both plasmids.

To improve the endogenous generation of PCB (alternatively PΦB), several engineering attempts have been tested. The initial step in the synthesis of all bilin chromophores is the ring-opening reaction that also results in the removal of the central iron ion. As this reaction is performed by HO in combination with redox-active enzymes, involving molecular oxygen and NAD^+/NADH-switching enzymes, authors probed the minimal component composition for biosynthesis. The combination of HO-PcyA (heme oxygenase + PCB:ferredoxin oxidoreductase) was sufficient for PCB synthesis *in vitro* combined with Fd (ferredoxin) as a one electron carrier.[170] In this study, also simpler systems were tested comprising only a heme oxygenase and a PCB:ferredoxin oxidoreductase, e.g., HO-PcyA from *Thermosynechococcus elongates* (even without considering the compatibility with the host organism). However, this approach yielded only low to moderate amounts of chromophore-furnished phytochromes in *E. coli*. Insertion of the heme to PCB bioconversion pathway to mammalian cells initially raised hurdles caused by the compartmentalization of the heme synthesis pathway between cytosol and mitochondria, but several adjustments made these approaches reliable (see section 4.1.3).[100]

4. OPTOGENETIC APPLICATIONS

The intention to introduce phytochromes and related proteins into the field of optogenetics had a bumpy start. Plant phytochromes, the most prominent representatives, were long seen as rigid and unexciting proteins with a large molecular mass and a virtually nonexisting fluorescence. Furthermore, their physiological functions, though paramount for plant development, were never quite clearly understood. When later, light-regulated pythochrome transport into the nucleus was found (besides other, even more obscure activities in the cytosol),[147] their function appeared even more complex. Other photoreceptor classes followed the initial popularity of channelrhodopsins much earlier, preferentially the flavin-based LOV-, BLUF-, and cryptochrome proteins. These photoreceptors showed functional and structural properties more feasible to manipulate, and just considering LOV domains, genetic modifications including the generation of hybrid proteins appeared straightforward (complied by Losi et al.).[1]

Screening the literature for phytochrome-based optogenetic applications reveals a phenomenon also known from other classes of photoreceptors: the number of published proposals, perspectives, or reviews surpasses the number of publications reporting original experimental work. Many reviews discuss, e.g., the possibility of orthogonal applications combining the long wavelength-absorbing phytochromes with (preferentially) blue light-absorbing, flavin-based photoreceptors. A number of contemporary reviews provide overviews on selected topics highlighting for instance bacterial phytochromes and their use both as fluorescent markers[25] and light-regulators of enzyme activities such as dinucleotide cyclases[140] or cyanobacterial phytochromes,[171] including the most recently discovered class of cyanobacteriochromes.[172,173] A well-structured, comprehensive review by Hughes introduces the reader to nearly all fields of optogenetic variation, covering most of the relevant literature, but also discusses potential obstacles and drawbacks.[174]

The far-reaching impact of initial optogenetic applications using other photoreceptors leveraged phytochromes to slowly conquer their place in the field and catalyzed an "explosion" of newly characterized phytochromes with entirely unexpected properties during the last two decades. After better understanding their physiological functions, these features served as a blueprint for the employment into three branches. First, the interaction of the tandem PAS motif in plant phytochromes with interacting factors offers countless possibilities to design light-responsive protein-interaction switches. Second, the C-terminally located enzyme activities in bacterial phytochromes, intensively studied and well understood in other bacterial systems, were ready to be exploited. Third, protein engineering attempts led to phytochrome variants with fluorescent properties suitable for their use as fluorescent probes. In the following sections we review the potential of phytochromes for use in optogenetics, capitalizing on their activities as light switches for controlling protein homo- and heterodimerization, as well as enzymatic activities. Besides that, we further discuss their use as fluorescent reporters in the near-infrared light spectral region. As an overview, the various applications are schematized in Figure 6. The different photoreceptors utilized for the development of various optogenetic applications are summarized in Table 1.

We dedicate the beginning of this section to applications that explicitly address gene expression control, as most of the Phy-based applications dwell on this fundamental process. The following (sub)sections describe optogenetic influences of downstream signaling or other physiological processes, transport of or into individual cell organelles, or recruitment to particular compartments. We are aware that Phy-based applications are just about to leave their infancy; thus, many optogenetics applications are still proof-of-principle reports, even though in some examples red/far-red regulations have been demonstrated in living animals or plants (in contrast to, e.g., the channelrhodopsins that are already fully immersed in medical applications in animals and even in humans).

4.1. Transcriptional Gene Expression Control

Most applications of transcriptional control rely on the very early proposed system based on the interaction between PhyB of *A. thaliana* and one of the several PIF proteins, preferentially PIF3 or PIF6,[149] discussed in the following subsections for yeast, animal, and plant cells (sections 4.1.2, 4.1.3, and 4.1.4). A number of applications followed this approach in a similar way, utilizing the canonical cyanobacterial phytochrome Cph1 and other interacting proteins instead of the PIF proteins.

A second branch of phytochrome-based applications in optogenetics for gene expression control makes use of the naturally existing, light-driven signaling cascade found in bacterial phytochromes that functions via light-regulated enzyme activities. Bacterial phytochromes are rich in histidine kinases (HKs) transmitting signals to response regulators (the classical '"two component signal transduction system" best characterized as the Che-components). But also, adenylyl and guanylyl cyclases, and the couple of enzymes that adjust the concentration of cyclic-di-GMP, diguanylyl cyclases and phosphodiesterases, are functionalities found widespread. These light-regulated enzyme activities are of particular interest for selective use in mammalian cells, as HKs are found sparsely in

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Table 1. Phytochromes Used in Optogenetic Applications[a]

Name/Origin	Chromophore/λ	Mode of function	Application
Plant/cyanobacterial			
$PhyB_{650/908}$/*A. thaliana*	PΦB/PCB (650−750 nm)	PPI	**Protein localization** animal,[250,266,268] yeast;[256] **Membrane recruitment** animal;[250,268] **Gene expression** yeast,[149,188,190,256] animal,[192,198,199] plant;[168,199,223] **Gene editing** yeast,[191] animal;[193] **Viral infectivity** animal;[216] **Cell SOS signaling** animal;[259,260] **Protein splicing** yeast;[275] **Actin assembly** *in vitro*;[249] **Actin cytoskeleton modulation** animal;[250] **PIP_3production** animal;[252,253] **T-cell antigen receptor activation** animal;[178] **Cell cycle control** yeast;[256] **Budding control** yeast;[270] **Protein inheritance** animal;[268] **Endosome transport** animal;[271] **Protein purification** *in vitro*;[152] **Biomaterial protein caging** *in vitro*;[286] **Pattern formation** *in vitro*;[287] **Cell adhesion** animal;[288] **Cell sorting** animal;[289] **Smart biomaterials** *in vitro*;[291] **Viral gene delivery** animal[217]
PhyA/*A. thaliana*	PCB (660/730 nm)	PPI	**Gene expression** yeast[189]
$Cph1_{\Delta HK}$/*Synechocystis PCC6803*	PCB (660/720 nm)	PPI	**Kinase signaling activation** animal;[257] **Biohybrid materials** *in vitro* and animals[290]
Cph8 (Cph1/*Synechocystis PCC6803* − EnvZ-OmpR/*E. coli* chimera	PCB (650/705 nm)	ICC	**Gene expression** bacteria[175−177,181,184]
CcaS/*Synechocystis PCC6803*	PCB (535/672 nm)	ICC	**Gene expression** bacteria[176,182,184,185,294,296,297]
$CcaS_{\Delta PAS}$/*Synechocystis PCC6803*	PCB (535/672 nm)	ICC	**Gene expression** bacteria[248,285]
UirS/*Synechocystis PCC6803*	PCB (334/652 nm)	ICC	**Gene expression** bacteria[186]
Bacterial			
BphP1/*R. palustris*	BV (760/680 nm)	PPI	**Protein localization** animal;[220] **Gene expression** bacteria,[187] animal[203,204,206] **Kinase signaling activation** animal;[167] **Photoacoustic computed tomography** animal[277]
DGCL (PDE-deficient *R. sphaeroides* BphG1)	BV (700 nm)	Enzyme (c-di-GMP)	**Gene expression** animal[236]
BphS (BphG1/*R. sphaeroides* - *Slr1143/Synechocystis sp.* chimera)	BV (712/756 nm)	Enzyme (DGC)	**Gene Expression** bacteria;[226] animal;[214,215,230] **Bioprinting** bacteria[280]
BphG1/*R. sphaeroides* - CyaB1/*Nostoc sp.* chimera	BV (700 nm)	Enzyme (AC)	**Locomotion** animal[231]
BphP/*D. radiodurans* PCM	BV (630/780 nm)	Heat release	**Photoacoustic computed tomography** animal[277]
BphP/*D. radiodurans* - Cya2/*Synechocystis PCC6803* chimera		Enzyme (AC, GC)	**Locomotion** animal[232]
BphP/*D. radiodurans* - *Rv1264/M. tuberculosis*	BV (670/760 nm)	Enzyme (AC, GC)	**Gene expression** animal[233]
Bphy/*Pseudomonas aeruginosa* − PDE2A/*H. sapiens*	BV (690/850 nm)	Enzyme (PDE)	**cAMP and cGMP hydrolysis** animal;[238] **Sperm motility** animal;[282] **Cilia growth** animal[284]
BphPs/PDEs chimera variants	BV (670/780 nm)	Enzyme (cNMP-specific PDE)	**Ca^{2+} signaling** animal[240]
Cyanobacteriochromes			
Slr1393g3 GAF3/*Synechocystis PCC6803* - *Microcoleus chthonoplastes* PCC7420AC chimera	PCB (539/650 nm)	Enzyme (AC)	**cAMP production** bacteria[243]

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Table 1. continued

Name/Origin	Chromophore/λ	Mode of function	Application
PixJ GAFs/*Anabaena sp.* - CyaB1/*Anabaena PCC7120* chimera	PCB (540/650 nm)	Enzyme (AC)	**cAMP production** *in vitro*[244]

[a]Abbreviations: AC, adenylate cyclase; DGC, diguanylate cyclase; GC, guanylate cyclase; ICC, intramolecular conformational change; PCM, photosensory core module; PDE, phosphodiesterase; PPI, protein−protein interaction.

higher organisms, and also c-di-GMP is a common second messenger in prokaryotic organisms. Considering the c-di-GMP applications, gene expression control could be coupled also to these enzyme activities, as it was found that several transcription factors can modulate their affinity to individual DNA sequences through binding c-di-GMP. These applications will be discussed in section 4.2.

4.1.1. Systems in Bacteria. The first chimeric optogenetic switch to control gene expression in bacterial systems was a red/far-red tool developed and implemented in *E. coli* cells by Levskaya and collaborators in 2005.[175] They engineered a transcriptional system termed Cph8 that combines the cyanobacterial red/far-red light-sensitive photoreceptor Cph1 from *Synechocystis* PCC6803 with the EnvZ histidine kinase domain of the EnvZ-OmpR histidine kinase and response-regulator two component signaling system (TCS) from *E. coli.* Cph8 is phosphorylated in the dark, relaying the phosphate to OmpR that in turn activates expression of a target gene from an ompC promoter. Red light (650 nm) converts the Cph8 photoreceptor into the inactive (unphosphorylated) form, while far-red light (705 nm) leads to dephosphorylation and activation. As a proof of principle, the authors projected a light pattern onto a lawn of cells engineered for LacZ expression, thus yielding a bacterial photograph, as LacZ produces a black precipitate in the presence of S-Gal.[175,176] Further work later integrated Cph8 combined with a synthetic quorum sensing system into a genetically encoded edge detection algorithm.[177] The engineered cells were able to recognize an image/light pattern, process the information (light vs dark), and communicate it to neighboring cells by releasing diffusible signals. Through Boolean logics processing, the cells produced a corresponding visual output of the light/dark edges. This mathematical modeling-guided experimental approach employing optogenetic tools opened up novel perspectives for the engineering of pattern forming and cell-based image recognition/processing systems, synthetic ecosystems, and the study of complex logic and cell−cell communication-dependent regulatory networks.

The pioneering work of Levskaya et al. impressively demonstrated the potential of using phytochromes to regulate cell signaling events in bacterial cells.[175] It also generated awareness of obstacles and the notion that follow-up experiments would require careful design precautions. One paramount challenge is the design of the linker between a photosensory unit (PSU) and a HK when generating a hybrid protein. There is excellent systematic research in the field of the LOV-domain proteins.[178] However, apparently there is not a true 1:1 dubbing of this design principle for phytochromes. The group of J. Wu[179,180] designed a full catalogue of chimeric proteins, reported in two successive publications. In all cases, the authors used the PSU from Cph1 and combined it with a collection of 16 HKs from *E. coli*. It was claimed that for each of these new chimeras five constructs for the linker had been designed and realized. As reporter, a fluorescent protein was placed under the control of the corresponding response regulator. However, as shown for one example (NarX/NarL, a TCS involved in nitrate/nitrite regulation), none of the five linker variants showed convincing discrimination between light and dark conditions (at best a ca. 3-fold increase of marker protein expression under illumination).

The Cph8-EnvZ-OMR showed its usefulness for fermentation processes. Lee et al.[181] demonstrated ON- and OFF-gene

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expression in a (photo)-bioreactor and developed a protocol for routine procedures of protein expression.

A second approach toward light-regulated gene expression control made use of another CBCR of said *Synechocystis* PCC6803, namely CcaS, the sensor of the light-regulated CcaS/CcaR TCS. Direct implementation of CcaS/CcaR in *E. coli* provided reversible green (535 nm)/red (672 nm) light control over gene expression from a CcaR target promoter (cpcG2).[176] A detailed analysis of this system was presented by Davidson et al., implementing pulse-width modulation (PWM)—the response pace to light pulses of various length and intensities.[182] The authors extended the application to the control of bacterial growth by placing *metE* under light control. *metE* encodes an essential methyl transferase converting homocysteine into methionine. Green light induced the enzyme, whereas red light impeded further bacterial growth of the *metE*-genomic deletion strain. A customized version of the system found subsequent application in *Synechocystis* PCC6803 cells for the production of biofuel and biomaterials.[183] Introducing the *Synechocystis ho1* and *pcyA* genes required for PCB biosynthesis from heme into *E. coli* enabled the production of functional phytochromes, not necessitating exogenous PCB addition.[99,175]

These two optogenetic systems based on engineered TCS depict the capabilities and potential in terms of precise spatiotemporal and quantitative control of gene expression. However, these early examples suffered from low dynamic ranges and leaky basal expression levels. In addition, their otherwise pioneering work required combining up to three plasmids in order to bring all necessary components together and is incompatible with strains utilizing *tetR* in genetic circuits, because *cph8* was constitutively transcribed from the *PLtetO-1* in *E. coli*, lacking *tetR*.[184] Thus, in order to streamline the systems and improve their applicability, Schmidl et al. redesigned the light switches.[184] The genetic refactoring strategy included exchange of promoters, optimization of expression conditions, and combination of components into single plasmids, which resulted in lower leakiness and an enhanced ON/OFF switching from about 10-fold up to 72- and 117-fold dynamic ranges for Cph8 and CcaS, respectively, especially by reducing the leakiness. In addition, their improvement also yielded a broader range of applicability and target bacterial strains.[184]

A follow-up study by the same group significantly extended the toolbox of two-component systems.[185] Starting with both systems, Cph8 and CCaS/R, domains between these two systems were swapped and combined by extended mutagenesis to improve the communication between the two domains and the binding capacity to given DNA sequences by addressing the DNA-binding domains (DBDs). The improved OmpR domain was then fused to a set of other DNA binding domains from *B. subtilis* and *Synechocystis*. In these constructs, the variability of the DNA recognition sequences was probed by a series of mutations. A further optimization was achieved by exchanging the sensors of these TCS systems, e.g., by utilizing the sensing domains from *S. oneidensis*. This bacterium responds to extracellular electron acceptors through its inherent TCS. As an output from these screening efforts, a catalog of DBDs was developed with a wide range of possible applications.

The Tabor group expanded the toolbox and chromatic palette of photoreversible light gene switches with a UV-violet/green and a red/near-infrared system. The former uses the same CBCR TCS-based principle, in this case the UirS/UirR pair and the cognate promoter *csiR1* and constitutes the most blue-shifted photoreversible optogenetic tool in bacteria.[186] The latter, in turn, relies on the near-infrared bacterial phytochrome *Rp*BphP1, a bathy bPhy from the purple photosynthetic bacteria *Rhodopseudomonas palustris*. *Rp*BphP1 binds and inactivates the transcriptional repressor *Rp*PpsR2 upon exposure to 760 nm light (BphP1 has a particularly red-shifted absorption maximum in the parental state), and this interaction is interrupted with red light (660 nm). The BphP1/PpsR2 pair was combined with a customized strong target *E. coli* promoter, resulting in the optical switch with the longest activation wavelength among existing optogenetic tools in bacteria.[187]

A number of applications, including gene expression, employ bacterial (BV-binding) phytochrome photoreceptors (bPhys). As all signaling functions of bPhys derive from the C-terminally located enzyme activities, they are discussed in section 4.2.

4.1.2. Systems in Yeast. The first optogenetic tool for the control of cellular processes, namely gene expression, was developed and implemented in yeast. Quail's group harnessed the split transcription factor principle known from yeast-2-hybrid systems to engineer a reversible red/far-red light-inducible gene switch in *Saccharomyces cerevisiae*.[149] Plant phytochrome B (PhyB) and PIF3 were fused to the GAL4 DNA-binding and activation domains, respectively. Gene expression was then driven from a synthetic Gal4-responsive promoter ($Gal1_{UAS}$-LacZ) upon irradiation with 660 nm light and in the presence of exogenously added PCB. The illumination conditions (intensity, pulse duration, etc.) and incubation conditions were later further optimized by Hughes et al.[188] More recently, Sorokina and colleagues engineered an improved system based on phytochrome A and FHY1/FHL (FHY1, far-red-elongated hypocotyl 1; FHL, FHY1-like) with higher induction levels and reduced leakiness compared to the original PhyB/PIF3 pair.[189] Recently, the PhyB-based switch was combined with a customizable synthetic TALE DNA-binding domain to achieve control over the expression of endogenous or heterologous promoters (PhiReX system).[190] The same group further utilized the light switch to establish a Cre recombinase regulation system (L-SCRaMbLE) capable of inducing the activity of a split Cre in response to red light.[191] By substituting a previous chemical estradiol inducer with red light, the authors achieved 179-fold induction of recombination upon light exposure. However, the recombination activity depends on the time and chromophore concentration.

In addition to these highlighted examples, there are several other, more specific applications that will be discussed in the following subsections.

4.1.3. Systems in Animal Cell Culture and *In Vivo* (Animals). The first red/far-red light-reversible transgene expression switch for animal cells transferred the concepts developed in yeast and bacteria to mammalian cells with some important modifications.[192] Relying on the light-regulated interaction of *A. thaliana* PhyB and PIF6, the bistable toggle switch followed the split transcription factor design and consisted of the following: (i) the N-terminus of PIF6 comprising residues 1−100, including the active phytochrome binding (APB) domain sufficient for selective binding to the Pfr form of PhyB, fused to the C-terminus of the *E. coli* tetracycline repressor TetR, (ii) the PAS-GAF-PHY domains of PhyB (residues 1−650) fused to the VP16 transactivation domain from the *Herpes simplex* virus, and (iii) a synthetic reporter construct with repeats of the TetR-specific tetO operator cloned upstream of a minimal human cytomegalovirus immediate early promoter ($P_{hCMVmin}$) driving the expression of a gene of interest. Red light-mediated PhyB/PIF6 interaction reconstitutes the

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functional transcription factor, which is tethered to the promoter by TetR to trigger transcription. A deep characterization of the system showed its broad applicability, among efficient control in different mouse-, human-, hamster-, and monkey-derived cell lines as well as human primary cells. The system further benefits from a high dynamic range of expression induction (ca. 60-fold), low leakiness, and adjustable gene expression profiles through light dose and chromophore concentration dependency. The system provides spatiotemporally resolved control and is fully reversible, and the neglectable dark reversion in animal cells leads to a bistable toggle switch-like behavior. Moreover, the system acted in a tissue engineering approach for the control of neovascularization and angiogenesis upon spatiotemporally resolved red light-induced expression of human vascular endothelial growth factor (hVEGF) in CHO-K1 cells applied over chorioallantoic membranes of chicken embryos.[192]

Another sophisticated PhyB/PIF6 system was designed by Yen et al. for light-controlled DNA manipulation.[193] The authors brought the Cre/*lox*P function under red light control. Depending on the experimental conditions, Cre/*lox*P (Cre = cyclic recombinase) can promote excision or inversion of *lox* sequence-flanked DNA. Still, the control of this function in time and space required further improvement. The method of choice to achieve light-guided control has already been demonstrated in mouse brain tissue and in *Drosophila* utilizing a blue light-regulated switch.[194,195]

Yen et al. split the Cre enzyme into its N- and C-terminal parts and fused them individually to PhyB and PIF6, thereby generating a PhyB-CreC/PIF6-CreN system called CreLite. Recombination of DNA sequences was successfully demonstrated in zebrafish embryos by injection of recombinant mRNAs and PCB early into the one-cell stage. Identification of the individual animals was accomplished by whole-body expression of a red fluorescent protein, whereas spatially localized Cre activity was demonstrated by activating expression of genes encoding fluorescent proteins emitting at different colors in selected LED illuminated light spots. The authors state that their method allows (i) deep tissue penetration for gene expression due to the utilization of red light and (ii) live imaging after Cre-mediated induction.

Injection of PCB in order to generate a functional optogenetic *in vivo* system is accompanied by significant drawbacks caused by the mechanical disturbance of the experimental setup. Biosynthesis of PCB in various cell types was accomplished, e.g., in mammalian cells, complementing the expression system by avoiding the need to supply the chromophore exogenously (see section 3)[100,101,196] and also providing better conditions of apoprotein and chromophore assembly. Transfer of the PCB biosynthesis pathway from bacteria (see section 2.2.1) to mammalian cells initially raised issues due to the compartmentalization of the heme synthesis pathway between cytosol and mitochondria. This problem was previously solved by genetic engineering of HO and PcyA for mitochondrial targeting, where the final steps of heme synthesis occur.[100] Short hairpin RNA (shRNA)-mediated knockdown of biliverdin reductase A (BVRA) that degrades biliverdin IXa to bilirubin and PCB to phycocyanorubin, further boosted the bioavailable cellular PCB concentration in mammalian cells. In a follow-up study, the addition of genes encoding a ferredoxin and a ferredoxin:-$NADP^+$ reductase (Fd and FNR) to this enzymatic machinery additionally optimized this bioconversion pathway.[101] Exploiting their improved PhyB/PIF6 complexing system, the authors dubbed former work targeting cell protrusion formation based on a blue light-activated effect;[197] see section 4.4. Kyriakakis et al.[196] followed this approach and again investigated the bottleneck in the reduction reactions after the generation of BV. Concentrating on PCB synthesis, two different enzymes were used originating from *Synechococcus* PCC 7002 or from *Thermosynechococcus elongatus* termed sPcyA or tPcyA, respectively. The minimal combination was complemented with two further genes of mitochondrial origin, encoding Fd and FNR (ferredoxin:$NADP^+$ reductase) leading to improved PCB yields. The possibility to either rely on exogenous addition of the chromophore or employ a biosynthetic pathway provides additional flexibility. Intracellular generation and immediate incorporation of PCB might be advantageous in many experiments, as it supports proper folding of the protein. Alternatively, if cell culture experiments were performed where samples are usually exposed to environmental (continuous) light, these would not require protection from accidental activation prior to the addition of the chromophore.

In most cases, the engineering of optogenetic tools requires precise knowledge of the functional and dynamic properties of the involved molecular components to facilitate a streamlined design along with testing of different variants and configurations. As exemplified for the the red light-regulated gene switch, the maximal induction rates and leaky expression levels in a given cell type strongly depend, among other cues, on factors and parameters (i) at the target construct: the configuration of the synthetic promoter (number of repeats of the operator sequences and their separation distance from the minimal promoter region) and the reporter gene of choice and (ii) on the structure of the switch itself: domains of the photoreceptor (e.g., PhyB residues 1−650, alternatively employing a fragment spanning amino acids 1−908), fusion of NLS (nuclear localization signal) to it, and expression from individual plasmids vs transcription from a bicistronic expression unit (use of IRES sequences, etc.).[192,198] For instance, replacement of TetR by the *E. coli* macrolide repressor protein (E-protein) or the pristinamycin repressor PiP led to an increase of 2.5- and 1.8-fold in expression of reporter levels in comparison to the TetR-based system in CHO-K1 cells, respectively.[199] Also, alternative interaction partners including orthologs or truncated and mutated variants are possible.[151,200] PhyB-interacting PIF3 and PIF6 can be reduced to around 23−25 residues. The smaller size facilitates the construction of tandem repeat motifs, which may enhance light-dependent responses.[151] This indicates how one can tune the dynamic responses of the switch through modification/exchange of the different constituent modules allowing customization of the system ad-hoc for use in different cellular contexts. It is therefore possible to adjust the expression of the target protein of interest in terms of leakiness and amounts needed. Examples include the expression control of transcription factors for the study of signaling pathways. Here, a tight system is most desired (OFF state should be really off), whereas for the production of a biopharmaceutical in a bioreactor, or the expression control of an enzyme, high levels are usually required. Along these lines, the development of mathematical models parametrized with experimental and available literature data provides a quantitative understanding of the dynamics of the light-inducible systems and can be used to guide experimental designs in terms of the molecular configurations and induction conditions (light intensity, irradiation protocols, pulsing schemes, etc.), resulting in optimal induction characteristics for the application and cell system of choice.[192,201,202]

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Also, the photobiological and functional properties of the bacterial *Rp*BphP1-*Rp*PpsR2 pair were exploited for the engineering of optogenetic tools in animal systems. In particular, *Rp*BphP1 uses the chromophore biliverdin, readily available in eukaryotes, and responds to light of longer wavelength in the far-red/NIR spectral region. The ground state of *Rp*BphP1 (Pfr) photoconverts to the active Pr state, at the highest sensitivity with light of 740−780 nm. Light of ca. 636 nm returns the photoreceptor into the inactive Pfr state, but also dark relaxation contributes significantly to this process. Both forms, however, show a considerable spectral overlap, making periods of darkness essential for full dissociation.[203] Following the approach of the PhyB-based split transcription factor switch, *Rp*BphP1 fused to TetR and *Rp*PspR2 to VP16 with an NLS sequence acted as gene expression system combined with a $tetO_7$ operator upstream of a minimal promoter. Illumination with light of 740 nm led to ca. 40-fold induction rates of expression of a reporter in transiently transfected HeLa cells. The interaction can be interrupted upon incubation in the dark and accelerated with additional 636 nm light. HeLa cells transfected with the system and subcutaneously injected into the interscupulum area of hydrodynamically transfected mice or livers showed the applicability of the system for *in vivo* use, profiting from the deeper tissue penetration and lower toxicity of far-red light.[203] The basic system was later improved by shortening *Rp*PpsR2 to a single-domain, Q-PAS1, which is sufficient for efficient interaction with *Rp*BphP1 and shows reduced oligomerization.[204,205] The BphP1-Q-PAS1 switch was combined in different molecular configurations with the Gal4-UAS system to achieve far-red-light-controlled inhibition of transcription by disruption of DNA binding and to regulate chromatin epigenetic states upon targeted recruitment of an epigenetic modifier to a reporter gene.[204,206]

Recently, a near-infrared optogenetic transcription regulation system has been developed based on a single evolved photosensory core module of a bacterial phytochrome from *Idiomarina sp.*[207] The development termed iLight is relatively small, thus encouraging viral delivery strategies, as demonstrated by the authors using adeno-associated viral vectors. iLight was employed to control gene transcription repression in bacteria and activation in cultured mammalian cells, primary isolated neurons, and intact mouse tissues *in vivo*. Of note, iLight may be used in combined (spectral multiplexed) optogenetic experiments with blue/green light-responsive systems such as channelrhodopsins.[207]

Identification of the CRISPR-Cas9 system revolutionized gene editing experiments. It is a powerful tool to manipulate both DNA and RNA sequences, to adjust the expression of endogenous genes via recruitment of activation or repression factors, or to change epigenetic DNA patterns. Shortly after the initial reports of applications utilizing CRISPR in 2013,[208,209] new studies grew exponentially in number. The outstanding capabilities of this system called for further designs that would elegantly allow control of temporal activities. In fact, systems were developed that converted the "gene-scissors" into optogenetic tools. Bubeck et al. brought the expression of "anti-CRISPR" proteins under control of the LOV2 protein from *A. sativa*, thereby regulating the activity of the CRISPR/Cas system by blue light.[210] Gao et al. compared the chemical activation of a CRISPR/dCas system to a blue light-regulated one. Interestingly, the authors reported a test experiment employing the PhyB/PIF system in their supplementary data.[211] Nihongaki et al. generated a blue light-regulated CRISPR/Cas system,[212] and Polstein and Gersbach presented a similar approach.[213] The first red light-driven activation of the CRISPR/Cas system was reported by Shao et al.[214] In their application, they employed light-regulated enzymes targeting a signaling network to induce gene expression. For this, the far-red/red bPhy of *R. sphaeroides*, void of its PDE domain, was used. Upon illumination, this switch generates c-di-GMP, and when combined with a constitutively active PDE (YhjH), the c-di-GMP concentration is kept at minimum in the dark. For downstream control, the transcription factor BldD from *Streptomyces coelicolor* served as template for a hybrid trans-activator that in the presence of c-di-GMP dimerizes and binds to its tailored, chimeric promotor to initiate the expression of a gene encoding an MS2-p65-HSF2 protein. Together with a constitutively expressed dCas9 variant (d = dead, a mutant lacking endonuclease activity), MS2 recruits the p65-HSF2 transcriptional activator to the DNA target site by binding to an engineered gRNA that harbors the MS2 recognition sequence. Implemented in human HEK-293 cells, far-red light (FRL)-activated CRISPR-dCas9 effector (FACE) went through further rounds of optimization enabling a series of applications. The changes include the MS2 proteins from the synergistic activation mediator (SAM) for enhancement of stem-loop structures. An advantage of the red light-driven system over the formerly described blue light-controlled variants is a deeper penetration of the light in living animals. This became evident when genetically engineered cells, embedded into hollow fibers, were inserted into the dorsum of living mice. A nearly 200-fold increase of the red light system over a 9-fold increase using blue light proved the advantages. The authors also succeeded in activating simultaneously the expression of several genes of interest. Some specifically designed constructs allowed targeting genes for, e.g., muscle mass and regeneration; other constructs yielded promising results in iPSCs (induced pluripotent stem cells), where after adapting the FACE system selectively, NEUROG2 (neurogenin-2, transcription factor involved in neural differentiation) could be up-regulated by red light. Following this treatment, a number of other neuronal markers were expressed. The same group extended the applications of their far-red light activated gene-expression system to gene editing by generating a split Cas9 protein.[215] Once again under control of the c-di-GMP-generating GGDEF domain from the bathy-phytochrome BphS (from *Rhodobacter sphaeroides*), the authors split Cas9 into an N- and a C-terminal half, each separately fused to two proteins from *Clostridium thermocellum*, Coh2 (N-terminal portion of Cas9), and DocS (C-terminal portion) that are known to form a tight heterodimeric complex. The N-terminal portion of Cas9 was furnished with an NLS motif. This construct, NLS-Cas9(N)-Coh2 was brought under expression control of a light-driven c-di-GMP regulated system, whereas expression of the DocS-Cas9(C)-NES construct was driven from a constitute promoter P_{hCMV}. The system had very low background activity in the dark, and a comparison between blue light and (far-) red light irradiation yielded an at least 5-fold better penetration for the long wavelength light source. Application examples chosen by the authors were gene editing in mouse liver hepatocytes and in the PLK1 oncogene using a mouse xenograft tumor model. This proof-of-principle might open the door for promising future research on somatic and stem cell differentiation, *in vivo* gene-editing, and even applications in the field of regenerative medicine.[214,215]

Tabor and co-workers pursued the strong interaction between PhyB and PIF6 and developed a light-controlled system for

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transport of virus particles (adeno-associated virus, AAV) into the nucleus of a mammalian cell.[216] AAV serves as a most useful vehicle for many different subcellular transport processes. The viral genome was furnished with the PIF6-encoding gene (several variations) to expose PIF6 motifs on the capsid surface. Cells expressing the interaction partner PhyB containing an NLS served as a suitable target. With these modifications, the authors found an increase up to 600% of viral particle entry into the nucleus of HEK-293T cells. Considering the well-documented transport properties of AAV, the authors propose their system as well suited for therapeutic research efforts.

In a recent, further improved approach avoiding the need to engineer target cells, Hörner et al. devised a red light-controlled gene delivery strategy based on engineered adeno-associated viral vectors.[217] The developed OptoAAV system allows highly spatially resolved control of gene delivery to mammalian cells, extending to single-cell resolution. The design relies on displaying the active phytochrome binding domain of PIF6 on the viral capsid, by genetic fusion to the N-terminus of the viral capsid protein VP20. Simultaneously, specific mutations ablate the natural tropism of the virus. Light-controlled virus transduction using 660 nm red light into mammalian cells is accomplished using a PhyB-fused adapter protein to mediate specific cell binding to cell surface proteins, e.g., utilizing DARPins (designed ankyrin repeat proteins) as fusion partners. The authors demonstrated sequential and spatially defined delivery of different transgenes into native mammalian cell lines and primary cells with single cell resolution.[217]

4.1.3.1. Orthogonal Control of Cellular Functions: Combining Photoreceptors of Different Wavelengths. Given the fact that biological photoreceptors cover the entire visible light spectrum and beyond, extending to the UV and infrared regions, systems that combine various photoreceptors can generate differentiated responses to individual light conditions, just like plants do. Multichromatic expression systems have been reported for bacteria, combining red and green two-component systems (section 4.1.1).[176] To enable multichromatic gene-expression control in mammalian cells, Müller et al. first developed a strong mammalian cell transgene expression system based on the *Arabidopsis* photoreceptor UV resistance locus 8 (UVR8) protein and then combined it with their PhyB-based expression system as well as a previously described blue light-responsive *Neurospora crassa* Vivid (VVD)-based expression tool (LightON).[201,209] An analysis of the individual subsystem guided by a mathematical model assisted in the identification of multiplexed illumination regimes that enabled multichromatic multigene control by differentially expressing three genes in a single cell culture. The authors applied the combinatorial setup to reconstitute biological processes of neovascularization and angiogenesis. They used blue (465 nm) light to express $VEGF_{165}$ (165-residue splice variant of the human vascular endothelial growth factor) in engineered HEK-293T cells for initiating the formation of new blood vessels in a transwell-connected monolayer of endothelial cells emulating the blood vessel wall. The next day, UVB (311 nm) light was applied for producing angiopoietin 1 (Ang1), leading to blood vessel maturation. Measuring the permeability of the cell monolayer using fluorescently labeled dextran revealed the biological effect of this sequential production of secreted biomolecules: an initial increased permeability correlated with the blue light-mediated signaling of $VEGF_{165}$. The following day, the maturation-inducing effect of UVB-induced Ang1 prevailed and triggered a subsequent decrease in monolayer permeability. Due to overlapping absorbance spectra of the involved photoreceptors, control of the individual targets, however, only works in a sequential time order, starting with the longest wavelength. Shorter wavelength illumination sources would trigger induction of the longer-wavelength photoreceptors (e.g., UV light would also excite VVD- and PhyB-based systems, but red and blue light have no effects on the UVR8-based system). The crosstalk between Phys and other photoreceptors has to be carefully taken into account. It is evident from Figure 1 that the strong absorbance of the Soret band of Phys shows a significant overlap with flavin-based photoreceptors. As illumination into the Soret band has been demonstrated to initiate photochemistry in Phys, a selection of excitation wavelengths in combined applications is essential to clearly discriminate between processes caused by two different photoreceptor systems. To address the bottleneck of the sequential time order and render UV-, blue, and red-/far-red-based gene expression systems truly orthogonal to each other, the authors applied a mathematical model to analyze the requirements for orthogonal triple-wavelength expression control, resulting in the following modifications.[169] First, additional application of 740 nm far-red light suppressed the PhyB system under UV or blue light illumination. Second, orthogonality between the UV and blue systems required the development of a novel blue light system with rapid dark reversion, replacing the VVD-based LightON. The authors identified that light induces the reconstitution of the VVD TF with the on-rate $k_{on}I$ where I is the light intensity. Associated TFs activate the expression of the target gene and dissociate with a constant off-rate k_{off}. Since the time scale of association and dissociation is fast compared to gene expression, the light-dependent TF formation lies in a chemical equilibrium that depends only on the light intensity, on the association constant $K_b = k_{on}/k_{off}$, and on the total number of monomers. The model identified that a decreased association constant of the blue system would result in a lower cross activation by UV light. As a reduced association constant can be achieved either by lowering the k_{on} and/or by increasing k_{off}, the authors resorted to the development of a novel blue-light system. They made use of the TULIP system with rapid reversibility and an active-state half-life time of only 17 s.[218] TULIPs consist of an engineered variant of the LOV2 domain from *Avena sativa* phototropin 1 with a C-terminal epitope tag fused to its LOV2 Jα-helix (LOVpep) and activate through complex formation with a high-affinity PDZ domain upon blue light illumination. Combined with pulsed UV light administration (2 min every 30 min) the authors achieved orthogonal, three-color, individual reporter responses.

Signaling pathways were also independently controlled using photoswitches responding to different light colors. Kramer et al. combined the PhyB/PIF optoSOS system with a CRY2 blue light clustering approach to orthogonally control RAF/ERK and the AKT signaling pathways in mammalian cells.[219]

Verkhusha and co-workers further completed the phytochrome-based toolbox for optogenetic applications by improving the specificity of interacting proteins. They achieved this by combining the BphP1/QPAS1 system with "intrabodies" (iBs).[220] iBs comprise shortened fragments of antibodies of various sizes which are specific in epitope recognition. iB applications without light regulation are well studied. Estimations count about 10^6 antibodies covering approximately 90% of the human proteome and 1.5×10^3 single domain antibodies, thereby representing a library of enormous extent that according to these authors is awaiting photoreceptor control. Adding the absolute specificity of antibodies, the

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authors unreel the entire scenario of optogenetic applications. As a light-gated tool, the above-discussed system BphP1/PpsR2 from *R. palustris* was utilized (section 4.1.1).[221] PpsR2 has been down-sized and made monomeric. The authors demonstrated recruitment of proteins to the plasma membrane and to other cellular compartments, as well as import into and export from the nucleus using NLS and NES fusions, respectively, in HeLa cells. As performed in former studies, the authors present orthogonal applications adding a blue light-activated system to the red-/far-red-responding bacterial phytochrome. They demonstrated that the engineered iBs do not cross-react in this dual photoreceptor setup. In addition, the orthogonal illumination allowed, in a construct combining a *Pps*R2 domain with an NLS-LOV domain, its nuclear import upon blue light illumination. Red light illumination, however, activated membrane-tagged BphP1, leading to translocation of the bifunctional construct to the membrane instead. The specificity of iB-binding was exploited by selective binding of antibody fragments to various fluorescent proteins or cellular factors (example given vhhGFP4 binds to *A. victoria* GFPs but not mCherry) which were genetically tagged to proteins of interest. Finally, the authors also followed former investigations of actin movement control and, as was shown using blue light activation, the guiding of an endogenous GTPase.

4.1.4. Systems in Plant Cells. The implementation of inducible systems for the control of gene expression is essential for the study of plant signaling and metabolic networks. There is a set of chemically regulated switches available. However, as discussed above, these systems suffer from inherent limitations, in particular the poor spatiotemporal resolution and quantitative control and lack of reversibility. The superior properties of light as an inducer have not yet been widely exploited in plant systems, most probably due to the fact that plants need light to monitor their environment and as a source of energy, for growth and development. Ambient light therefore would activate the optogenetic systems. Due to these reasons, the implementation of optogenetic tools in plant systems lags behind in comparison to microorganisms and animal systems. Until recently, only two alternative approaches overcame the experimental limitations and showed proof of principle applications. One is based on a bacterial green light receptor, CarH, and another on PhyB.[199,222] The former can make use of the reduced interference of green light on endogenous light signaling networks, while the latter can be activated by red light but actively turned off with far red-light. The red/far-red light-responsive split-transcription factor switch developed for use in mammalian cells (see section 4.1.3) was customized and optimized for expression in plant cells (promoters, DNA-binding protein, plasmids) and introduced in *Arabidopsis*, *Nicotiana tabacum*, and the moss *Physcomitrella patens* protoplasts.[199,223] The system exhibits a high dynamic range of induction (ca. 400-fold), is very tight in the OFF-state in darkness or under far red-light, and is highly sensitive (high activation rates at light intensities as low as 0.5 μE m^{-2} s^{-1}). A proof of principle application demonstrated the implementation as a tool for studying signaling networks, such as the phytohormone auxin-perception machinery. The light switch permitted the interrogation of the sensing system by controlling the levels (overexpression or downregulation upon expression of an antisense miRNA) of the auxin receptor TIR1, and in combination with a genetically encoded sensor for the phytohormone auxin, it was possible to quantitatively and noninvasively monitor the effects of the manipulation of the system and to understand the fine-tuning of the auxin response.[199] In addition, the system can be used for the noninvasive, chemical inducer-free production of biopharmaceuticals, as shown in this case for the production of hVEGF in *P. patens* protoplasts, a biotechnologically relevant host cell system, orthogonal to most other employed organisms for biosynthesis.[199] The implementation of optogenetic switches based on Phys opens up interesting perspectives for the control of cellular processes in plant cells, as the possibility to revert between ON and OFF states (toggle switch behavior) with two different wavelengths of light permits keeping the system off just by supplementing far-red light in the plant chamber.[224]

A completely alternative engineering strategy was just implemented to overcome the ambient light dilemma. A system termed PULSE (Plant Usable Light-Switch Elements) is insensitive to broad-spectrum white light and activates specifically in red light.[168] In order to tolerate the portion of red light present in ambient light conditions, it combines transcriptional repression and activation of a synthetic bipartite promoter using two different photoswitches (Figure 7). A "Blue-OFF module" uses the LOV-based transcription factor, EL222, that dimerizes and binds to its target DNA sequence under blue light.[225] When fused to the EAR repressor domain designated SRDX, EL222 can inhibit transcriptional activation.[168] Due to the fact that blue light is a significant component in ambient light, the promoter is repressed under standard cultivation conditions, thereby preventing unintended activation. The "Red-ON module" complements the dual-controlled promoter and overwrites the repression of the "Blue-OFF module" when intended. The "Red-ON module" consists of the above-mentioned PhyB/PIF6 photoswitch, so that monochromatic red light specifically activates the promoter. PULSE thus enables effective optogenetic manipulation of plant gene expression under standard growth conditions and has already been successfully applied in plant cells and whole plants. An accompanying mathematical model contributed to a description of the experimental behavior and was used to define conditions for targeted perturbation of a gene of interest with desired amplitude and period. The possibility to perform optogenetic experiments in whole plants despite white light interference gives access to a variety of potential applications, including light-dependent control of plant immunity, development, and CRISPR/Cas9-based technologies.

4.2. Red/Far-Red Light-Regulated Enzyme Activities in Bacterial Phytochromes

The discovery of a bPhy with a dual enzymatic motif embedded in the C-terminus by Gomelsky and co-workers raised strong interest in developing optogenetically controlled enzymes. The seminal paper by Tarutina et al. reported a diguanylyl-cyclase and phosphodiesterase in the bPhy from *Rhodobacter sphaeroides*.[48] Both enzyme activities, designated as GGDEF- and EAL domains based on highly conserved sequence motifs, balance the intracellular concentration of the bacterial second messenger molecule cyclic-di-GMP (c-di-GMP) formed from two molecules of GTP (GGDEF domain) and hydrolyzed by the EAL domain into two 5′-GMP molecules. The c-di-GMP signaling pathway is nearly exclusive for bacteria and might by itself provide relevant properties for its application. In fact, many bacteria, among them human pathogens including staphylococci, streptococci, enterococci, and mycoplasma, do not contain c-di-GMP-dependent pathways. Gomelsky et al. thus suggested to introduce the light-controlled c-di-GMP control into those bacteria to allow an orthogonal control of bacterial

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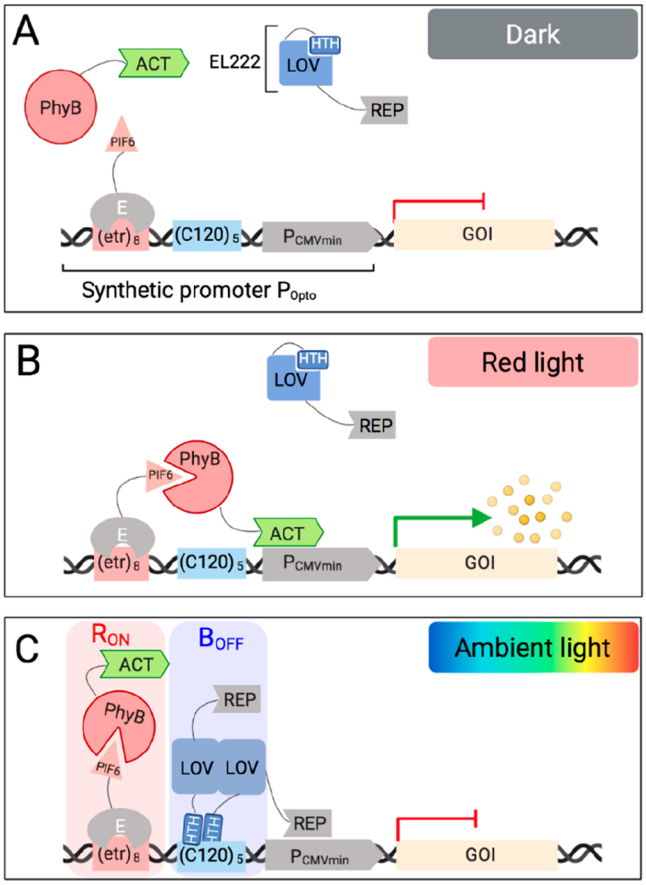

Figure 7. Mode of function of the PULSE system under different illumination conditions. PULSE constructs. The B_{OFF} (B, blue light activated) module is based on EL222 (LOV domain protein). A repressor protein (REP) is fused to its Jα-helix and is set free upon blue light illumination. This B_{OFF} is combined with R_{ON} (R, red light activated), consisting of the first 650 amino acids of the PhyB photoreceptor ($PhyB_{1-650}$) fused to the transactivation domain (ACT) and the DNA-binding protein E (MphR(A)) fused to the first 100 amino acids of PIF6 (PIF_{1-100}). The B_{OFF} and R_{ON} modules are constitutively expressed (promoter $P_{CaMV35S}$). A synthetic promoter P_{Opto}, comprising the target sequence of the protein E and of EL222, $(etr)_8$, and $(C120)_5$, and the minimal promoter $P_{hCMVmin}$ drive expression of the gene of interest (GOI). (A) No illumination applied (Dark): neither of both light-regulated systems is active. (B) Red light: illumination causes binding of PIF6 to PhyB, thereby guiding the ACT domain to the minimal $P_{hCMVmin}$ promoter initiating expression of the GOI. (C) Ambient light (this condition equals selective blue light illumination, not shown) activates the blue light-regulated system that overrides the PhyB/PIF6 system by binding to its genuine C120 operator and blocks the promoter via REP; adapted with permission;[168] copyright with the author.

activity,[226] and they also laid the basis for employment of the GGDEF-EAL domain couple for optogenetic applications (Elbakush et al.[227]). In a most detailed manner, the authors identified the c-di-GMP-signaling pathway in the human pathogen *Listeria monocytogenes*. By demonstrating up- or down-regulation of an entire virulence regulon including PrfA, the master regulator of virulence, they introduced this particular signaling pathway as a target for future optogenetic applications.

This fundamental knowledge on light-regulated GGDEF/EAL enzyme activities served as a blueprint for the generation of several hybrid proteins. In a series of papers, the Gomelsky group expounded a wide variation using c-di-GMP-governed physiological processes. They generated additional flexibility as they brought both enzyme activities under control of two different light qualities. The cyclase activity remained red/far-red light-regulated, whereas the phosphodiesterase function became blue light-regulated.[228] Two homologous proteins from either *Allochromatium vinosum* or *Magnetococcus marinus*, both composed of an EAL- (PDE-function) and a BLUF domain, were studied (BLUF, blue light sensing using FAD, flavin-based photoreceptors). The *M. marinus*-derived protein showed a superior, ca. 30-fold activity increase upon illumination. The authors also proposed the c-di-GMP system for therapeutic applications[229] (see also Shao et al.[230] further below).

Gomelsky and co-workers designed a hybrid protein by fusing the adenylyl cyclase CyaB1 from *Nostoc punctiforme* with the red-/far-red-switching photosensory domain of *Rs*BphP1 GGDEF/EAL.[231] They demonstrated the function of this designed protein by a light-controlled cAMP-dependent behavior change in the nematode *C. elegans*. In this paper, the authors point to the difficulties in combining two domains originating from different proteins, again by heavily varying the length of the connecting linker. In addition, adenylyl cyclases (ACs) of three different origins were exploited. For all linker and AC variations, the photochemical properties were robust and remained unchanged. The architecture of the helical linker originates from LOV domains regulating HK enzymes, although ACs adopt a dimeric arrangement with head to tail orientation.

Etzl et al. functionally exchanged the GGDEF/EAL-couple in the bPhy of *D. radiodurans* with an A-/GMP cyclase from *Synechocystis* PCC6803, Cya2.[232] Cya2 is annotated as a guanylyl-cyclase (GC). Yet, few amino acid exchanges increase its capability to act also as an AC. In this effort generating hybrid proteins, the authors redesigned the photo-to-enzyme linker and could confirm the formerly reported importance of maintaining the helical turn degree for proper function. More than a dozen constructs with different linker lengths were prepared, revealing again the linker of natural length and also with addition of seven amino acids (n+7 helicity motif), leading to highest activity and best light-dark response. Overall, the best dynamic range (ratio of enzyme activity in light vs dark) was just about 4-fold for the AC and 14-fold for the GC hybrid protein (*in vitro* experiments). As further valuable information, the authors also provided a crystal structure of this hybrid protein. This structural information is of particular relevance, as the photosensory module shows a parallel arrangement of the PAS-GAF-PHY domains of both protomers, whereas the cyclase part, as also known from other cyclases, adapts a head-to-tail arrangement. The AC capacity to activate nematode locomotion was then compared to the construct formerly prepared by Gomelsky et al., IlaC22 k27, by generating transgenic animals expressing either cyclase in cholinergic motor neurons (it is worth mentioning here that Gomelsky and co-workers have further improved the yield and velocity of cAMP production of these "Ila"-series of bPhy-induced AC tools).[232,233] Both hybrid cyclases showed comparable light-induced stimulation of the locomotion in the nematodes.

Light-regulation of c-di-GMP-dependent processes using bPhys is remarkably beneficial, as c-dinucleotides precisely regulate important physiological pathways. Gomelsky and co-workers further developed this signaling pathway to control gene expression by switching between red and far-red light.[226] They modified the interplay between guanylyl cyclase and phosphodiesterase by a number of variations, including selected mutations that impact on the photochemical function of this

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protein. Furthermore, they replaced the GGDEF domain by another, more potent cyclase, Slr1143 from the cyanobacterium *Synechocystis* PCC6803. A gene encoding an *E. coli*-derived PDE (YhjH) was additionally added to the bPhy-BV-encoding operon, yielding a nearly complete suppression of the c-di-GMP concentration in the dark. Upon irradiation, the level of c-di-GMP increased more than 50-fold. Having improved the lit-dark ratio, the authors coupled this modified photoreceptor separately to two different gene expression-regulating factors, to the Crp-like protein Clp from *Xanthomonas axonopodis* and to MrkH from *Klebsiella pneumoniae*. Whereas the affinity of Clp to its genuine promotor decreases upon c-di-GMP binding, MrkH has an opposite effect, leading to increased binding. Functionality and light control of both systems could be demonstrated.

The dependence of bacterial lifestyle on the intracellular c-di-GMP concentration was probed by the group of Gomelsky, applying the above-described bicolor approach[228] to the microaerophilic bacterium *Azospirillum brasilense*.[234] The setup evades potential interactions between GGDEF- and EAL-domains by bringing the enzyme activities under control of two different light qualities and thereby offers maximal flexibility for behavioral manipulation. *A. brasilense* actively follows an oxygen gradient and accumulates in thin layers at microaerobic concentrations, yielding a clear readout of physiological responses. Gomelsky and co-workers identified a maze of c-di-GMP-regulated components including transcriptional regulators, riboswitches, metabolic enzymes, and signal transduction proteins. Among those, also the chemoreceptor Tlp1 was identified, which is involved in regulating tactic activity in *A. brasilense*. Tlp1 showed reduced activity in the presence of c-di-GMP, leading to the proposal by the authors that binding of c-di-GMP to Tlp1 via the PilZ domain causes an allosteric conformational change and thereby a reduced sensitivity of Tlp1 toward carbon sources (tested with malate and fructose).

Tabor and co-workers addressed the bottlenecks of the concept from Gomelsky that couples the c-di-GMP concentration to gene expression, among the dependence of approximately 200 *E. coli* genes on c-di-GMP.[187] Instead, they bypassed the action of c-di-GMP and directly coupled a bacterial photoreceptor to gene expression through a very short regulating element. *Rp*BphP1 from *Rhodopseudomonas palustris* is a member of the bathy-phytochromes (adopting the Pfr form as parental state) with one of the most red-shifted absorption maxima (λ_{max} = 760 nm). Instead of utilizing an enzyme activity as devised by Gomelsky et al. in the above-discussed publication, the authors found that this bPhy sports a two-helix motif at its C-terminal end (HOS motif) which interacts with the transcriptional repressor PpsR2 upon irradiation in the Pr state. Binding of the HOS motif inhibits the repressor function of PpsR2 and releases it from the promotor site, thereby allowing selective gene expression. Adaptation of various *E. coli*-inherent promotor sequences to this system led to gene expression control with an outstandingly wide dynamic range reaching up to an 80-fold induction comparing dark and lit states.

The report of direct gene expression control through this two-helix-signaling motif resulted in a remarkable publication by Bellini and Papitz, with initial identification and structural characterization.[235] In the crystal structure (2.9 Å resolution), *Rp*BphP forms an antiparallel homodimer such that the two chromophore-binding PAS-GAF-PHY domains are distant from each other, whereas the two HOS (2-helix output sensors) arrange directly antiparallel. This structural element gave an explanation for the function of *Rp*BphP: it can form a complex with the repressor *Rp*PpsR2 under far-red light and release this protein from the promoter to induce expression of photosynthesis genes under low oxygen pressure or microaerobic conditions.[231] The structure-based proposal of the authors offers two pathways for the Pr form of *Rp*BphP1: either it forms a heterodimer with *Rp*PpsP2, or it remains homodimeric, thereby delimiting expression of photosynthetic genes. This alternative fate was thus termed "light-induced promoter swap" (for details see section 4.4).

A creative experiment performed by Fussenegger and co-workers exemplifies the futuristic potential of optogenetics for integration into complex regulatory biological networks (see section 4.5.4 for details).[236] This approach contradicts the long-standing assumption that cyclic or open-chain dinucleotides were seen as second messengers selectively active in bacteria. However, so far few, very specialized pathways controlled by these dinucleotides have been identified in mammals. Therefore, bacterial phytochromes such as those found in *Rhodobacter sphaeroides* (*Rs*BphG1) may intervene the control of such events via red/far red light-dependent formation and degradation of c-di(G/A)MP. *Rs*BphG1 was thus employed to use light in order to keep c-di-GMP levels low and to override the function of the inherent human PDE activity. This setup enabled the authors to control STING (stimulator of interferon genes) activity, a human sensor for c-di-GMP that in turn regulates a strong response of the immune system with remarkable outcome for future biomedical applications (see section 4.5.4).

Generation of hybrid proteins based on bPhys was also targeted in a study performed by the Möglich group. They had previously reported a detailed analysis on the linker region between the photosensory and the signaling domains in artificial photoreceptors, initially outlined in blue light-sensing histidine kinases.[237]

In another study, Gasser et al.[238] presented one of the still few examples of a hybrid optogenetic tool composed of the photosensory part of a bPhy (*Pa*BphP from *Pseudomonas aeruginosa* and in follow-up experiments from the bPhy of *Deinococcus radiodurans*[239]) and a human enzyme. The authors noted a structural similarity between the histidine kinase module of *Pa*BphP and the human phosphodiesterase *Hs*PDE2A with cAMP/cGMP hydrolysis activity.

Originally driven by small chemical ligands that bind in one of two N-terminal GAF domains, an intramolecular stimulus transmitted through a coiled coil structure-linking sensor and effector activates the PDE, reminiscent of the communication between the light-sensing unit and the histidine kinase domain in the bacterial phytochrome. Despite a high similarity between the linker domains of both full-length proteins, the fusion constructs suffered from insolubility issues that the authors solved by changing the photosensory unit of *P. aeruginosa* to that from *D. radiodurans*. Linker elements of various length showed a clear functional preference when maintaining helicity. Following the *in vitro* functional characterization, the authors succeeded in demonstrating light-regulated hydrolysis in CHO cells using a fluorescence-based assay and finally in Zebrafish embryos. The dynamic range (dark vs light) was about six, still a moderate number; albeit, the finding strongly resembles naturally occurring enzyme activities in phytochromes. Interestingly, no exogenous addition of the BV chromophore was required.

In a recent publication, the same group generated a "zoo" of LAPD-offsprings.[239] C-terminal sequences of some 15 PDE photosensory units were analyzed with N-terminal sequences from five PDEs of different origins for the formation of novel

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hybrid proteins. In the case of the photosensory units, special emphasis was given to a maximal conversion (dependent on the distance of the absorption maxima of the Pr- and Pfr-maxima), thermal stability of the photoproducts, and completeness of photochemical recovery. After *in vitro* characterization, novel hybrid LAPDs were tested in HEK-TM cells in a sophisticated assay: genes encoding various LAPDs were tagged with mCherry-encoding DNA and brought into HEK cells expressing a cNMP-gated Ca-selective channel. The cells were then loaded with the Ca-sensitive dye Fluo4-AM and treated with the forskolin-derivative NKH477 that stimulates intracellular cAMP synthesis. Under these conditions, the Ca-sensitive cNMP-gated channel opens, allowing a Ca-influx into the cells leading to an increase in Fluo4-AM fluorescence. The assay is now based on a reduction of the Ca-dependent fluorescence by illuminating the expressed LAPD-derivatives that cause light-dependent cAMP hydrolysis.

4.3. Applications Employing Cyanobacteriochromes (CBCRs) as Enzyme Regulators

Although representing a protein class discovered just about 15 years ago, with many properties still remaining not fully understood, the wide variety of absorption maxima found in CBCRs has called the attention of scientists to employ the separately expressed CBCR-GAF domains (ca. 150−180 amino acids in length) for generating hybrid proteins. Such approaches may possess several advantageous characteristics of optogenetic applications: the wavelengths of absorption are more red-shifted than those of other photosensory units, e.g., the LOV- and the BLUF-domains, and many of these GAF domains show good to moderate thermal stability of their photoproduct, thereby allowing experiments to continue in the dark after a short light pulse for activation. Most CBCR proteins carry either a HK or a GGDEF-/EAL-enzymatic activity.[172] Hybrid proteins were generated by exchanging these enzymatic domains for an AC domain following impressive results obtained with blue light-regulated ACs.[240,241] A major challenge in combining two domains originating from different proteins resides in an optimally designed helical linker region between both domains. Examples were reported for LOV-domain-containing hybrid proteins.[178,237,242] The combination of a red-green switching CBCR-GAF domain (Slr1393g3 from *Synechocystis* PCC6803) and an AC derived from the cyanobacterium *Microcoleus chthonoplastes* PCC7420 showed a 3-fold enhanced AC activity in the green-absorbing (photoproduct) state.[243] The increase in activity is moderate but comparable to that of the native protein.[241] A similar increase by a factor of 3 for cAMP formation between red and green irradiation has been reported for a hybrid protein composed of a red/green-switching CBCR-GAF domain (from AnPixJ) and the AC domain of CyaB1 from *Anabaena* PCC7120.[244] This CBCR carries three chromophore-binding GAF domains (g2, g3, and g4),[245] of which GAF2 showed functional discrimination for the two wavelengths of irradiation. Interestingly, a blue/green-switching CBCR has recently been reported that carries a blue light-inducible AC function in its natural composition.[246]

The employment of the red/green-switching CcaS/CcaR system by the Tabor group is of relevance (see section 4.1.1).[176] The response regulator of this histidine kinase system, CcaR, directly binds to a promotor site and up-regulates in its native host *Synechocystis* PCC6803 the bilin chromophore production for the antenna system.[247] In this contribution, Tabor et al. used CcaR in a proof-of-principle experiment with the LacZ promotor.[176] This system has been recently improved by the same authors through a series of PAS-domain deletions and point mutations, yielding a third-generation photoswitch.[248] An interesting observation about the minimal size of optogenetic systems was reported here. The CcaS protein is composed of three GAF/PAS domains, out of which only the first one carries a PCB chromophore. However, it can be shortened by removing the two intermittent PAS domains and directly connecting the HK domain to the chromophore-bearing GAF domain. These variations influenced both dark state leakage and green state activation, resulting in a strong dynamic range with an almost 600-fold increase in activity comparing both states.

4.4. Subcellular Localization and Control of Signaling Pathways

Nowadays, the PhyB/PIF system is well established in a broad range of applications. This happened gradually, owing to the experimental characterization of the interaction that allowed fine-tuning, depending on the intended experiments. Rosen and co-workers[249] provided a classical description of the hetero-dimerization parameters and their effect on one of the most fundamental processes in living cells, actin filament formation. Besides providing information on holophytochrome formation (zinc blot), they determined complex formation between an N-terminal PhyB fragment and the APB of PIF3 by gel filtration chromatography along with quantitative binding parameters using ITC-titration (ITC, isothermal calorimetry, K_D = 575 nM). The chosen proof-of-principle application depicted an important signaling pathway that demonstrated the applicability of the PhyB/PIF3 couple, with profound relevance for future experiments. Cdc42 is a Rho-family GTPase known to form a complex with WASP (Wiscott-Aldrich-Syndrome-Protein). In this complexed form, WASP stimulates actin assembly and filament formation through its target, Arp2/3. Arp2/3 is a multiprotein complex serving as a kind of scaffold or chaperon for the attachment of actin molecules to the existing filament. Rosen et al. generated the PhyB chromophore, PΦB, *in vivo* in yeast allowing the production of holo-PhyB. They fused Cdc42 to a truncated version of PhyB (residues 1−651). The PIF3 APB domain comprised the first 100 residues and was fused to WASP. Under regular conditions, WASP undergoes a conformational change upon complexation with Cdc42 and thereby activates the Arp2/3 complex. The same effect, now under rapid light control through the PhyB/PIF3 interaction, could be shown in this *in vitro* experiment. Red light illumination overrides the inherent WASP activation, whereas far-red light brings the system to its regular physiological activity. Actin assembly was followed by monitoring the fluorescence of the added pyrene, a known fluorescent actin-intercalating agent.

The discussed gene expression control experiments based on the interaction of PhyB and PIF proteins (performed by Shimizu-Sato et al. in yeast)[149] were further refined by Levskaya et al. with the purpose of inducing light-driven morphological changes of living mammalian cells.[250] Fusion to the APB domain of PIF6 enabled recruitment of the yellow fluorescent protein (YFP) to plasma membrane-anchored PhyB (residues 1−908). In order to localize PhyB to the membrane, the photoreceptor contained a C-terminal genetic fusion to the CAAX box prenylation motif derived from the Kras protein. By alternating spatial laser excitation with 650 and 760 nm light, the authors demonstrated reversible YFP membrane recruitment over >100 cycles. Reminiscent of experiments previously performed using a blue light-activated system,[197] red light could provoke Rac-

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mediated cellular protrusions (that remained stable after the light was switched off). In this application, PH-DH tandem domains from the Tiam protein fused to YFP and PIF caused lamellipodia formation upon red light illumination (Tiam, T-lymphoma invasion, and metastasis-inducing protein; PH-DH domains, pleckstrin homology-Dbl homology domain; the latter domain is synonymous with the Rho-GEF domain).

Alternatively, when the target proteins were exchanged (ITSN-DH-PH, intersectin, and Cdc42-G-protein), filopodial extrusions could be generated. By employing PhyB constructs of different length, the authors concluded a redundancy for the C-terminal portion in this application. However, in their use case, reversible PIF-binding required the PhyB tandem PAS domains. The authors also proposed to combine their system with intracellular PCB synthesis, an idea which has repeatedly been addressed.[100,101] They dubbed a seminal blue light-regulated work by Hahn, Schlichting, and co-workers, who originally brought Rac1, a small GTPase stimulating actin reorganization and lamellipodia formation, under the control of a LOV protein.[197] Activation with blue light releases a regulating "Jα"-helix. Uda et al. adapted this concept to red light regulation to demonstrate the formation of cell protrusions.[101]

Another contribution of the Verkhusha group demonstrated the utility of bacterial phytochromes for the monitoring of G-protein activity (Shcherbakova et al.).[251] Using a series of rational design approaches led to the development of the monomeric near-infrared (NIR) FP miRFP720, derived from bPhys of *Rhodopseudomonas palustris* (in particular PAS-GAF-truncated forms of *Rp*BphB2). miRFP720 is an offspring of the formerly generated miRFP670 obtained by several rounds of mutagenesis. Both 670 and 720 nm light-emitting proteins constitute a matching NIR FRET couple which is compatible with CFP-YFP imaging and blue-green optogenetic tools. The authors used their probes to build an NIR biosensor for the Rac1 GTPase that allowed combined usage with optogenetic blue light systems to control Rac1 activation in real time. Further on, Shcherbakova et al. combined these near-IR FRET acceptors with various other FP-derived donor proteins to tag interaction pathways, again employing the FRET effect. This setup allowed studying GTP/GDP exchange (i.e., G-protein OFF function) and the interplay between GEFs (G-nucleotide exchange factors) and GDI (G-nucleotide dissociation inhibitors). Multiplexing the light-sensitive tools (i.e., combined use without interference), antagonistic activity of Rho and Rac1 could be demonstrated. Diving deeper into this regulatory network, the authors disclosed the activity of ROCK (Rho kinase activities); also, these kinases are paramount for the precise regulation of Rho and Rac proteins. The long wavelengths for excitation and emission of these bacterial phytochromes facilitated combined application with a blue light-absorbing LOV domain system.

Graziano et al. made use of PhyB for membrane recruitment of PIF fusion proteins to control PIP_3 (phosphatidyl-inositol-(3,4,5)-trisphosphate) signaling pathways.[252] Instead of directly controlling GTPases such as Rac1, the authors focused on the downstream events caused by the β,γ-heterodimer after dissociation of the G-protein to initiate the phosphorylation of PIP_2 to PIP_3 via recruitment of the phosphoinositide-3 kinase (opto-PI3K). To achieve this aim, they fused PIF to an iSH2 domain that binds the regulatory p83 domain of PI3K. A relevant activity under control of this cascade is the guanine-exchange factor P-Rex1 that activates Rac and opens the door to actin polymerization. Their approach made use of pulsing or continuous light stimulation, an advantage intrinsic to optogenetic systems. The injection of chemicals, for example, permits such stimulation regimes because chemicals suffer diffusion and the stimuli are difficult to remove once added. Interestingly, persistent light-driven generation of PIP_3 resulted in a transient increase of selective Rac activity, a clear finding for adaptative circuits regulating this biological system.

Toettcher et al. employed the opto-PI3K system for establishing a computer-aided real-time feedback control system that adjusts the local light intensity to compensate for cell to cell variability.[253] Their computer-aided feedback controller first performs microscopic monitoring of the extent of membrane PIF recruitment via fluorescence. As this process happens instantly, the controller then automatically adjusts the local intensity of the 650 nm LED via proportional integral control, generating a customized activity across an area, thereby compensating biological variation.

Yousefi et al. used the PhyB/PIF6 photoswitch for extracellular stimulation of T-cells, major constituents of the immune system.[200] The T-cell antigen receptor (TCR) on the surface of T-cells recognizes foreign molecules derived from pathogens with high specificity. According to the kinetic proofreading model, T-cells discriminate self from foreign ligands by the different ligand binding half-lives to the TCR. The authors used the reversible interaction of the photoswitch to challenge the kinetic model by defining the time a ligand may bind to the receptor using red and far-red light control. Here, the light intensity determines the cycling rate and thus the binding duration. They genetically modified the TCR by fusion of a short PIF6 fragment to the extracellular receptor moiety and irradiated the cells with light of 660 nm and/or 740 nm while using a tetrameric variant of the PhyB photoreceptor, which was recombinantly produced in *E. coli* (see also section 4.5.5). Assisted by a mathematic model, the authors found that the kinetic proofreading at the TCR occurs with a half-life time of 8 s. Additionally, the authors could reveal a cross-TCR antagonism using their optogenetic assay in a follow up publication.[254] Their work was further accompanied by a detailed protocol describing the experimental steps required to produce the tetrameric ligand for the opto-TCR system, to measure ligand−TCR binding by flow cytometry, and to quantify T-cell activation by calcium influx.[255]

Yang et al. used the budding yeast as work horse for a systematic approach to improve and fine-tune the PhyB/PIF couple.[256] The authors reduced the size of both proteins (PhyB: residues 1−908, PIF6: residues 1−100) and selected 20 different organelle-specific proteins as targets for anchoring PhyB (tagged for identification with mCherry). Twenty different locations were chosen, including plasma membrane, cytoplasm, nucleus, nucleolus, bud neck, myosin ring, spindle pole body (SPB), endosome, and peroxisome. Nine were selected for further work, as the other candidates caused growth behavior or insufficient anchor capacity. Light-regulated ON and OFF reactions took place within seconds with slight retention when the anchor position was at the inner side of the nuclear membrane. This may be due to the fact that the fluorescent tags themselves show a relatively large molecular mass and the nuclear pores allow ready passage of the components. The complex formation could even be followed in a quantitative manner by "titrating" the reaction with various ratios of red and far-red light (650 nm over 750 nm). Interestingly, anchoring of the PIF construct was successful also within the endosome (targeting protein Snf7), but light-induced release from PhyB was not observed; the authors

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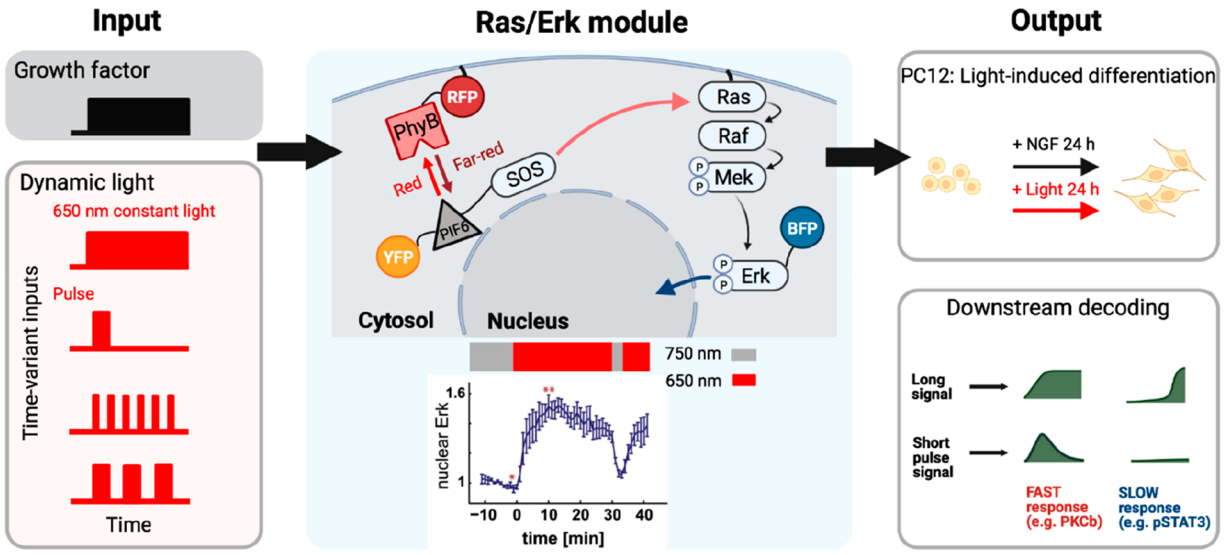

Figure 8. Opto-SOS: Engineering a light-gated switch to drive Ras activation. Left (Input): Activation of the Ras/Erk pathway either by addition of a growth factor or by light in continuous or pulsed mode (time variant inputs). Center (Ras/Erk module): Light drives the heterodimerization of membrane-localized PhyB with a cytoplasmic PIF6-tagged SOScat construct, leading to Ras activation and nuclear translocation of BFP-Erk2; PhyB, PIF6, and Erk are tagged with red, yellow, and blue fluorescent proteins. Right (Output): top, stem cells (PC12 Opto-SOS) differentiate after stimulation with either 100 ng/mL NGF or red light, measured by neurite outgrowth after 24 h; bottom, selective activation of individual modules by the Ras/Erk pathway, PKCβ, or pSTAT3, depending on the mode of light activation (long-time signal or pulsed signal); PKCβ is a member of the canonical MAPK cascade proteins that shows prompt response upon MAPK activation, and STAT3 is member of the STAT family, responding slowly to Ras/Erk signals; adapted with permission from Johnson et al.[261] Copyright *Cell*, Elsevier.

speculate that the PIF protein is retained in the endosome. Yang et al. also applied their location control system to regulate gene expression by making use of the galactose (Gal) signaling pathway. Gal80 is a transcriptional repressor for Gal-responsive genes. The presence of galactose removes the repressor from the promoter, initiating transcription of Gal-responsive genes. In the absence of galactose, Gal80 rapidly shuttles between cytoplasm and nucleus. To control this metabolic pathway, the authors bound PhyB to the plasma membrane and linked Gal80 to PIF. In fact, dependent on the wavelength applied, Gal80 localized inside or outside of the nucleus and Gal-responsive genes were switched on or off.

In this same publication,[256] the authors accomplished cell cycle of *S. cerevisiae* by fusing Clb2 to PIF. Clb2 is the primary mitotic cyclin in budding yeast, which accumulates during G2 and M phases and degrades at the end of mitosis. Clb2 is widely distributed in the entire cell. It forms a complex with Cdk1 (Cdc28), which controls a wide range of substrates to coordinate the early stages of mitosis and progression from the metaphase-to-anaphase transition. In brief, when Clb2 is recruited to the nucleus, it has an impact on the nuclear fission essential for delivering genetic material to the daughter cell. Clb2 regulates this process together with several other factors (e.g., Clb1). In a manipulated strain, thus, light control regulated the activity of Clb2. Under far-red light (system OFF) these cells divided as expected. Under red light illumination, however, more than 83% of all cells failed to split into two nuclei. Similar effects could be triggered by recruiting PIF-linked Clb2 to the spindle pole body. Using the well-characterized budding yeast, the authors demonstrated impressively the facilities of the PhyB/PIF complex.

Reichhart et al.[257] found a strict switch between monomeric and dimeric forms of Cph1 (after removal of the inherent C-terminal histidine kinase module) following red/far-red light irradiation and exploited this property to light-regulate an important pathway in human cells. They codon-optimized the Cph1-encoding gene, yielding an improved expression without cytotoxicity in HEK-293 cells and fused it to the fluorescent protein mVenus. Encouraged by these results, they targeted the important, strictly dimerization-dependent signaling pathway of receptor tyrosine kinases (RTKs). These receptors are known as major regulatory components in cell growth control and important targets for cancer treatment therapies when malfunctioning, e.g., due to mutations. As RTKs carry a membrane-intrinsic element, the Cph1 portion was fused to the C-terminal, intracellular part of two ligand-dependent tyrosine kinases (the fibroblast growth factor receptor 1 and the neurotrophin receptor trkB). This led to a significant light-dependent activation of the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) or phosphatidylinositol-3 kinase/Akt (PI3K/Akt) pathway by more than a factor of 20, compared to the recorded value in the dark. To make the receptor inert to its natural stimulus, the authors replaced the extracellular domain of the receptor with a myristoylation domain.[257] As a control, the authors compared their Cph1-based system with a dimerization-inducing modified FK506 ligand. They found that no FK506-driven dimerization occurred in the dark, but in light they clearly documented the phytochrome-catalyzed effect. Also, they demonstrated the preference of red over blue light-activated systems using synthetic tissues mimicking muscle, skin, and skull models.

Ras and Erk proteins became a hot spot for optogenetic control, due to their paramount relevance for regulating important physiological processes. Also the Toettcher group addressed the complex, again employing the PhyB/PIF system (a detailed protocol has recently been published).[258] The authors developed a screen to assay transcription and downstream control of the involved proteins.[259] In mammalian cells, the Ras/Erk pathway modulated the expression of immediate early genes (IEGs) at one of several hierarchically arranged

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nodes. The expressed gene products have been identified as key components of several responses, including learning capacity and memory, cell proliferation, and initiation of cancer formation. Transcription of these genes rapidly peaks ca. 1−2 h after initiation, making them well-suited targets for light-induced regulation. Several stimuli influence this pathway, among them a genuine activator platelet-derived growth factor (PDGF). Stimulation of the RAS/Erk system by light using the membrane-attached PhyB construct and a PIF-tagged Ras activator (Opto-SOS)[260] yielded nearly exclusive stimulation of the Ras/Erk pathway. A comparison with an alternative activation using the PDGF growth factor showed that both stimuli caused comparable transcription levels of early genes. Adding dose−response studies and frequency−response experiments, the authors presented a very prescient project (Figure 8). As discussed above, an advantage of optical systems is the possibility to apply pulsed illumination. Interestingly, regular light pulses yielded stronger responses of the expressing cells compared to continuous illumination or irregularly applied pulses. For comparative experiments, the authors relied on population-averaged RNA sequencing.[260] This method did not suffice to produce data for individual genes, leading the authors to assay also the gene products of several IEGs. Here, they could clearly identify additional regulatory mechanisms conducted through regulating the phosphorylated or unphosphorylated state of the Erk protein, which initiates a negative feedback mechanism. Such experiments that unravel outstandingly complex and interlaced regulations of gene expression and protein fate again demonstrate the potential of phytochrome-based optogenetic tools.

Interestingly, in a follow-up study, Toettcher et al. compared their previous PhyB/PIF-based experiments with a blue light-driven setup.[261] In *Drosophila* embryos, their results revealed that the blue light system, which was based on a LOV domain from *Avena sativa*, yielded better control with superior precision and reproduction than the Phy-based setup. The authors followed embryogenesis during the first 4 h after fertilization, driven—as in their former study—by the master regulators for development, the Ras/Erk proteins. The authors state the low and unstable maturation of the phytochrome component accompanied by the challenge to inject the PCB chromophore as drawbacks of the red light system.

The paramount role of tyrosine kinases in a number of physiologically relevant pathways and the attempt to bring these major regulators under light control has also been picked up by Verkhusha and co-workers,[167] who have recently discussed the pros and cons of the ensemble of light-activated kinases in two comprehensive reviews.[262,263] The authors fused two different receptor tyrosine kinases to the photosensory module (PAS-GAF-PHY) of the bPhy from *D. radiodurans*. Alternatively, they extended the photosensory part by adding the coiled coil motif of the C-terminal histidine kinase. The chosen RTKs, TrkA and TrkB, as most other members of this protein family, are membrane-associated proteins with at least one α-helical transmembrane motif (Trk, tropomyosin receptor kinase). They activate through homodimerization, accomplished upon ligand binding to the extracellular portion, in particular neurotrophic growth factors. In the active form, Trks then trigger downstream phosphorylation cascades and have been shown to initiate tumor formation upon loss of regulation. Here, the authors circumvented the dimerization through ligands but instead brought the cytosolic, enzymatically active fraction of the Trks under red/far-red light control. They generated "opto-kinases" with activities comparable to their native, ligand-gated siblings. It was possible to reversibly light-regulate the PI3K pathway, calcium signaling, induce neurite outgrowth in PC6-3 cells, and activate MAPK/ERK signaling in cells implanted in mice. Moreover, the TrkA-based opto-kinase could induce apoptosis in neuroblastoma and glioma cells but not in other cell types, including neurons. As a proof of principle, the authors added the *As*LOV2 blue light receptor to demonstrate multiplexing two optogenetic tools in the same cell.

The numerous published applications that employ the PhyB/PIF system to recruit proteins to membranes or intracellular compartments for controlling diverse cellular activities corroborate the versatility of the approach. Rapid photoresponse and dual-wavelength-controlled reversibility to precisely control ON and OFF states, combined with a high binding constant of the couple and the possibility to combine it with UV to green light-responsive tools, are major hallmarks of the phytochrome optogenetics.

The key property to reversibly switch phytochromes between the Pr and Pfr state, and thus the PIF interactions with dual-wavelength control, stimulated the employment of these red light systems for a broad range of applications where reversible control is of advantage among the control of protein localization across organelle borders. In natural signaling cascades, proteins often shuttle between cytosol and cell nucleus depending on the signaling status. The nuclear pores thereby serve as a barrier separating proteins with nuclear from cytosolic activity. For example, transcriptional regulators or modulators thereof often utilize this regulatory principle. In fact, phytochrome signaling in plants involves light-regulated nuclear/cytosolic shuttling of the photoreceptors themselves. This was most prominently studied for PhyA and the associated transport facilitators FHY1 (far-red elongated HYpocotyl 1) and FHY1 Like (FHL).[264] But also PhyB undergoes a translocation into the nucleus upon binding to PIF proteins; this has successfully been demonstrated *in vitro* using isolated nuclei of the unicellular green algae *Acetabularia acetabulum*.[265] These findings inspired the use of the natural nuclear transport mechanism in mammalian cells.[266,267] Here, coexpression of PIF3 combined with red light illumination resulted in nuclear import of PhyB (residues 1−908) fusion proteins either containing mCherry or an artificial TetR-based transcription factor. Interestingly, PIF3 formed nuclear bodies of enriched local concentration that colocalized with nuclear imported PhyB. These substructures were resolved after illumination with far-red light. Further engineering of PhyB with a nuclear export sequence allowed reversible nuclear protein transport using sequential illumination with red and far-red light to repeatedly shuttle proteins between cytoplasma and nucleus over several cycles. The authors made use of the nuclear import-based gene expression system to demonstrate spatially confined expression of an FP using a cellphone-to-cell communication. For this, the authors placed cells on a smartphone display to project a red image. Their work also documented the first application of the PhyB/PIF system *in vivo* in zebrafish embryos.

Buckley and co-workers addressed in great detail the challenging question of vertebrate development control by light[268] by delineating a work protocol using zebrafish embryos as a model system.[269] Here, they pushed further the question of spatiotemporal control, while still facing the drawback to inject the chromophore PCB externally. Tagged with fluorescent proteins, these authors furnished a PhyB fragment (PAS-GAF-PHY) with the CAAX membrane attachment to show

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recruitment of PIF-fused constructs upon red light illumination.[269] In addition, they demonstrated the similarly rapid and efficient release from the membrane, initiated by far-red light to switch the system off. The authors selected Pard3 to apply the localization control with light. Pard3 is a major regulator in animal (zebrafish) embryo development contributing to epithelial development in many systems. It localizes gradually to the tissue midline prior to lumen formation during neural tube development. A Pard3-EGFP-PIF6 fusion protein localized to apical rings in neuro-epithelial cells and enveloped layer cell membranes, similarly to endogenous Pard3. Red light illumination induced sequestration of the Pard3 construct to plasma membrane-localized PhyB and concurrent depletion from its previous location. Pard6, a natural binding partner of Pard3, colocalized to the recruitment spots, suggesting that the PIF-fusion did not influence their natural interaction. The authors further interfered into cell division by asking whether cells would inherit this Pard3 protein asymmetrically upon irradiation. Localizing the Pard3 protein through its PIF tag to one selected location of the cell membrane during cell division led to passage of a majority of the Pard3 constructs to only one of the daughter cells. Optogenetic control of protein inheritance during mitosis provides one example of ample possibilities to gain precise control over cell and developmental biology processes *in vivo* and even in multicellular organisms.

The increasing number of highly variable applications that make use of the PhyB/PIF couple witnesses the versatility of the optogenetic approach. Allard et al. published a further example targeting an entire complex of cell development regulations.[270] They observed that under certain conditions, cells grow larger than normal, for example in tumors, when cells lack functional size-homeostasis pathways. The authors hypothesized that they could obtain control over cell size regulation in the yeast *S. cerevisiae* by transiently blocking bud initiation. In yeast, the process of budding initiation (daughter cell formation) is relatively well understood and likely regulated while passing through cell cycle states (S/G1/G2/M). A main regulator in cell size homeostasis is Bem1, a cell polarity factor that localizes to one pole of the cell initiating budding. The authors fused Bem1 to PIF6 and furnished PhyB with a mitochondrial membrane anchor, aiming to purposely mis-localize Bem1 upon red light illumination. As control for their experiments served a temperature-sensitive *cdk1* allele (cyclin-dependent kinase 1), known to lack the capacity to enter the S-phase. In fact, red light illumination of the cells recruited PIF-tagged Bem1 (opto-Bem1) to the mitochondrial membrane and caused continuous isotropic growth instead of initiating the budding process. Switching to far-red light released optoBem1 within seconds into the cytoplasm and rapidly (within minutes) caused return to cell homeostasis and initiation of budding and cytokinesis. Controlling illumination conditions at various different time points led the authors to conclude that besides a cell size marker, a timer must also exist that regulates the S/G2/M duration across the full range of daughter sizes.

The group of Kapitein addressed the transportation of cell organelles using size-reduced variants of PhyB and PIF6.[271] The authors fused the two proteins of the light switch to kinesin and dynein motor components. PhyB connected to Rab-11, a small GTPase that induces cell trafficking, and PIF6 was fused with kinesin-3 derived Kif1. Using red light, the authors distributed recycling endosomes that were tagged with the PhyB-Rab-11 fusion construct in the cytosol of COS-7 and U2OS cells. More than 96% of all tested cells showed indications of cargo repositioning in response to red light. Exchange of Kif1 by another member of this protein family yielded similar results. Fusing PIF to BICD, which adheres to the nuclear membrane and recruits dynein, caused accumulation of the endosomes at the microtubule organizing center (MTOC). Additionally, combination with the LOV domain-based TULIP system[218] enabled independent repositioning of different organelles.

A recent study demonstrated cell differentiation in an *E. coli* model based on the CcaS/R system developed by Tabor et al.,[176] proposing that this proof-of-principle work will yield valuable information also in other, higher organized organisms.[272] Bowman, Gomelsky, and co-workers employed *E. coli* as an organism that under regular conditions generates daughter cells identical to the mother cell. In contrast, many bacteria commonly show differentiation upon cell division such that, e.g., a supporting mother and spore cell (*B. subtilis*) or a sessile mother and motile daughter cell are generated, as is the case in *C. crescentus*. Such asymmetry can be pinned to the number of proteins that are located in one or the other end: 383 in *B. subtilis* or 135 in *C. crescentus*. The authors of this study speculate that it might be possible to induce asymmetry upon cell division and furthermore to control that process by light. A master regulator in *C. crescentus* is PopZ, a pole-forming scaffolding protein that forms self-aggregates and recruits other proteins to one pole of the future daughter cell. Also, in *Cc*PopZ-transformed *E. coli*, PopZ aggregates locate close to one pole (under conditions of moderate to low concentrations). In order to control the concentration of c-di-GMP, the authors added two additional components to the assay, a diguanylyl cyclase (DGC) and a phosphodiesterase (PDE). The latter gene was fused to *popZ* and brought under control of a light-dependent promoter. The authors selected the c-di-GMP concentration as tunable tool, as many physiological processes are controlled by this second messenger, making a control of the intended asymmetry readily detectable. It was thus possible to generate daughter cells with high or low c-di-GMP concentrations. A first biotechnological application of induced asymmetry—after addition of two genes essential for the synthesis of neutral lipids to the system (*acyl-Co-A reductase* and *wax ester synthase* from *A. baylii*)—was the generation of "normal" cells that generated neutral lipids. The above-described PopZ-PDE construct was also cloned under control of the *CcaR* promoter of the red-green switching CBCR-system, CcaS/CcaR. The test for optogenetic control of asymmetric cell division yielded more than 80% of cells with asymmetric behavior upon activation with green light (pulsed, over 3 h). Applying red light to deactivate the CcaS/R system left less than 10% of cells in a differentiating state. A further proof for asymmetric development was performed with a complemented nonmotile Δ*motA-motB E. coli* strain (Mot proteins are components of the flagellar stator). Against this background, *motA* and *motB* were transformed under CcaS/R control and again rescued motility upon continuous exposure to red light.

4.5. Synthetic Materials, Proof-of-Principle Experiments, and Other Applications

4.5.1. Light-Induced Protein Splicing. Protein splicing is a natural phenomenon where an intervening protein sequence (intein) excises itself autocatalytically from a precursor protein under concomitant ligation of the flanking sequences (exteins). Inteins also exist as split couples where each intein fragment carries one extein, giving rise to protein *trans*-splicing leading to the ligation of independent polypeptides with a conventional peptide bond. *Trans*-splicing intein fragments generally possess

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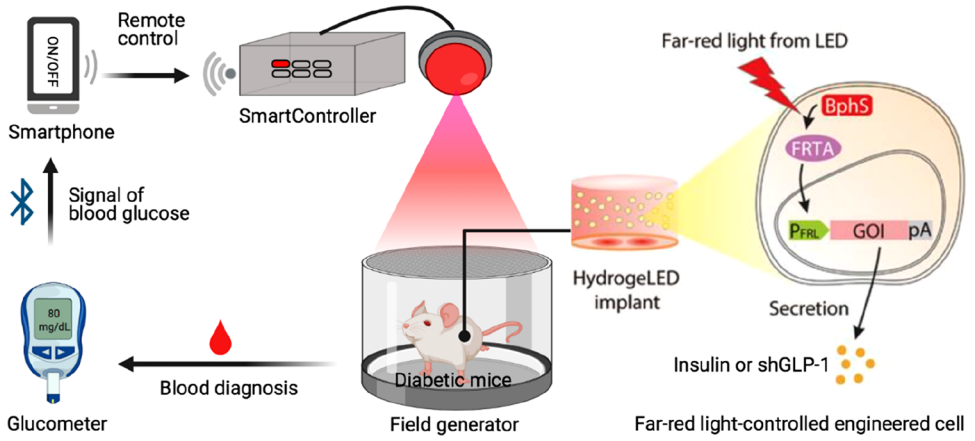

Figure 9. Proof of principle for remote control of insulin levels. Center, far-red light-emitting LEDs were hydrogel-implanted intraperitoneally into diabetic mice allowing them to move freely. Cells with a light-responding insulin-expressing pathway were embedded into hollow fibers that were implanted subcutaneously into the animals. The light-regulated expression system (right-hand part) is based on BphS (FR-activated c-di-GMP cyclase from *Rhodobacter sphaeroides*) and the constitutively active human phosphodiesterase YhjH. BphS in its activated form induces synthetic FRTAs (synthetic far-red light responsive <mammalian> transactivators) that bind to the promoter region and induce expression of the GOI, here insulin or a short variant of GLP-1 (shGLP-1). Remote control (left-hand part): Schematic representation of a programmable smartphone-regulated electronic control system. Freely moving mice exposed to a field generator chamber are blood-controlled for glucose levels; results are transmitted to the smartphone; upon reaching a threshold, the software activates FR-emitting implanted LEDs. Figure adapted with permission (Shao et al.).[230] Copyright: the American Association for the Advancement of Science.

a high binding affinity to each other, so that *trans*-splicing occurs upon reconstitution of the so-called Hedgehog/INtein (HINT) fold. For example, for the two naturally split fragments of the generally well-splicing *Npu*DnaE intein from *Nostoc punctiforme*,[273] a binding affinity of 1.2 nM was reported.[274] However, under certain circumstances split intein fragments may have a lower binding affinity or are less active. Tyszkiewicz and Muir used the PhyB/PIF3 switch to enhance the splicing efficiency of the artificially split *S. cerevisiae* vacuolar ATPase (VMA) intein in yeast cells upon red light illumination.[275]

4.5.2. Photoacoustic Tomography Employing Bacterial Phytochromes. Li and co-workers introduced an interesting application of bPhy proteins that simply capitalizes on their long wavelength absorption and the high efficiency of heat delivery by phytochromes. In fact, even in *in vitro* experiments using plant PhyA, a strong heat release could be determined in the forward reaction upon excitation of the Pr state.[276] The light absorption is not directly detected, but rather the heat release accompanying photoisomerization allows visualization by a variety of opto- or photoacoustic (PA) methods.[277] The authors combined the rapid photoresponse of a bPhy from *Rhodopseudomonas palustris* (*Rp*BphP1) with photoacoustic tomography (PAT).[278] *Rp*BphP1 is a member of the bathy-phytochromes (adopting the Pfr state as the parental state and forming the Pr state as photoproduct) with negligible fluorescence. The authors took advantage of several properties of this system: (i) the most bathochromic absorption properties intrinsic to *Rp*BphP1 with respect to all other chromophores in mammalian cells, (ii) the large extinction coefficients of this bPhy, (iii) the use of ubiquitous BV as chromophore, (iv) the fact that tissue penetration of red light is particularly deep compared to light of shorter wavelength, and (v) the very low photochemical conversion yield, i.e., the very high efficiency of heat release. The Pfr state (λ_{max} = 750 nm) determined the ON state, readily obtained in short time with 630 nm laser light, whereas the Pr state (λ_{max} = 700 nm) switched the protein into the OFF state, obtained by 780 nm irradiation. A direct comparison with other signal-generating components, e.g., oxyhemoglobin (HbO_2), immediately demonstrated the advantage of *Rp*BphP1: at the two irradiation wavelengths (630 and 780 nm), the extinction coefficients of bPhys are approximately 40- and 70-fold larger than those of HbO_2. The *R. palustris* protein also excels compared to other red-shifted derivatives of bPhy, e.g., the nonphotoswitchable iRFP720.

Comparison with other signaling components was initially performed in cell culture (U87 human glioblastoma cells). PAT yielded stronger signals than comparable signaling components, reached deeper into tissues, and enabled identification of an induced tumor in the kidney of mice. Even second-generation metastases could be identified. The method may detect tumors with a minimal size of about 300 μm. This corresponds to the detection of approximately 3000 U87 cells, based on an average U87 cell size of ca. 10 μm.

In a follow-up study, again dedicated to PA, the authors improved the instrumental setup and compared the constructs derived from the *Deinococcus* bPhy with those from *Rhodopseudomonas*.[277] In the same publication, the authors further developed the photoacoustic application by generating a split derivative of the *Deinococcus* bPhy, where the PAS- domain was separated from the GAF-PHY domains. Light-regulated reassembly of this variant allowed monitoring the interaction between target proteins using photoacoustic methods.

By changing the photoswitching protein in PA to Agp1 (bacterial phytochrome from *Agrobacterium tumefaciens*) and improving the excitation and analysis parameters, Märk and coworkers[279] obtained high resolution 3D images of tumors in cultures of human cells. Advanced hardware that involved a constantly switching dual-wavelength irradiation combined with a computed signal analysis significantly contributed to their success. As the authors point out, permanent switching between two states requires high quantum yields, which makes Agp1 with a factor of >10 for the "forward" and the "backward" photoreaction yields well suited for this application.

4.5.3. Bioprinting. Huang et al. devised a method for optogenetic bioprinting of microbes. They employed one of the genuine functions of c-di-GMP, i.e., controlling biofilm

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formation.[280] Separately controlling the cyclase and phosphodiesterase activities in the *Pseudomonas aeruginosa* bPhy, with red and blue light, respectively, yielded a bicolor-regulated c-di-GMP switch which guided aggregation of *P. aeruginosa* according to the projected illumination patterns. As single cells could be addressed, the authors estimate reaching a spatial resolution of ca. 10 μm.

4.5.4. Designing Future Medical Applications. The first medical applications relying on optogenetic control of c-di-GMP-dependent pathways were recently reported by Shao et al.[230] These authors presented a state of the art study: they combined the bPhy-based c-di-GMP synthase, which shows nearly no activity in the dark, together with a constitutively active phosphodiesterase YhjH. The resulting c-di-GMP regulation system highlights extremely low cyclic messenger concentrations in the dark.[230] This system was combined with a second module comprising a c-di-GMP-binding transcription factor BldD fused to a trans-activation domain. This construct binds to DNA in response to increasing c-di-GMP concentrations. The following medical application was designed outstandingly sophisticated: cells expressing this light-regulated system were embedded into hollow fibers of hydrogel capsules, which were subcutaneously injected into a diabetes-developing mouse model. The implant further contained far-red light-emitting LEDs, which could be activated in an electromagnetic field. Having cloned the genes responsible for the generation of mouse insulin and the human GLP-1 protein under the control of the above-described transcription factors, the authors succeeded in decreasing the blood glucose concentration upon irradiation with far-red light (Figure 9). With a futuristic vision of smartphone-assisted semiautomatic treatment of diabetes, the same authors (Shao et al.)[230] coupled a digital glucometer to a smartphone activation- and control-software for regulating illumination conditions. Gomelsky[229] foresees people roaming on the streets wearing fashionable LED wristbands under the control of a smartphone irradiating implanted cells engineered to produce genetically encoded drugs.

A fascinating study, alluded to briefly in section 4.2 with far-reaching future application potential, was presented by Fussenegger and co-workers.[236] These scientists employed a PDE-deletion variant of a bacterial phytochrome from *Rhodobacter sphaeroides* (*Rs*BphG1). This protein binds BV as chromophore and entails both enzymatic activities, a diguanylyl cyclase, and a phosphodiesterase (GGDEF and EAL domains). Thus, the formation and degradation of c-di(G/A)MP can be controlled via red-/far red illumination. Removal of the bacterial PDE domain ensured that no cross-talk between the generating and degrading domains of *Rs*BphG1 might interfere with the designed experiment. In fact, control experiments clearly demonstrated a light intensity-dependent generation of c-di-GMP that, after switching the light source off, rapidly returned to a minimal basal level. This was apparently caused by inherent non light-regulated PDEs. In contrast to the long-standing notion that cyclic-dinucleotide-dependent pathways are characteristic for bacteria, there are di(G/A)MP-dependent processes in human cells. Cyclic-di(G/A)MP molecules are formed in human cells by enzymatic activity of cGA synthase that identifies cytosolic DNA as a substrate, thereby generating an "alarm" signal.[281] The subsequent pathway responding to these c-dinucleotide-signaling molecules belongs to the human innate immunity that responds through STING (stimulator of interferon genes), a cyclic-di nucleotide sensor. In brief, activated STING triggers phosphorylation of the interferon-regulatory-factor-3 (IRF3) by the tank-binding kinase-1, upon which IRF3 translocates to the nucleus to bind to IRF3-specific operators and induces type-1 interferon promoters. This regime seemed promising to probe the function of a bacterial phytochrome in mammalian cell cultures or in living mammals. Thus, the authors inserted *Rs*BphG1 into one such pathway instead of relying on the inherent, human nonlight-regulated PDEs, thereby maintaining the c-di-GMP concentration light-controlled at a basal level. Using this engineered light-controlled pathway, the authors constructed a sophisticated experimental setup based on the light-regulation of the c-di-GMP second messenger. An EEG (electroencephalography) head-cap captured brain activities of pretrained volunteers and forwarded them to a computer interface for translation into a correlating electrical current controlling connected LEDs for adjusting the activity of the optogenetic system. Test persons were trained for biofeedback (keeping a value of the mediation-meter in a defined limit), concentration (computer gaming), and relaxing (meditation). This cybernetics BCI (brain−computer interface) then enabled mind-controlled regulation of transgene expression depending on the mental state of the person. Further refinement of the experiment through combination with a wireless-powered optogenetic implant containing designer cells provided proof-of-concept data for secreted embryonic alkaline phosphatase (SEAP) reporter expression *in vivo*, controlled by the human interferon (hIFN) promoter in a mouse model. This is for sure a proof of principle work, but the futuristic vision of the authors proposes that patients could either learn to generate specific mental states (e.g., pain relief), or rely on disease-related brain activities (epilepsy, neurodegenerative disorders, etc.) to trigger therapeutic implants producing corresponding doses of protein pharmaceuticals in real time.

Another cyclic nucleotide-dependent signaling pathway gave rise to optogenetic control of mammalian sperm beating.[282] A former study demonstrated that sperm motility can be adjusted by blue light employing the light-dependent adenylyl cylase, bPAC.[283] Recent work from the groups of Wachten and Möglich now pushed further these studies on cAMP-controlled signaling pathways by inserting the light-dependent cAMP-degrading phosphodiesterase LAPD into mice sperms.[238,282] These investigations bear high social significance, as in humans approximately 15% of all couples planning to conceive suffer from infertility. In about 30−40% of these cases, the cause of male infertility remains unknown (idiopathic male infertility).

Wachten and Möglich also applied blue light up- and red light down-regulation of cAMP for a functional analysis of primary cilia that are present in nearly all vertebrate cells.[284] Nonmotile (primary) cilia can be considered as antennae that translate sensory stimuli into a physiological cell response and are essential for vital processes in cells. Accordingly, ciliar misfunctions (ciliopathies) are usually severe and cause loss of essential functions. An example given by the authors is AC3, a transmembrane adenylyl cyclase that is involved in many actions of cilia. Mutations or loss of expression of the *adcy3* gene encoding AC3 cause serious consequences, such as severe obesity and high risk of developing type-2 diabetes. A major limitation in understanding ciliary functions is the lack of tools to probe selective regions of the cilium by a certain assay without simultaneously affecting the entire cell body. In this study, the authors overcame this bottleneck and achieved selective control of primary cilia with nanobodies directed against fluorescent proteins which were connected to trafficking epitopes that guide them to the primary cilium. The photoactivatable cyclases

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(PAC) and phosphodiesterases (LAPD) were fluorescently tagged and thus bound by the nanobody without compromising their light-controlled function. The researchers could document that without nanobodies, both PAC and LAPD localized to the cell body. The transport and localization of the light-controlled enzymes into the cilium were also accomplished with nanobodies, where the above-described manipulation of the cAMP concentration could be performed. After demonstrating the successful ciliary localization of the light-controlled enzyme functions in cilia of HEK cells, the authors went one step further to an *in vivo* system: employing zebrafish embryos, they showed the precise localization of the light-controlled activities in zebrafish cilia. Further attempts to regulate the linear growth of cilia by controlling the cAMP concentration were so far not fully convincing.

Another recent study describes optogenetic control of gut bacteria in order to promote the longevity of the host.[285] In this work, Hartsough et al. genetically engineered an *E. coli* strain that secretes colanic acid under the quantitative control of light within the gut of *Caenorhabditis elegans* using the CcaS/R system to induce the colanic acid operon with combined PCB biosynthesis. The compound produced by the non-natural symbiont provokes a local effect in protecting intestinal mitochondria of *C. elegans* from stress-induced hyperfragmentation, which resulted in extension of the host lifespan in correlation with the intensity of green light under which the organisms were cultivated.

4.5.5. Biotechnological Applications and Optogenetic Biomaterials. An "analytical" tool, e.g. for mass-spectroscopic *in vitro* workflows based on the PhyB/PIF6 couple, has been developed by Hörner et al.[152] The analytical protein purification system can maintain identical buffer compositions throughout the purification, thereby minimizing the coelution of weakly bound components. As purification tag for proteins of interest (POIs) served a size-reduced PIF6 protein containing only 22-amino acids that remains capable of binding to PhyB. A PhyB-functionalized chromatography resin captured PIF6-POI constructs under red light (immobilization step), similar to most commonly used affinity chromatography materials. However, the elegant option to release the PIF6-tagged POI with far-red light represents a particularly mild elution condition, yielding highly pure eluates. These highly selective elution conditions are advantageous, e.g., for mass-spectroscopic analysis of cobinders to determine the identity of eventual complexes. As proof of concept, the authors selected a tyrosine kinase (ZAP70), expressed in ZAP70-null Jurkat T-cells, purified, bound to, and released from the column, to identify ZAP70-associated proteins.

An alternative approach contrasting previously mentioned examples to control cellular activities with light relies on the construction of light-regulated biomaterials that interface cellular systems. Due to the dual-wavelength response, the PhyB/PIF photoswitch excellently serves as functional component for developing cell-compatible artificial extracellular matrices that can respond to light, e.g., by releasing embedded factors or changing their mechanophysical properties. The group around W. Weber capitalized on their experience with both optogenetic systems as well as functional biomaterials and developed a generic light-controlled "opto-trap", capable of reversibly binding and releasing biomolecules with high spatiotemporal control.[286] The authors coupled PhyB to various material scaffolds (agarose, glass, hydrogels) and fused cargo proteins directly or via adapter proteins to a PIF6-tag. In this way, they could reversibly release and recapture cargo proteins with spatiotemporal control or subsequently trigger mammalian cell responses that resulted from cargos activating extracellular receptors. The approach also opens new design concepts for the design of complex therapeutics and integrative diagnostic devices that require deliberate control of biomolecule abundance and activity.

Following similar paths, Jia et al. coupled PhyB to artificial extracellular membranes serving as a scaffold for guiding pattern formation and structural assembly processes *in vitro*.[287] The authors used red light to print among other components the bacterial self-assembling FtsZ protein on these membranes and studied nucleation and assembly into filaments and large-scale structures such as artificial rings.

The PhyB/PIF switch has further enabled adhesion control of entire mammalian cells.[288] In this paper, the authors describe how cells expressing a modified integrin cell adhesion surface receptor (OptoIntegrin) adhere to a tailored PhyB-furnished extracellular matrix (OptoMatrix) upon red light activation. The so activated integrins were capable of inducing downstream mechanosensory signaling pathways. As the authors conclude, the PhyB/PIF interaction creates a mechanical force rendering the integrin conformation active.

Yüz et al. also bridged soft and hard matter. The group established a bicolored cell-sorting regime via adhesion to solid surfaces.[289] In a most straightforward manner, they chose the CRY/CIBN for blue, and the PhyB/PIF6 couple for red light selection. CIBN is a truncated helix-turn-helix-CRY2-interacting protein. MDA-MB-231 cells were chosen to demonstrate their design. This cell line is commercially available and well characterized. The cells were stably transfected with pDisplay vectors that anchor POIs, here either CRY2 or PhyB, via the secretory pathway to the cell surface. Via His-tags, the interacting partners CIBN or PIF6 were immobilized to glass surfaces that were functionalized with Ni-NTA groups. The remainder of the glass surface was coated with nonadhesive PEG molecules. Binding of both CIBN and PIF6 was demonstrated selectively through the Ni-NTA/His-tag interaction. All four interacting proteins were fluorescence-tagged to detect their localization. Most elegantly, selective red or blue light irradiation brought either PhyB or CRY to the glass surface; imidazole triggered their subsequent release. The system demonstrated reasonable amplification upon illumination: dark vs light adhesion was enhanced by a factor of approximately 5.5 for the blue light-triggered system and about 4-fold for the PhyB system. The selectivity was also tested: blue light illumination brought about 30% red light-sensitive cells to the surface; the opposite light regime (red light illumination) adhered ca. 15% of the blue-sensitive cells.

To purely control the mechanical properties of polymer materials, Hörner at al. utilized the cyanobacterial phytochrome 1 (Cph1) as the functional component cross-linking poly(ethylene glycol) hydrogels.[290] The synthesized hydrogel materials reversibly respond to red/far-red light exposure with significant changes in their stiffness. Precisely adjusting these parameters served as tool for the study of mechano-signaling pathways responses in human mesenchymal stem cells or to control the migration of primary immune cells in 3D. The approach opened a novel avenue for investigating fundamental questions regarding the way cells react to dynamic mechanical environments and provide a platform for designing remote-controlled tissue engineering devices such as optically controlled drug depots.

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In a synthetic-biological design that combines different protein logics on a material scaffold, the concerted interplay of an "AND"-gate, memory device, and time-delay module yielded a circuit which rendered biomaterials capable of counting light pulses.[291] Calibrated by a mathematical model, the smart biomaterial contains protein cargos embedded via light- and protease-cleavable linkers, allowing the discrimination of initial versus repeated exposure to far-red light. These materials can process input light stimuli and respond with differentiated outputs, a behavior reminiscent to electronics or living systems. The authors demonstrated the release of different proteins which were embedded in the material, controlled by the light pulse counting state of the material. This way, a sequential availability of enzymes released by the material could be controlled using the same stimulus (here far-red light).

The synthesis of phytochrome-based biomaterials usually requires high quantities of recombinantly produced photoreceptors. In order to obtain these amounts, bacterial production systems commonly serve as suitable hosts due to their widespread use, fast growth, simple implementation of chromophore synthesis, and ability for high cell density fermentation. A recent protocol greatly facilitated the production pipeline by optimizing production conditions, yielding multigram scales of recombinant phytochrome photoreceptor protein per 10 L fermentation.[292] In another detailed protocol, the authors further describe the large-scale production of the used biotinylated variant of the photosensory domain of *A. thaliana* PhyB (PhyB-AviTag) in *E. coli* for use in extracellular optogenetics with a single, optimized expression plasmid that combines the phytochrome gene with the genes required for PCB biosynthesis.[293]

Also, the CBCR-based green light-inducible TC-system CcaS/CcaR entered the biochemical toolbox. Nakajima et al.[294] placed the Ag43 gene (encoding Antigen 43, an *E. coli*-derived self-aggregation system) under control of a modified *cpcG2* promoter, addressable by CcaR. Ag43, an autotransporter protein from *E. coli*, is an essential protein for aggregation and biofilm formation during infection. Ag43 is composed of three domains: a signal peptide for secretion into the periplasmic space, a β-domain that forms a selective channel in the outer membrane to transfer an α-domain for extracellular display, and the α domain, which is a linker for self-aggregation. High affinity among the α-domains triggers self-aggregation, which leads to cell precipitation. Green light activated the system and triggered precipitation of cells, visible by OD_{600} reading after 20 min with a plateau after 60 min. This study was performed following the two-plasmid-approach, i.e., generating the CcaS chromophore PCB *in vivo*.

The CcaS/CcaR system seems to gain increasing attention in biotechnological applications. The group of Tabor turned their interest to *B. subtilis*, a well-established production host for generating important proteins and relevant intermediate products targeting pharmaceutical processes.[295] However, employment of *B. subtilis* as biochemical generator requires distinct knowledge of its complex and time-dependent genetic circuits. Tabor and co-workers considered that *B. subtilis* required a set of improvements in order to serve a solid factory for light-controlled bioproduction.[296] While initially working on Cph1, the authors employed the CcaS/R system and boosted PCB production by a set of mutations that altered the promoter site and improved expression of the PCB-generating enzymes. Next, they coupled the optimized photoreceptor system to several well-characterized, *B. subtilis*-specific promoters that were routinely used in biotechnological applications. The authors succeeded in enhancing the fluorescence of a fluorescent protein by a factor of 70 in green over red light-illuminated samples.[296]

The capture of carbon dioxide and discussions on biomass and biofuel generation place cyanobacteria in the center of current ecological and economical green energy projects and topics associated with global warming. The inherent photoreceptor variety of cyanobacteria (here: *Synechocystis* PCC6803) opened a proof-of-principle experiment ready for transfer to other PCB-producing microorganisms. The current application targets photosynthetic organisms. Miyake et al. presented an amazing application employing the genes encoding holin and endolysin under control of the *cpcG2* promoter (see also other applications in section 4.1.1).[297] Holin and endolysin were originally found in the T4 phage and cause bacterial cell lysis upon induction. The optogenetic approach induces the expression through the green light-activated Ccas/CcaR system (see sections 4.1.1 and 4.4). As a result, the authors observed a clearly visible decrease in the growth rate. This was accompanied by a significant increase of cellular phycocyanin release into the medium and a considerable fraction of dead cells. Combined red and green light illumination provoked these effects. However, red light alone did not suffice to induce the system. Performing the experiments directly in cyanobacteria circumvented the need for PCB supply. A preceding publication by the same group described in detail the fine-tuning of the *cpcG2* promoter for the CcaS/CcaR couple utilizing a fluorescent protein (GFPuv).[183] Here, combined red and green light illumination caused a clear increase in product fluorescence compared to red light illumination alone.

4.6. Fluorescent Tools

4.6.1. Bacterial Phytochromes as Fluorescent Proteins (FPS). Phytochromes and related proteins possess an only moderate fluorescence quantum yield compared to commonly used fluorescent proteins (FPs). However, their yields could be increased by changing the chromophore (e.g., PEB for PCB in Cph1 and in the Phy from *Mesotaenium caldariorum*, see also section 2.2.2) or by mutagenesis following a directed evolution protocol.[96,298] These early findings opened the path to further improvement, so far preferentially entered with bPhys. Especially the employment of bPhys as fluorescent markers—despite their low quantum yield—is due to other properties. First, their fluorescence lies in the most red-shifted spectral range within the "transparent window" (Figure 1). Second, they bind biliverdin as chromophore which is available, e.g., in mammalian cells. The fluorescence quantum yield of wt-bPhys, exclusively in their Pr state, is around 3−8%. Mutagenesis approaches led to improvements of nearly 100% (between 8 and 15%).[69] However, the overall fluorescence quantum yield remained low, though applications employing the fluorescence of bPhy (-derivatives) had been performed and recently presented in a comprehensive review.[25]

Since then, improved variations of these bPhys were generated, and new systems were detected and successfully employed worthy of updating the collection of applications.[299] It was particularly the long-wavelength emission of bPhy-derived FPs that allowed combination with other FPs and tissues or cellular compartment-specific multicolor detection. This was accomplished by Cook et al., who used "Brainbow" for their studies, a set of multicolor FPs.[300] They performed screening assays in developing brain regions of zebrafish embryos to

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identify optimally suited far-red-emitting FPs. Part of their investigation concerned photobleaching effects of selected far-red emitting FPs, an often neglected aspect.

With all variations, one may assume that the upper end of fluorescence quantum yields for bPhys is probably in the order of 15%. Thus, further enhancement of fluorescence yield, together with their outstandingly large extinction coefficient in the order of $8 \times 10^4 - 10^5$, should yield a remarkable brightness and thus useful tools. Stepanenko et al. chose the opposite way, keeping in mind the low fluorescence of BV-binding bPhys.[301] Instead of capitalizing on the BV-binding properties of bPhys, the authors chose to enable the utilization of PCB in favor of BV by performing a mutagenesis approach on iRFP713 from *R. palustris*, formerly presented by the Verkhusha group.[302] They succeeded in generating proteins with clearly enhanced fluorescence quantum yields (between 0.45 and 0.5), however with shorter emission maxima due to the use of PCB. There is another inherent drawback to overcome when employing bPhys as fluorescent proteins. Because bPhys, just like other canonical phytochromes, tend to dimerize, the resulting fusion constructs are relatively large. Accordingly, efforts have been made to generate monomeric derivatives that retain strong fluorescence emission, but also other systems have been introduced during recent years.[303,304]

In fact, the Verkhusha group succeeded in tailoring a bPhy from *Rhodopseudomonas palustris* into a single monomeric GAF domain.[305] This construct was employed as the activation domain of a calcium sensor in a FRET experiment.[306] The single bPhy GAF domain was fused to the calcium-binding domain of the calmodulin/M13 peptide and subjected to several rounds of improvement by extensive mutagenesis. As fluorescence could be screened on Petri-dishes, improved variants were conveniently identified. In addition, the emission peak at 764 nm makes this fusion protein the most bathochromically emitting fluorescent calcium sensor. Application of bPhys with focus on animal systems was recently well presented in the review by Verkhusha and co-workers.[25] The composition was recently updated by the same authors, including multicolor excitation/emission and monitoring of FRET-based physiological processes such as cell protrusions and even discrimination of dividing from nondividing cells.[307]

4.6.2. Fluorescent Proteins Derived from Cyanobacterial Phycobiliproteins or from BV-Binding CBCR-GAF Domains. Recent reports initiated the development of fluorescent tools with cyanobacterial origin using linear tetrapyrroles as chromophores. Two classes of bilin-binding proteins came into focus: the cyanobacteriochromes (CBCRs) and derivatives of the phycobiliproteins originating from cyanobacterial antenna complexes (phycobilisomes).

CBCRs exhibit the remarkable advantage to unite all major properties of canonical phytochromes in a single GAF domain: chromophore lyase function, photochromicity, and signal generation by, e.g., enzyme activity control. Moreover, many GAF domains from CBCRs tolerate elevated temperature and pH changes and, in addition, show a fluorescence quantum yield larger than that of bPhys and their derivatives. Their usefulness for fluorescence microscopy has been demonstrated upon expression in *E. coli*.[57,98] CBCR-GAF domains could even be engaged in a FRET experiment: a hybrid protein composed of a flavin-binding LOV domain (λ_{max} = 450 nm, λ_{em} = 525 nm, Φ_{fl} = 0.3) and a CBCR-GAF domain (Slr1393g3 from *Synechocystis* PCC6803, λ_{max} = 650 nm, λ_{em} = 675−680 nm, Φ_{fl} = 0.3), connected by a flexible linker, showed a FRET efficiency of approximately 22%.[308]

Screening of CBCR-GAF domains from *Nostoc punctiforme* to identify proteins with novel absorption properties led—besides the identification of several red-/green- and also orange-/green-switching proteins—to the discovery of a photoinactive GAF domain that instead showed a strong fluorescence with emission peaking around 680 nm (GAF5 in a multi-GAF CBCR, *Np*F2164g5).[309] The authors propose an energy-transducing function (a FRET process) to neighboring GAF domains in the same protein for this GAF domain. The same protein originally binding PCB was further modified for BV. Again, it showed a strong fluorescence, now with a significantly longer emission maximum around 700 nm.[310]

A comprehensive study on a selected CBCR-GAF domain by Oliinyk et al. probably opens the door further for these proteins to be employed in optogenetics and as fluorescence tags.[311] The authors systematically modified a CBCR-GAF domain from the cyanobacterium *Nostoc punctiforme* (NpR3784) for fluorescence applications. They demonstrated conversion into BV binding, multiplexed application, and FRET properties. In addition, the authors determined the crystal structure (1.95 Å) of the widely mutated protein and demonstrated *in vivo* experiments in animals.

Also Wu et al. presented promising properties of a CBCR-GAF domain from *Spirulina subsalsa*.[312] This protein showed a relatively large fluorescence quantum yield of ca. 14% with a slight increase after mutagenesis.

Zhao and co-workers could further increase the fluorescence quantum yield of CBCR-GAF domains by incorporating PEB into GAF domains. Their examples include All2699g1, Slr1393g3, Tlr0911, and others, thereby combining the small molecular size of these GAF domains with the high extinction coefficient and fluorescence quantum yield of PEB or PUB.[71] These two bilins are chromophores of the antenna components, but they are not naturally occurring as a chromophore in phytochrome(-related) proteins, as they carry a single bond (C15−C16) instead of the double bond essential for photoisomerization.[88] Reports characterized PEB as a strongly fluorescent chromophore when incorporated into recombinant oat PhyA,[313] and together with its high extinction coefficient, the authors could generate bright proteins emitting in the orange spectral range.[71]

Of special mention are CBCRs that under natural conditions bind BV as an adaptation to life conditions in selectively red-shifted ecological niches.[102] One can imagine that these proteins serve as a further improved unit to shape CBCR-GAF domains for long wavelength-absorbing and -emitting optogenetic tools.

Verkhusha et al. emphasized advantages of CBCRs over the other class of bilin-binding proteins originating from the cyanobacterial antenna complexes, the phycobilisomes.[311] However, detailed analysis and design efforts yielded some phycobiliproteins with remarkably favorable properties. These antenna proteins, each of small molecular mass (mostly 15−20 kDa), aggregate under natural conditions into large light-harvesting antenna complexes of hundreds of subunits forming the light harvesting phycobilisome. Each monomer bears, depending on the species considered, one or even two covalently bound PCB- or PEB-chromophores.[95] Thermostability, long wavelength absorption and emission, and their large extinction coefficient and fluorescence quantum yield predestine these antenna proteins for optogenetic applications, yet their tendency to noncovalently aggregate into supercomplexes stands against

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their employment. The problem of amphipathic protein sections causing aggregation has recently been overcome for several of these proteins, e.g., ApcE, by removing distinct sequences including a transmembrane peptide.[314] These now soluble proteins represent a valuable addition to the palette of bright fluorescent tags.

More phycobiliproteins have been studied and optimized with great success by the group of Zhao. A very promising approach to enlarge the toolbox of strongly fluorescent proteins followed a formerly introduced gene-fusion protocol of the same group that yielded an *in vivo* assembled red-green-switching ("RGS") CBCR protein, Slr1393g3 from *Synechocystis*.[57] Using their knowledge of all enzymes that convert biliverdin into various modified bilins (Figure 2), these scholars fused the gene encoding the apoprotein with the genes for the enzymes generating various bilins and thereby applied this gene-fusion approach also to phycobiliproteins.[315] As, in contrast to the phytochromes, not all phycobiliproteins carry a lyase function required for autoligation of the chromophore, three genes were fused encoding a "super"-enzyme, consisting of a heme oxygenase and a BV-reducing, PCB- or PEB-generating enzyme, from which selective lyases specific for the phycobili-apoprotein were employed to generate PUB or PVB. A strict 1:1 genetic ratio led to success: a catalog of soluble, small, and fluorescent proteins was generated with emission maxima ranging from 506 to 641 nm, making these proteins promising tools for orthogonal applications. By this approach of fused/combined gene expression, the yield of chromophore loading was, for the best constructs, 2.7-fold higher than the loading yield obtained from the expression of both genes independently. This finding of an apparently optimized, mutual control of protein and chromophore biosynthesis might be of particular importance, also for other biological photoreceptors, for instance, when performing functional optogenetic experiments in hosts devoid of the required chromophore. Considering the high extinction coefficient of bilins (in the range of 10^5 M^{-1} cm^{-1}) and the high fluorescence quantum yield (up to 99%), outstandingly bright fluorescent tools were reported.[315]

In a follow-up study, Zhao and co-workers successfully incorporated PEB as chromophore into homo- and heterodimeric and -trimeric biliproteins multiplying the fluorescence of each single monomer.[316] Their multiple chromophorylation generated proteins with remarkably high brightness. The combination of two different phycobiliproteins provided binding of two different chromophores (with two distinct absorption maxima), allowing even multicolor irradiation or applications where large Stokes shifts are necessary. The authors referenced an FP-derivative, iRFP670, and compared the brightness of these two proteins in HEK-293T cells, in which the diad of the biliprotein outperformed the FP-derivative by a factor of 5.9. The favorable absorption and emission parameters of biliproteins allowed Zhao and co-workers to generate a fused protein composed of mCherry, a "classical" FP, and a biliprotein in which the emission of the FP optimally overlapped with the absorption of the biliprotein, thereby generating a FRET couple with a large Stokes shift of 79 nm (λ_{max} of mCherry, 587 nm; emission maximum λ_{em} of the biliprotein, 666 nm).[317] Again, this construct was compared to iRFP670 and outperformed this protein by a factor of 4.2 in mammalian cells. The authors consider this protein as excellently suited for multicolor applications.

It shall be mentioned that very few proteins, either from CBCRs or from phycobiliproteins, have been reported that bind biliverdin as chromophore in their natural form in order to meet the transparency window. Here, one would preferentially refer to those cyanobacteria that have adapted to an ecological niche with long wavelength irradiation, an approach that has already been followed successfully.[102,318] Interestingly, nature found also another way: as an alternative, antenna proteins have been identified from equally long wavelength-adapted cyanobacteria that incorporate PCB noncovalently (carrying a tyrosine at the position of the PCB-binding cysteine),[319] thereby further shifting absorption and fluorescence by about 30 nm.[22] These proteins originate from a thermophile, *Chroococcidiopsis thermalis*, offering a greater thermal stability for a variety of applications. Interestingly, the wild-type protein did not incorporate BV. Thus, the authors asked whether one might be able to convert the long wavelength-absorbing, PCB-incorporating protein into an even further red-shifted BV-binding derivative: several rounds of mutagenesis yielded a derivative that binds BV covalently, significantly shifting absorption and emission maxima to longer wavelengths.[320] Binding of the BV chromophore causes a slight reduction in the fluorescence quantum yield compared to the wild-type protein, but considering the large extinction coefficient, relatively bright fluorescence tags were presented. A random mutagenesis approach yielded a number of proteins, all with relatively long absorption and emission maxima extending up to 720 nm (Table 1 in Ding et al.[22]). Some of these derivatives even served as FRET couples. The authors succeeded in generating monomeric forms, overcoming the above-mentioned drawbacks of aggregation for intracellular applications. This protein from *C. thermalis*, for further work termed BDFP1.0 (λ_{max} = 709 nm, ε = 68,200 M^{-1} cm^{-1}, Φ_{fl} = 0.056), showed a brightness half that of IFP2.0 (taken as reference). IFP2.0 was generated by Yu et al. from the bacterial phytochrome of *D. radiodurans* by a series of DNA-shuffling and random mutagenesis experiments that first yielded monomeric forms of this bPhy and further improved its brightness.[321] Interestingly, these brightness comparisons with other FPs did not hold when used in, e.g., HEK cells, probably due to differences in chromophore maturation and availability. The stability of these *C. thermalis*-derived proteins at low pH was demonstrated by expression in *Lactobacillus lactis*. Employing other hosts, these proteins could be localized by fluorescence microscopy when used as tags to selected proteins in HEK cells and also in the nematode *C. elegans*. The properties of these "long wavelength-acclimated" proteins were further improved in a follow-up publication with special emphasis on combining several proteins in FRET experiments.[322]

The usefulness of such novel fluorescence tags was further demonstrated by fusing them to various proteins indicative of individual cellular compartments and structural elements, e.g., histones, nuclear localization sequences, actin, keratin, or TOMM20, a mitochondrial import-receptor subunit.[320] The protein employed here (BDFP1.9) was improved with respect to monomer/dimer formation by designed mutagenesis. Comparing crystal structures of this protein to data from former work,[314] the authors identified amino acids that promote dimerization. Akin to phytochromes, also biliproteins exhibit a large extinction coefficient of their bilin chromophore. Their intrinsically high fluorescence quantum yields, resulting in an extreme brightness, make them promising candidates for future generations of fluorescent proteins. In fact, recent applications of these modified biliproteins benefit from their properties for direct application in mammalian cell cultures and even in an animal system.[322]

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The currently most optimized proteins from this "pipeline" are further improved monomeric derivatives with large extinction coefficients and a noticeable fluorescence quantum yield (ε up to 130,000 M^{-1} cm^{-1}, Φ_{fl} up to 0.01−0.012). The intrinsic excitation and emission wavelengths are within the transparency window of mammalian cells,[323] and in fact, developed FRET triad fusions were 5-fold brighter than IFP2.0 in HEK cells.

It might well be that CBCR proteins could be tailored to higher fluorescence in both parental and photoproduct states. Due to their photochromicity, they might become suitable for nanoscopy applications such as FPALM, STET, or STORM, as preliminary experiments with the GAF3 domain of Slr1393 indicated.[98] A recent application based on derivatives of mCherry illustrates the path to follow.[324] Fluorescence images with the highest resolution were presented, and one can imagine that the favorable parameters intrinsic to the small CBCR-GAF domains with respect to photostability, extinction coefficient, and quantum yield may guide future applications.

It appears as if fluorescence-based applications employing bPhy- and phycobiliprotein-derived tools have no limits. It has been demonstrated that phycobiliproteins could be engineered to monomeric strongly fluorescent proteins utilizing internally available BV with emissions extending up to 720 nm. Concerns initially raised about the properties of these proteins that may stand against their use for optogenetics and fluorescence experiments have clearly been solved or demonstrated as nonrelevant in a series of proof-of-principle experiments. Solutions to these problems have been depicted, and the structural elements that convey di- and oligomerization have been identified. Similar strategies including structure-based mutagenesis or further genomic analyses will likely also identify novel CBCRs and provide novel assets to the infrared optical tool box. A comprehensive overview brings together the wide spectrum of applications, even proposing most fascinating future investigations.[325]

5. WEBSITE PLATFORMS, MODELING, AND RELATED HARDWARE

During the past decade, literature databases have increasingly accumulated published second generation optogenetic approaches with a rapid pace. Keeping an overview of all existing basic approaches, identifying suitable tools for experimental problems, and differencing neuronal ion transporter systems from modern optogenetic tools is challenging when relying on a conventional literature search, especially when freshly entering the field. To overcome these initial hurdles, the online platform "OptoBase" (https://www.optobase.org/) generates a guided entry point and assists in identifying appropriate optogenetic systems. The web page maintains an up-to-date compendium of relevant literature and compiles search algorithms to orient interested researchers to identify the best suitable candidates under consideration of user input restraints.

Nevertheless, optogenetics remains a scientific discipline with many variables to take into account. Often optimizations aim to reduce noise (activity in the dark state) or to improve the dynamic range of light induction requiring large numbers of parallel experiments. In particular, if hybrid proteins were designed from a light-sensing module of organism A, and a signaling domain of organism B, the connecting linker sequence usually requires thorough experimental testing and optimization. Tabor and co-workers engaged themselves in providing hardware at a reasonable price to enable precisely this: a wide range of wavelengths for irradiation and a wide dynamic range of intensities,[326] even running computer-programmable irradiation programs allowing oscillation at various frequencies.[202] Their hardware platform, called IRIS, provides parallel treatment of a 24-well plate, to which, independent to each of the wells, the Light Plate Apparatus (LPA) delivers two wavelengths selectable between 315 and 1550 nm with an intensity control over 3 orders of magnitude and with millisecond resolution. IRIS has been benchmarked using a red-/green-switching system originating from *Synechocystis* PCC6803 cyanobacteriochrome (CcaS/CcaR[176,247]), with a Cry2-based (blue light regulation) system, and with the PhyB/PIF6 system. All three test experiments yielded impressive precision and control of the reaction conditions. A likewise programmable "light box" has been described providing an irradiation range with eight LEDs from 385 up to 850 nm.[327] The authors, however, modified a Tecan plate reader, thereby setting the price of this device much higher. The IRIS system has recently been further improved including thorough calibration protocols and a better and more precise validation of dynamic responses.[328]

In a follow-up approach, Möglich and co-workers further developed their hardware tools, by generating a programmable switchboard that allows continuous or pulsed illumination with LEDs at several wavelengths.[329] In this manuscript, the authors also provide a detailed building plan and several fully automated protocols for pulsed or continuous illumination. Their setup was built using their former devices for blue light illumination, now including several applications of phytochromes. Test runs for long wavelength irradiation were performed with a hybrid photoreceptor protein. Based on a construct called PaaC, in which the photosensory module of *D. radiodurans* was fused to the photoactivated adenylyl cyclase Cya2,[232] Möglich and co-workers exchanged the PAS-GAF-PHY module to the one from *Deinococcus deserti* that showed more favorable properties (light/dark contrast, lower dark activity) than the *Dr* part.[330] For further applications it is worth mentioning that Cya2 can be readily changed from GTP- to ATP-selectivity by exchanging just a few amino acids.

To facilitate high-throughput data generation using up to three-color optogenetic experiments, Bugaj et al. devised an open-sourced illumination hardware and software platform, termed optoPlate-96.[331] The device enables simultaneous and individual illumination of common red- and blue-light-sensitive optogenetic systems in 96-well microwell plates or in groups of wells in 384-well plates. Each of the 96-well positions individually harbors three light sources which can be addressed via an Arduino microcontroller, generating convenient ways of producing results from samples treated with many different illumination protocols. The entire instrument can be assembled from commercially available and 3D-printed parts in about 3−4 h without the need of specialized equipment, vastly contributing to generating alternatives to otherwise highly specialized and expensive illumination sources. A software developed by Thomas et al. provides a graphical user interface for code-free programming of simple and advanced optoPlate-96 illumination schemes.[332]

Another microcontroller-based illumination device called "light activation at variable amplitudes" (LAVA) for 24- and 96-well culture plates has been developed by Repina et al.[333,334] LAVA can mount LEDs of a single wavelength suitable to perform temporal as well as spatially confined optogenetic experiments due to the possibility to optionally insert a photomask. The authors used LAVA to induce patterns of

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Wnt signaling in human embryonic stem cells using a Cry2-based photoclustering approach (optoWnt), leading to LRP6c oligomerization and the transcription of β-catenin target genes. A graphical user interface facilitates LAVA programming, and building instructions are deposited and documented in the form of a protocol.[334]

Olson and Tabor extended the similarity between components of synthetic biology, hierarchically organized regulators, and complex nodes, at which cells distinguish pragmatically between several options with a mathematically controlled computing device.[335]

Progress in focusing illumination in tissues, thus overcoming further the inherent problem of light scattering, has recently been presented in a living mouse brain tissue. Based on channelrhodopsins, which allow facile excitation and recording of neuronal activity, the group of C. Yang from Caltech set up a sophisticated optical system specialized for in-depth light penetration and spot focusing in such living tissues.[336] Optical and functional comparisons to more conventional techniques clearly demonstrated the advantage of a technique called "wavefront shaping" or "wavefront engineering", known for a while but not employed in optogenetics so far. The authors demonstrated activation of a channelrhodopsin in a neuron of an excised brain patch at a depth of 800 μm. In a follow-up experiment, the group of Yang teamed-up with the group of Verkhusha and adapted this technique for a bPhy (PAS-GAF-PHY domain from *D. radiodurans*).[337] Initially demonstrating the working principle in an artificial highly scattering material, the researchers laid out the focusing advantage of this technique for comparable applications in mouse brains and tumor biology.

6. CONCLUSIONS AND PERSPECTIVES

Phytochromes, especially the bacterial representatives, took time to gain ground in optogenetic applications. The survey for naturally occurring, phytochrome-interacting proteins, performed very early on, offers great opportunities for selective activation of genes or gene clusters.[338] The many members of bHLH proteins identified in plant genomes (of which only the PIF were exploited to a larger extent) provide candidates with so far unknown, distinct activities regulated by light.

The PhyB/PIF couple still holds multiple potentials for applications, albeit so far not fully exploited. The many functional and flexibly used size variants that have been accomplished for both proteins make this system highly versatile. In addition to note is that often one or both proteins carry fluorescent tags. The number of applications in which this protein couple engages permanently grows and has already become equally versatile as the LOV domain-based systems. Both photoswitches show the simplicity of being activated by remote control. However, both have their own strengths and weaknesses. The PhyB system entails the unique property of dual-wavelength regulation, contrasting the relatively slow processing photocycle of LOV domains that only in a single case was demonstrated to be light-activated in both ON and OFF directions.[339] In addition, Phys and phycobiliproteins stand out over other photoreceptors in presenting inherently long wavelengths in absorption and fluorescence emission that cannot be accomplished in the flavin-based photoreceptors. Blue light systems, instead, often benefit from readily available chromophores, whereas phytochromes often require either exogeneous addition of the bilins or engineering of a suitable chromophore biosynthesis pathway in the target organism.

Detailed characterization of the properties of cyanobacteriochromes is being performed in various laboratories and will most certainly provide additional input to foster optogenetic applications. In fact, knowledge of their full functional repertoire is still in its infancy. CBCRs complement the Phy-based applications in an optimal manner: they are distinguished by their small size and their wide range of absorption maxima facilitating multicolor applications. These proteins are expected to enrich the pool of optogenetic and fluorescent tools with further-reaching applications, particularly in combination with modern (super-resolution) microscopy. At the current state, it is worthwhile to consider both optogenetic switches and optical imaging functions for further improvement.

The engineering of Phys into fluorescent proteins has just begun, but beneficial properties can already be identified. A bright near-infrared fluorescence biolabeling is paramount for observing and tracing individual molecular events over an extended period of time. Here, the large extinction coefficient and the high fluorescence of bilin chromophores is probably unbeatable.

In the future, we expect optogenetic systems to mature further, increasingly transitioning into applied biosystems and meaningful medical applications that may, in the long term, significantly reshape biomedicine and basic research. Bilin-binding proteins have been shown to possess potential for such applications. Mind-controlled or monitoring and immediate feedback of medical applications are surely far-reaching at present. However, their principal functionality has been demonstrated in test cases. Especially future profiling, molecular engineering, design of hybrid proteins composed of domains from different proteins, and the discovery of novel photoswitches with distinct properties may pave the way in generating robust and convenient optogenetic systems. These approaches will see parallel progress in the development of hard- and software to make the technology easily accessible. Due to the ever-growing knowledge of the individual photoswitches, we foresee a rising number of orthogonal and multiplexed applications in optogenetics. All these future developments will further improve the principal idea to spatiotemporally stimulate manifold biological processes, solely guided by light in a noninvasive manner, without interfering with the delicate metabolic equilibria of living systems.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrev.1c00194.

Sequence alignment of selected phytochromes (PDF)

Special Issue Paper

This paper is an additional review for *Chem. Rev.* **2018**, *118*, issue 21, "Optogenetics and Photopharmacology".

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Notes

The authors declare no competing financial interest.

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Kun Tang studied microbiology at the Huazhong Agriculture University, China, and received her Ph.D. in 2015. During this time (2013−2015), she was a CSC (Chinese Scholar Council) exchange student studying cyanobacterial photoreceptors in the group of W. Gärtner at the Max Planck Institute for Chemical Energy Conversion, Mülheim, Germany. After graduation, she took a postdoctoral position in China at the Southern University of Science and Technology, Shenzhen, identifying protein-mediated interactions and molecular machinery assembly in biological processes. Since October 2018, she joined the Institute of Synthetic Biology headed by M. Zurbriggen at the University of Düsseldorf. Her current project focuses on the design and application of optogenetic tools.

Hannes M. Beyer studied Biology at the University of Freiburg, Germany, and Uppsala, Sweden. He obtained his Ph.D. from the University of Freiburg in Synthetic Biology where he worked on optogenetic phytochrome-based pathways in mammalian cells and biohybrid materials. After three years of postdoctoral studies at the University of Helsinki, Finland, investigating and bioengineering self-splicing proteins and their structural properties, he moved to the University of Düsseldorf, Germany, to continue his work on optogenetics and establish his own group.

Matias D. Zurbriggen did his undergraduate studies in Biotechnology at the University of Rosario and IBR, Argentina, followed by a joint work on plant biotechnology together with the Institute of Plant Genetics and Crop Research Leibniz-IPK, Gatersleben, Germany, graduating in 2009. After two years of postdoctoral work between the IBR, IPK and John Innes Centre, Norwich, UK, he moved to the University of Freiburg and BIOSS as an Alexander von Humboldt Foundation Fellow to work on mammalian synthetic biology and optogenetics. He was appointed Assistant Professor and started his group in 2012, working on mammalian and plant synthetic biology. He was awarded a full Professorship in Synthetic Biology at the University of Düsseldorf and the Cluster of Excellence on Plant Sciences (CEPLAS) in 2015. He is a member of the steering board of the Study Group Systems and Synthetic Biology of DECHEMA and cofounding member of the Study Group Synthetic Biology at the German Society of Biochemistry and Molecular Biology (GBM) and of the Study Group Synthetic Biology (DECHEMA, GBM, DBG, GDCh). He is coordinator of the Research Area Synthetic and Reconstruction Biology at CEPLAS and organizes conferences and symposia on synthetic biology and optogenetics.

Wolfgang Gärtner studied chemistry at the Universities of Göttingen and Würzburg, Germany. He graduated in 1982. After two postdoctoral sojourns at the Max-Planck-Institute for Biochemistry in Martinsried (Germany) and the Biocenter of the University of Basel (Switzerland) he was research assistant at the University of Freiburg (Germany), before he joined in 1991 the Max-Planck-Institute for Chemical Energy Conversion (at that time MPI for Radiation Chemistry) as a group leader. He habilitated at the University of Duisburg in Bio-Organic Chemistry and became Professor adjunct for Biochemistry at the University of Düsseldorf. He was guest Professor at the Huazhong Agricultural University in Wuhan and was awarded in 2017 "Professor ad Honorem" at the University of Parma, Italy. He is a long-standing member of the American Society of Photobiology (ASP) where he was elected for two terms as member of the Society's Council. He serves since many years as Associate Editor to the Society's journal *Photochemistry and Photobiology* and is also an Editorial Board member to *Journal of Biological Chemistry*. He is retired since 2016 and continues his research as a guest Professor at the University of Leipzig (Germany).

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REFERENCES

(1) Losi, A.; Gardner, K. H.; Möglich, A. Blue-Light Receptors for Optogenetics. *Chem. Rev.* **2018**, *118*, 10659−10709.
(2) Hüll, K.; Morstein, J.; Trauner, D. In Vivo Photopharmacology. *Chem. Rev.* **2018**, *118*, 10710−10747.
(3) Tochitsky, I.; Kienzler, M. A.; Isacoff, E.; Kramer, R. H. Restoring Vision to the Blind with Chemical Photoswitches. *Chem. Rev.* **2018**, *118*, 10748−10773.
(4) Engelhard, C.; Chizhov, I.; Siebert, F.; Engelhard, M. Microbial Halorhodopsins: Light-Driven Chloride Pumps. *Chem. Rev.* **2018**, *118*, 10629−10645.
(5) Zhang, F.; Wang, L. P.; Boyden, E. S.; Deisseroth, K. Channelrhodopsin-2 and Optical Control of Excitable Cells. *Nat. Methods* **2006**, *3*, 785−792.
(6) Deisseroth, K.; Feng, G.; Majewska, A. K.; Miesenböck, G.; Ting, A.; Schnitzer, M. J. Next-Generation Optical Technologies for Illuminating Genetically Targeted Brain Circuits. *J. Neurosci.* **2006**, *26*, 10380−10386.
(7) Nagel, G.; Szellas, T.; Kateriya, S.; Adeishvili, N.; Hegemann, P.; Bamberg, E. Channelrhodopsins: Directly Light-Gated Cation Channels. *Biochem. Soc. Trans.* **2005**, *33*, 863−866.
(8) Mukherjee, S.; Hegemann, P.; Broser, M. Enzymerhodopsins: Novel Photoregulated Catalysts for Optogenetics. *Curr. Opin. Struct. Biol.* **2019**, *57*, 118−126.
(9) Li, F.-W.; Melkonian, M.; Rothfels, C. J.; Villarreal, J. C.; Stevenson, D. W.; Graham, S. W.; Wong, G. K.-S.; Pryer, K. M.; Mathews, S. Phytochrome Diversity in Green Plants and the Origin of Canonical Plant Phytochromes. *Nat. Commun.* **2015**, *6*, 7852.
(10) Rockwell, N. C.; Lagarias, J. C. Phytochrome Evolution in 3D: Deletion, Duplication, and Diversification. *New Phytol.* **2020**, *225*, 2283−2300.
(11) Duanmu, D.; Bachy, C.; Sudek, S.; Wong, C.-H.; Jimenez, V.; Rockwell, N. C.; Martin, S. S.; Ngan, C. Y.; Reistetter, E. N.; van Baren, M. J.; et al. Marine Algae and Land Plants Share Conserved

Phytochrome Signaling Systems. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 15827−15832.
(12) Fushimi, K.; Hasegawa, M.; Ito, T.; Rockwell, N. C.; Enomoto, G.; Win, N.-N.; Lagarias, J. C.; Ikeuchi, M.; Narikawa, R. Evolution-Inspired Design of Multicolored Photoswitches from a Single Cyanobacteriochrome Scaffold. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 15573−15580.
(13) Duanmu, D.; Rockwell, N. C.; Lagarias, J. C. Algal Light Sensing and Photoacclimation in Aquatic Environments. *Plant, Cell Environ.* **2017**, *40*, 2558−2570.
(14) Rockwell, N. C.; Lagarias, J. C. Phytochrome Diversification in Cyanobacteria and Eukaryotic Algae. *Curr. Opin. Plant Biol.* **2017**, *37*, 87−93.
(15) Song, J.-Y.; Lee, H. Y.; Yang, H. W.; Song, J.-J.; Lagarias, J. C.; Park, Y.-I. Spectral and Photochemical Diversity of Tandem Cysteine Cyanobacterial Phytochromes. *J. Biol. Chem.* **2020**, *295*, 6754−6766.
(16) Ulijasz, A. T.; Cornilescu, G.; von Stetten, D.; Kaminski, S.; Mroginski, M. A.; Zhang, J.; Bhaya, D.; Hildebrandt, P.; Vierstra, R. D. Characterization of Two Thermostable Cyanobacterial Phytochromes Reveals Global Movements in the Chromophore-Binding Domain during Photoconversion. *J. Biol. Chem.* **2008**, *283*, 21251−21266.
(17) Hontani, Y.; Shcherbakova, D. M.; Baloban, M.; Zhu, J.; Verkhusha, V. V.; Kennis, J. T. M. Bright Blue-Shifted Fluorescent Proteins with Cys in the GAF Domain Engineered from Bacterial Phytochromes: Fluorescence Mechanisms and Excited-State Dynamics. *Sci. Rep.* **2016**, *6*, 37362.
(18) Moreno, M. V.; Rockwell, N. C.; Mora, M.; Fisher, A. J.; Lagarias, J. C. A Far-Red Cyanobacteriochrome Lineage Specific for Verdins. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 27962−27970.
(19) Miyake, K.; Fushimi, K.; Kashimoto, T.; Maeda, K.; Ni-Ni-Win Kimura, H.; Sugishima, M.; Ikeuchi, M.; Narikawa, R. Functional Diversification of Two Bilin Reductases for Light Perception and Harvesting in Unique Cyanobacterium Acaryochloris Marina MBIC 11017. *FEBS J.* **2020**, *287*, 4016−4031.
(20) Salewski, J.; Escobar, F. V.; Kaminski, S.; von Stetten, D.; Keidel, A.; Rippers, Y.; Michael, N.; Scheerer, P.; Piwowarski, P.; Bartl, F.; et al. Structure of the Biliverdin Cofactor in the Pfr State of Bathy and Prototypical Phytochromes. *J. Biol. Chem.* **2013**, *288*, 16800−16814.
(21) Harris, D.; Bar-Zvi, S.; Lahav, A.; Goldshmid, I.; Adir, N. The Structural Basis for the Extraordinary Energy-Transfer Capabilities of the Phycobilisome. In *Sub-cellular biochemistry*; Springer: Singapore, 2018; Vol. *87*, pp 57−82.
(22) Ding, W. L.; Miao, D.; Hou, Y. N.; Jiang, S. P.; Zhao, B. Q.; Zhou, M.; Scheer, H.; Zhao, K. H. Small Monomeric and Highly Stable Near-Infrared Fluorescent Markers Derived from the Thermophilic Phycobiliprotein, ApcF2. *Biochim. Biophys. Acta, Mol. Cell Res.* **2017**, *1864*, 1877−1886.
(23) Rodriguez, E. A.; Tran, G. N.; Gross, L. A.; Crisp, J. L.; Shu, X.; Lin, J. Y.; Tsien, R. Y. A Far-Red Fluorescent Protein Evolved from a Cyanobacterial Phycobiliprotein. *Nat. Methods* **2016**, *13*, 763−769.
(24) Brock, H.; Ruzsicska, B. P.; Arai, T.; Schlamann, W.; Holzwarth, A. R.; Braslavsky, S. E.; Schaffner, K. Fluorescence Lifetimes and Relative Quantum Yields of 124-Kilodalton Oat Phytochrome in Water and Deuterium Oxide Solutions. *Biochemistry* **1987**, *26*, 1412−1417.
(25) Chernov, K. G.; Redchuk, T. A.; Omelina, E. S.; Verkhusha, V. V. Near-Infrared Fluorescent Proteins, Biosensors, and Optogenetic Tools Engineered from Phytochromes. *Chem. Rev.* **2017**, *117*, 6423−6446.
(26) Yang, X.; Kuk, J.; Moffat, K. Crystal Structure of Pseudomonas Aeruginosa Bacteriophytochrome: Photoconversion and Signal Transduction. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 14715−14720.
(27) Lindner, I.; Braslavsky, S. E.; Schaffner, K.; Gärtner, W. Model Studies of Phytochrome Photochromism: Protein-Mediated Photoisomerization of a Linear Tetrapyrrole in the Absence of Covalent Bonding. *Angew. Chem., Int. Ed.* **2000**, *39*, 3269−3271.
(28) Oka, Y.; Kong, S.-G.; Matsushita, T. A Non-Covalently Attached Chromophore Can Mediate Phytochrome B Signaling in Arabidopsis. *Plant Cell Physiol.* **2011**, *52*, 2088−2102.
(29) Quest, B.; Gärtner, W. Chromophore Selectivity in Bacterial Phytochromes. *Eur. J. Biochem.* **2004**, *271*, 1117−1126.
(30) Tasler, R.; Moises, T.; Frankenberg-Dinkel, N. Biochemical and Spectroscopic Characterization of the Bacterial Phytochrome of Pseudomonas Aeruginosa. *FEBS J.* **2005**, *272*, 1927−1936.
(31) Butler, W. L.; Norris, K. H.; Siegelman, H. W.; Hendricks, S. B. Detection, Assay, and Preliminary Purification of the Pigment Controlling Photoresponsive Development of Plants. *Proc. Natl. Acad. Sci. U. S. A.* **1959**, *45*, 1703−1708.
(32) Sage, L. C. *Pigment of the Imagination*; Elsevier, 1992.
(33) Casal, J. J. Photoreceptor Signaling Networks in Plant Responses to Shade. *Annu. Rev. Plant Biol.* **2013**, *64*, 403−427.
(34) Viczián, A.; Klose, C.; Ádám, É.; Nagy, F. New Insights of Red Light-Induced Development. *Plant, Cell Environ.* **2017**, *40*, 2457−2468.
(35) Lagarias, J. C.; Lagarias, D. M. Self-Assembly of Synthetic Phytochrome Holoprotein in Vitro. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 5778−5780.
(36) Hill, C.; Gärtner, W.; Towner, P.; Braslavsky, S. E.; Schaffner, K. Expression of Phytochrome Apoprotein from Avena Sativa in Escherichia Coli and Formation of Photoactive Chromoproteins by Assembly with Phycocyanobilin. *Eur. J. Biochem.* **1994**, *223*, 69−77.
(37) Zhang, Y.; Sun, J.; Xia, H.; Zhao, C.; Hou, L.; Wang, B.; Li, A.; Chen, M.; Zhao, S.; Wang, X. Characterization of Peanut Phytochromes and Their Possible Regulating Roles in Early Peanut Pod Development. *PLoS One* **2018**, *13*, No. e0198041.
(38) Anders, K.; Essen, L. O. The Family of Phytochrome-like Photoreceptors: Diverse, Complex and Multi-Colored, but Very Useful. *Curr. Opin. Struct. Biol.* **2015**, *35*, 7−16.
(39) Yeh, K. C.; Wu, S. H.; Murphy, J. T.; Lagarias, J. C. A Cyanobacterial Phytochrome Two-Component Light Sensory System. *Science* **1997**, *277*, 1505−1508.
(40) Hughes, J.; Lamparter, T.; Mittmann, F.; Hartmann, E.; Gärtner, W.; Wilde, A.; Börner, T. A Prokaryotic Phytochrome. *Nature* **1997**, *386*, 663.
(41) Hübschmann, T.; Jorissen, H. J. M. M.; Börner, T.; Gärtner, W.; De Marsac, N. T. Phosphorylation of Proteins in the Light-Dependent Signalling Pathway of a Filamentous Cyanobacterium. *Eur. J. Biochem.* **2001**, *268*, 3383−3389.
(42) Boylan, M. T.; Quail, P. H. Are the Phytochromes Protein Kinases? *Protoplasma* **1996**, *195*, 12−17.
(43) Fortunato, A. E.; Jaubert, M.; Enomoto, G.; Bouly, J.-P.; Raniello, R.; Thaler, M.; Malviya, S.; Bernardes, J. S.; Rappaport, F.; Gentili, B.; et al. Diatom Phytochromes Reveal the Existence of Far-Red-Light-Based Sensing in the Ocean. *Plant Cell* **2016**, *28*, 616−628.
(44) Davis, S. J.; Vener, A. V.; Vierstra, R. D. Bacteriophytochromes: Phytochrome-like Photoreceptors from Nonphotosynthetic Eubacteria. *Science* **1999**, *286*, 2517−2520.
(45) Bhoo, S. H.; Davis, S. J.; Walker, J.; Karniol, B.; Vierstra, R. D. Bacteriophytochromes Are Photochromic Histidine Kinases Using a Biliverdin Chromophore. *Nature* **2001**, *414*, 776−779.
(46) Han, Y.; Meyer, M. H. F.; Keusgen, M.; Klug, G. A Haem Cofactor Is Required for Redox and Light Signalling by the AppA Protein of Rhodobacter Sphaeroides. *Mol. Microbiol.* **2007**, *64*, 1090−1104.
(47) Wagner, J. R.; Brunzelle, J. S.; Forest, K. T.; Vierstra, R. D. A Light-Sensing Knot Revealed by the Structure of the Chromophore-Binding Domain of Phytochrome. *Nature* **2005**, *438*, 325−331.
(48) Tarutina, M.; Ryjenkov, D. A.; Gomelsky, M. An Unorthodox Bacteriophytochrome from Rhodobacter Sphaeroides Involved in Turnover of the Second Messenger C-Di-GMP. *J. Biol. Chem.* **2006**, *281*, 34751−34758.
(49) Moyano, L.; Carrau, A.; Petrocelli, S.; Kraiselburd, I.; Gärtner, W.; Orellano, E. G. Bacteriophytochromes from Pseudomonas Syringae Pv. Tomato DC3000 Modulate the Early Stages of Plant Colonization during Bacterial Speck Disease. *Eur. J. Plant Pathol.* **2020**, *156*, 695−712.
(50) Woitowich, N. C.; Halavaty, A. S.; Waltz, P.; Kupitz, C.; Valera, J.; Tracy, G.; Gallagher, K. D.; Claesson, E.; Nakane, T.; Pandey, S.; et al. Structural Basis for Light Control of Cell Development Revealed by Crystal Structures of a Myxobacterial Phytochrome. *IUCrJ* **2018**, *5*, 619−634.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(51) Vierstra, R. D.; Davis, S. J. Bacteriophytochromes: New Tools for Understanding Phytochrome Signal Transduction. *Semin. Cell Dev. Biol.* **2000**, *11*, 511−521.
(52) Ohmori, M.; Ikeuchi, M.; Sato, N.; Wolk, P.; Kaneko, T.; Ogawa, T.; Kanehisa, M.; Goto, S.; Kawashima, S.; Okamoto, S.; et al. Characterization of Genes Encoding Multi-Domain Proteins in the Genome of the Filamentous Nitrogen-Fixing Cyanobacterium Anabaena Sp. Strain PCC 7120. *DNA Res.* **2001**, *8*, 271−284.
(53) Rockwell, N. C.; Martin, S. S.; Gulevich, A. G.; Lagarias, J. C. Phycoviolobilin Formation and Spectral Tuning in the DXCF Cyanobacteriochrome Subfamily. *Biochemistry* **2012**, *51*, 1449−1463.
(54) Rockwell, N. C.; Lagarias, J. C. A Brief History of Phytochromes. *ChemPhysChem* **2010**, *11*, 1172−1180.
(55) Kehoe, D. M.; Grossman, A. R. Similarity of a Chromatic Adaptation Sensor to Phytochrome and Ethylene Receptors. *Science* **1996**, *273*, 1409−1412.
(56) Gottlieb, S. M.; Kim, P. W.; Rockwell, N. C.; Hirose, Y.; Ikeuchi, M.; Lagarias, J. C.; Larsen, D. S. Primary Photodynamics of the Green/Red-Absorbing Photoswitching Regulator of the Chromatic Adaptation e Domain from Fremyella Diplosiphon. *Biochemistry* **2013**, *52*, 8198−8208.
(57) Zhang, J.; Wu, X. J.; Wang, Z. B.; Chen, Y.; Wang, X.; Zhou, M.; Scheer, H.; Zhao, K. H. Fused-Gene Approach to Photoswitchable and Fluorescent Biliproteins. *Angew. Chem., Int. Ed.* **2010**, *49*, 5456−5458.
(58) Hirose, Y.; Rockwell, N. C.; Nishiyama, K.; Narikawa, R.; Ukaji, Y.; Inomata, K.; Lagarias, J. C.; Ikeuchi, M. Green/Red Cyanobacteriochromes Regulate Complementary Chromatic Acclimation via a Protochromic Photocycle. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 4974−4979.
(59) Lim, S.; Yu, Q.; Gottlieb, S. M.; Chang, C.-W.; Rockwell, N. C.; Martin, S. S.; Madsen, D.; Lagarias, J. C.; Larsen, D. S.; Ames, J. B. Correlating Structural and Photochemical Heterogeneity in Cyanobacteriochrome NpR6012g4. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 4387−4392.
(60) Rockwell, N. C.; Martin, S. S.; Lagarias, J. C. Identification of DXCF Cyanobacteriochrome Lineages with Predictable Photocycles. *Photochem. Photobiol. Sci.* **2015**, *14*, 929−941.
(61) Gottlieb, S. M.; Kim, P. W.; Chang, C. W.; Hanke, S. J.; Hayer, R. J.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Larsen, D. S. Conservation and Diversity in the Primary Forward Photodynamics of Red/Green Cyanobacteriochromes. *Biochemistry* **2015**, *54*, 1028−1042.
(62) Rockwell, N. C.; Njuguna, S. L.; Roberts, L.; Castillo, E.; Parson, V. L.; Dwojak, S.; Lagarias, J. C.; Spiller, S. C. A Second Conserved GAF Domain Cysteine Is Required for the Blue/Green Photoreversibility of Cyanobacteriochrome Tlr0924 from Thermosynechococcus Elongatus. *Biochemistry* **2008**, *47*, 7304−7316.
(63) Rockwell, N. C.; Martin, S. S.; Lagarias, J. C. Mechanistic Insight into the Photosensory Versatility of DXCF Cyanobacteriochromes. *Biochemistry* **2012**, *51*, 3576−3585.
(64) Rockwell, N. C.; Martin, S. S.; Feoktistova, K.; Lagarias, J. C. Diverse Two-Cysteine Photocycles in Phytochromes and Cyanobacteriochromes. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 11854−11859.
(65) Ma, Q.; Hua, H.-H.; Chen, Y.; Liu, B.-B.; Krämer, A. L.; Scheer, H.; Zhao, K.-H.; Zhou, M. A Rising Tide of Blue-Absorbing Biliprotein Photoreceptors - Characterization of Seven Such Bilin-Binding GAF Domains in Nostoc Sp. PCC7120. *FEBS J.* **2012**, *279*, 4095−4108.
(66) Rockwell, N. C.; Martin, S. S.; Lagarias, J. C. There and Back Again: Loss and Reacquisition of Two-Cys Photocycles in Cyanobacteriochromes. *Photochem. Photobiol.* **2017**, *93*, 741−754.
(67) Tros, M.; Bersanini, L.; Shen, G.; Ho, M.-Y.; van Stokkum, I. H. M.; Bryant, D. A.; Croce, R. Harvesting Far-Red Light: Functional Integration of Chlorophyll f into Photosystem I Complexes of Synechococcus Sp. PCC 7002. *Biochim. Biophys. Acta, Bioenerg.* **2020**, *1861*, 148206.
(68) Rockwell, N. C.; Martin, S. S.; Lagarias, J. C. Identification of Cyanobacteriochromes Detecting Far-Red Light. *Biochemistry* **2016**, *55*, 3907−3919.
(69) Bhattacharya, S.; Auldridge, M. E.; Lehtivuori, H.; Ihalainen, J. A.; Forest, K. T. Origins of Fluorescence in Evolved Bacteriophytochromes. *J. Biol. Chem.* **2014**, *289*, 32144−32152.
(70) Kuwasaki, Y.; Miyake, K.; Fushimi, K.; Takeda, Y.; Ueda, Y.; Nakajima, T.; Ikeuchi, M.; Sato, M.; Narikawa, R. Protein Engineering of Dual-Cys Cyanobacteriochrome AM1_1186g2 for Biliverdin Incorporation and Far-Red/Blue Reversible Photoconversion. *Int. J. Mol. Sci.* **2019**, *20*, 2935.
(71) Sun, Y.-F.; Xu, J.-G.; Tang, K.; Miao, D.; Gärtner, W.; Scheer, H.; Zhao, K.-H.; Zhou, M. Orange Fluorescent Proteins Constructed from Cyanobacteriochromes Chromophorylated with Phycoerythrobilin. *Photochem. Photobiol. Sci.* **2014**, *13*, 757.
(72) Blumenstein, A.; Vienken, K.; Tasler, R.; Purschwitz, J.; Veith, D.; Frankenberg-Dinkel, N.; Fischer, R. The Aspergillus Nidulans Phytochrome FphA Represses Sexual Development in Red Light. *Curr. Biol.* **2005**, *15*, 1833−1838.
(73) Consiglieri, E.; Gutt, A.; Gärtner, W.; Schubert, L.; Viappiani, C.; Abbruzzetti, S.; Losi, A. Dynamics and Efficiency of Photoswitching in Biliverdin-Binding Phytochromes. *Photochem. Photobiol. Sci.* **2019**, *18*, 2484−2496.
(74) Rensing, S. A.; Sheerin, D. J.; Hiltbrunner, A. Phytochromes: More Than Meets the Eye. *Trends Plant Sci.* **2016**, *21*, 543−546.
(75) Duanmu, D.; Casero, D.; Dent, R. M.; Gallaher, S.; Yang, W.; Rockwell, N. C.; Martin, S. S.; Pellegrini, M.; Niyogi, K. K.; Merchant, S. S.; et al. Retrograde Bilin Signaling Enables Chlamydomonas Greening and Phototrophic Survival. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 3621−3626.
(76) Rockwell, N. C.; Duanmu, D.; Martin, S. S.; Bachy, C.; Price, D. C.; Bhattacharya, D.; Worden, A. Z.; Lagarias, J. C. Eukaryotic Algal Phytochromes Span the Visible Spectrum. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 3871−3876.
(77) Ryter, S. W.; Alam, J.; Choi, A. M. K. Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications. *Physiol. Rev.* **2006**, *86*, 583−650.
(78) Kräutler, B. Phyllobilins-the Abundant Bilin-Type Tetrapyrrolic Catabolites of the Green Plant Pigment Chlorophyll. *Chem. Soc. Rev.* **2014**, *43*, 6227−6238.
(79) Gisk, B.; Wiethaus, J.; Aras, M.; Frankenberg-Dinkel, N. Variable Composition of Heme Oxygenases with Different Regiospecificities in Pseudomonas Species. *Arch. Microbiol.* **2012**, *194*, 597−606.
(80) Shah, R.; Schwach, J.; Frankenberg-Dinkel, N.; Gärtner, W. Complex Formation between Heme Oxygenase and Phytochrome during Biosynthesis in Pseudomonas Syringae Pv. Tomato. *Photochem. Photobiol. Sci.* **2012**, *11*, 1026.
(81) Scheer, H.; Zhao, K.-H. Biliprotein Maturation: The Chromophore Attachment. *Mol. Microbiol.* **2008**, *68*, 263−276.
(82) Dammeyer, T.; Frankenberg-Dinkel, N. Function and Distribution of Bilin Biosynthesis Enzymes in Photosynthetic Organisms. *Photochem. Photobiol. Sci.* **2008**, *7*, 1121.
(83) Beale, S. I.; Cornejo, J. Biosynthesis of Phycobilins. 15,16-Dihydrobiliverdin IX Alpha Is a Partially Reduced Intermediate in the Formation of Phycobilins from Biliverdin IX Alpha. *J. Biol. Chem.* **1991**, *266*, 22341−22345.
(84) Frankenberg, N.; Mukougawa, K.; Kohchi, T.; Lagarias, J. C. Functional Genomic Analysis of the HY2 Family of Ferredoxin-Dependent Bilin Reductases from Oxygenic Photosynthetic Organisms. *Plant Cell* **2001**, *13*, 965−978.
(85) Wu, S.-H.; McDowell, M. T.; Lagarias, J. C. Phycocyanobilin Is the Natural Precursor of the Phytochrome Chromophore in the Green Alga Mesotaenium Caldariorum. *J. Biol. Chem.* **1997**, *272*, 25700−25705.
(86) Jorissen, H. J. M. M.; Braslavsky, S. E.; Wagner, G.; Gärtner, W. Heterologous Expression and Characterization of Recombinant Phytochrome from the Green Alga Mougeotia Scalaris. *Photochem. Photobiol.* **2002**, *76*, 457−461.
(87) Rockwell, N. C.; Martin, S. S.; Li, F.; Mathews, S.; Lagarias, J. C. The Phycocyanobilin Chromophore of Streptophyte Algal Phytochromes Is Synthesized by HY2. *New Phytol.* **2017**, *214*, 1145−1157.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(88) Blot, N.; Wu, X.-J.; Thomas, J.-C.; Zhang, J.; Garczarek, L.; Böhm, S.; Tu, J.-M.; Zhou, M.; Plöscher, M.; Eichacker, L.; et al. Phycourobilin in Trichromatic Phycocyanin from Oceanic Cyanobacteria Is Formed Post-Translationally by a Phycoerythrobilin Lyase-Isomerase. *J. Biol. Chem.* **2009**, *284*, 9290−9298.
(89) Chen, Y.-R.; Su, Y.; Tu, S.-L. Distinct Phytochrome Actions in Nonvascular Plants Revealed by Targeted Inactivation of Phytobilin Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 8310−8315.
(90) Ikeuchi, M.; Ishizuka, T. Cyanobacteriochromes: A New Superfamily of Tetrapyrrole-Binding Photoreceptors in Cyanobacteria. *Photochem. Photobiol. Sci.* **2008**, *7*, 1159.
(91) Zhao, C.; Höppner, A.; Xu, Q.-Z.; Gärtner, W.; Scheer, H.; Zhou, M.; Zhao, K.-H. Structures and Enzymatic Mechanisms of Phycobiliprotein Lyases CpcE/F and PecE/F. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 13170−13175.
(92) Zhao, K.-H.; Wu, D.; Zhou, M.; Zhang, L.; Böhm, S.; Bubenzer, C.; Scheer, H. Amino Acid Residues Associated with Enzymatic Activities of the Isomerizing Phycoviolobilin-Lyase PecE/F. *Biochemistry* **2005**, *44*, 8126−8137.
(93) Böhm, S.; Endres, S.; Scheer, H.; Zhao, K.-H. Biliprotein Chromophore Attachment. *J. Biol. Chem.* **2007**, *282*, 25357−25366.
(94) Gossauer, A. Synthetic Methods in Bile Pigment Chemistry. *Isr. J. Chem.* **1983**, *23*, 167−172.
(95) Bar-Eyal, L.; Shperberg-Avni, A.; Paltiel, Y.; Keren, N.; Adir, N. Light Harvesting in Cyanobacteria: The Phyobilisomes. In *Light harvesting in photosynthesis*; Croce, R., van Grondelle, R., van Amerongen, H., van Stokkum, I., Eds.; Taylor & Francis: Boca Raton, FL, 2018; pp 77−93.
(96) Murphy, J. T.; Lagarias, J. C. The Phytofluors: A New Class of Fluorescent Protein Probes. *Curr. Biol.* **1997**, *7*, 870−876.
(97) Sineshchekov, V.; Mailliet, J.; Psakis, G.; Feilke, K.; Kopycki, J.; Zeidler, M.; Essen, L.-O.; Hughes, J. Tyrosine 263 in Cyanobacterial Phytochrome Cph1 Optimizes Photochemistry at the Prelumi-R→ lumi-R Step. *Photochem. Photobiol.* **2014**, *90*, 786−795.
(98) Pennacchietti, F.; Losi, A.; Xu, X. L.; Zhao, K. H.; Gärtner, W.; Viappiani, C.; Cella, F.; Diaspro, A.; Abbruzzetti, S. Photochromic Conversion in a Red/Green Cyanobacteriochrome from Synechocystis PCC6803: Quantum Yields in Solution and Photoswitching Dynamics in Living E. Coli Cells. *Photochem. Photobiol. Sci.* **2015**, *14*, 229−237.
(99) Gambetta, G. A.; Lagarias, J. C. Genetic Engineering of Phytochrome Biosynthesis in Bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 10566−10571.
(100) Müller, K.; Engesser, R.; Timmer, J.; Nagy, F.; Zurbriggen, M. D.; Weber, W. Synthesis of Phycocyanobilin in Mammalian Cells. *Chem. Commun.* **2013**, *49*, 8970−8972.
(101) Uda, Y.; Goto, Y.; Oda, S.; Kohchi, T.; Matsuda, M.; Aoki, K. Efficient Synthesis of Phycocyanobilin in Mammalian Cells for Optogenetic Control of Cell Signaling. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 11962−11967.
(102) Narikawa, R.; Nakajima, T.; Aono, Y.; Fushimi, K.; Enomoto, G.; Ni-Ni-Win; Itoh, S.; Sato, M.; Ikeuchi, M. A Biliverdin-Binding Cyanobacteriochrome from the Chlorophyll d-Bearing Cyanobacterium Acaryochloris Marina. *Sci. Rep.* **2015**, *5*, 7950.
(103) Heyne, K.; Herbst, J.; Stehlik, D.; Esteban, B.; Lamparter, T.; Hughes, J.; Diller, R. Ultrafast Dynamics of Phytochrome from the Cyanobacterium Synechocystis, Reconstituted with Phycocyanobilin and Phycoerythrobilin. *Biophys. J.* **2002**, *82*, 1004−1016.
(104) Kim, P. W.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Larsen, D. S. Dynamic Inhomogeneity in the Photodynamics of Cyanobacterial Phytochrome Cph1. *Biochemistry* **2014**, *53*, 2818−2826.
(105) Müller, M. G.; Lindner, I.; Martin, I.; Gärtner, W.; Holzwarth, A. R. Femtosecond Kinetics of Photoconversion of the Higher Plant Photoreceptor Phytochrome Carrying Native and Modified Chromophores. *Biophys. J.* **2008**, *94*, 4370−4382.
(106) Chizhov, I.; Zorn, B.; Manstein, D. J.; Gärtner, W. Kinetic and Thermodynamic Analysis of the Light-Induced Processes in Plant and Cyanobacterial Phytochromes. *Biophys. J.* **2013**, *105*, 2210−2220.
(107) Grombein, S.; Rüdiger, W.; Zimmermann, H. The Structures of the Phytochrome Chromophore in Both Photoreversible Forms. *Hoppe-Seyler's Z. Physiol. Chem.* **1975**, *356*, 1709−1714.
(108) Rüdiger, W.; Thümmler, F. Phytochrome, the Visual Pigment of Plants. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1216−1228.
(109) Ulijasz, A. T.; Cornilescu, G.; Cornilescu, C. C.; Zhang, J.; Rivera, M.; Markley, J. L.; Vierstra, R. D. Structural Basis for the Photoconversion of a Phytochrome to the Activated Pfr Form. *Nature* **2010**, *463*, 250−254.
(110) Rohmer, T.; Lang, C.; Bongards, C.; Gupta, K. B. S. S.; Neugebauer, J.; Hughes, J.; Gärtner, W.; Matysik, J. Phytochrome as Molecular Machine: Revealing Chromophore Action during the Pfr –> Pr Photoconversion by Magic-Angle Spinning NMR Spectroscopy. *J. Am. Chem. Soc.* **2010**, *132*, 4431−4437.
(111) Song, C.; Psakis, G.; Kopycki, J.; Lang, C.; Matysik, J.; Hughes, J. The D-Ring, Not the A-Ring, Rotates in Synechococcus OS-B' Phytochrome. *J. Biol. Chem.* **2014**, *289*, 2552−2562.
(112) Wiebeler, C.; Rao, A. G.; Gärtner, W.; Schapiro, I. The Effective Conjugation Length Is Responsible for the Red/Green Spectral Tuning in the Cyanobacteriochrome Slr1393g3. *Angew. Chem., Int. Ed.* **2019**, *58*, 1934−1938.
(113) Andel, F.; Hasson, K. C.; Gai, F.; Anfinrud, P. A.; Mathies, R. A. Femtosecond Time-Resolved Spectroscopy of the Primary Photochemistry of Phytochrome. *Biospectroscopy* **1997**, *3*, 421−433.
(114) Zhang, C. F.; Farrens, D. L.; Björling, S. C.; Song, P. S.; Kliger, D. S. Time-Resolved Absorption Studies of Native Etiolated Oat Phytochrome. *J. Am. Chem. Soc.* **1992**, *114*, 4569−4580.
(115) Gärtner, W.; Braslavsky, S. E. The Phytochromes: Spectroscopy and Function. In *Photoreceptors and light signaling*; Batschauer, A., Ed.; Royal Society of Chemistry: Cambridge, UK, 2003; pp 136−180.
(116) Buhrke, D.; Kuhlmann, U.; Michael, N.; Hildebrandt, P. The Photoconversion of Phytochrome Includes an Unproductive Shunt Reaction Pathway. *ChemPhysChem* **2018**, *19*, 566−570.
(117) Song, C.; Psakis, G.; Lang, C.; Mailliet, J.; Gärtner, W.; Hughes, J.; Matysik, J. Two Ground State Isoforms and a Chromophore D-Ring Photoflip Triggering Extensive Intramolecular Changes in a Canonical Phytochrome. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 3842−3847.
(118) Schmidt, P.; Gensch, T.; Remberg, A.; Gärtner, W.; Braslavsky, S. E.; Schaffner, K. The Complexity of the Pr to Pfr Phototransformation Kinetics Is an Intrinsic Property of Native Phytochrome. *Photochem. Photobiol.* **1998**, *68*, 754−761.
(119) Kneip, C.; Hildebrandt, P.; Schlamann, W.; Braslavsky, S. E.; Mark, F.; Schaffner, K. Protonation State and Structural Changes of the Tetrapyrrole Chromophore during the P(r) → P(Fr) Phototransformation of Phytochrome: A Resonance Raman Spectroscopic Study. *Biochemistry* **1999**, *38*, 15185−15192.
(120) Freer, L. H.; Kim, P. W.; Corley, S. C.; Rockwell, N. C.; Zhao, L.; Thibert, A. J.; Lagarias, J. C.; Larsen, D. S. Chemical Inhomogeneity in the Ultrafast Dynamics of the DXCF Cyanobacteriochrome Tlr0924. *J. Phys. Chem. B* **2012**, *116*, 10571−10581.
(121) Gottlieb, S. M.; Kim, P. W.; Corley, S. C.; Madsen, D.; Hanke, S. J.; Chang, C. W.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Larsen, D. S. Primary and Secondary Photodynamics of the Violet/Orange Dual-Cysteine NpF2164g3 Cyanobacteriochrome Domain from Nostoc Punctiforme. *Biochemistry* **2014**, *53*, 1029−1040.
(122) Kim, P. W.; Freer, L. H.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Larsen, D. S. Femtosecond Photodynamics of the Red/Green Cyanobacteriochrome NpR6012g4 from Nostoc Punctiforme. 1. Forward Dynamics. *Biochemistry* **2012**, *51*, 608−618.
(123) Kim, P. W.; Freer, L. H.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Larsen, D. S. Femtosecond Photodynamics of the Red/Green Cyanobacteriochrome NpR6012g4 from Nostoc Punctiforme. 2. Reverse Dynamics. *Biochemistry* **2012**, *51*, 619−630.
(124) Slavov, C.; Xu, X.; Zhao, K. H.; Gärtner, W.; Wachtveitl, J. Detailed Insight into the Ultrafast Photoconversion of the Cyanobacteriochrome Slr1393 from Synechocystis Sp. *Biochim. Biophys. Acta, Bioenerg.* **2015**, *1847*, 1335−1344.
(125) Ishizuka, T.; Shimada, T.; Okajima, K.; Yoshihara, S.; Ochiai, Y.; Katayama, M.; Ikeuchi, M. Characterization of Cyanobacteriochrome

TePixJ from a Thermophilic Cyanobacterium Thermosynechococcus Elongatus Strain BP-1. *Plant Cell Physiol.* **2006**, *47*, 1251−1261.
(126) Hardman, S. J. O.; Hauck, A. F. E.; Clark, I. P.; Heyes, D. J.; Scrutton, N. S. Comprehensive Analysis of the Green-to-Blue Photoconversion of Full-Length Cyanobacteriochrome Tlr0924. *Biophys. J.* **2014**, *107*, 2195−2203.
(127) Fukushima, Y.; Iwaki, M.; Narikawa, R.; Ikeuchi, M.; Tomita, Y.; Itoh, S. Photoconversion Mechanism of a Green/Red Photosensory Cyanobacteriochrome AnPixJ: Time-Resolved Optical Spectroscopy and FTIR Analysis of the AnPixJ-GAF2 Domain. *Biochemistry* **2011**, *50*, 6328−6339.
(128) Xu, X. L.; Gutt, A.; Mechelke, J.; Raffelberg, S.; Tang, K.; Miao, D.; Valle, L.; Borsarelli, C. D.; Zhao, K. H.; Gärtner, W. Combined Mutagenesis and Kinetics Characterization of the Bilin-Binding GAF Domain of the Protein Slr1393 from the Cyanobacterium Synechocystis PCC6803. *ChemBioChem* **2014**, *15*, 1190−1199.
(129) Essen, L.-O.; Mailliet, J.; Hughes, J. The Structure of a Complete Phytochrome Sensory Module in the Pr Ground State. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 14709−14714.
(130) Björling, A.; Berntsson, O.; Lehtivuori, H.; Takala, H.; Hughes, A. J.; Panman, M.; Hoernke, M.; Niebling, S.; Henry, L.; Henning, R.; et al. Structural Photoactivation of a Full-Length Bacterial Phytochrome. *Sci. Adv.* **2016**, *2*, No. e1600920.
(131) Gourinchas, G.; Etzl, S.; Göbl, C.; Vide, U.; Madl, T.; Winkler, A. Long-Range Allosteric Signaling in Red Light−Regulated Diguanylyl Cyclases. *Sci. Adv.* **2017**, *3*, No. e1602498.
(132) Burgie, E. S.; Bussell, A. N.; Walker, J. M.; Dubiel, K.; Vierstra, R. D. Crystal Structure of the Photosensing Module from a Red/Far-Red Light-Absorbing Plant Phytochrome. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 10179−10184.
(133) Nagano, S.; Guan, K.; Shenkutie, S. M.; Feiler, C.; Weiss, M.; Kraskov, A.; Buhrke, D.; Hildebrandt, P.; Hughes, J. Structural Insights into Photoactivation and Signalling in Plant Phytochromes. *Nat. Plants* **2020**, *6*, 581−588.
(134) Wahlgren, W. Y.; Golonka, D.; Westenhoff, S.; Möglich, A. Cryo-Electron Microscopy of Arabidopsis Thaliana Phytochrome A in Its Pr State Reveals Head-to-Head Homodimeric Architecture. *Front. Plant Sci.* **2021**, *12*, 663751.
(135) Takala, H.; Björling, A.; Berntsson, O.; Lehtivuori, H.; Niebling, S.; Hoernke, M.; Kosheleva, I.; Henning, R.; Menzel, A.; Ihalainen, J. A.; et al. Signal Amplification and Transduction in Phytochrome Photosensors. *Nature* **2014**, *509*, 245−248.
(136) Cornilescu, C. C.; Cornilescu, G.; Burgie, E. S.; Markley, J. L.; Ulijasz, A. T.; Vierstra, R. D. Dynamic Structural Changes Underpin Photoconversion of a Blue/Green Cyanobacteriochrome between Its Dark and Photoactivated States. *J. Biol. Chem.* **2014**, *289*, 3055−3065.
(137) Narikawa, R.; Ishizuka, T.; Muraki, N.; Shiba, T.; Kurisu, G.; Ikeuchi, M. Structures of Cyanobacteriochromes from Phototaxis Regulators AnPixJ and TePixJ Reveal General and Specific Photoconversion Mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 918−923.
(138) Burgie, E. S.; Wang, T.; Bussell, A. N.; Walker, J. M.; Li, H.; Vierstra, R. D. Crystallographic and Electron Microscopic Analyses of a Bacterial Phytochrome Reveal Local and Global Rearrangements during Photoconversion. *J. Biol. Chem.* **2014**, *289*, 24573−24587.
(139) Burgie, E. S.; Zhang, J.; Vierstra, R. D. Crystal Structure of Deinococcus Phytochrome in the Photoactivated State Reveals a Cascade of Structural Rearrangements during Photoconversion. *Structure* **2016**, *24*, 448−457.
(140) Gourinchas, G.; Etzl, S.; Winkler, A. Bacteriophytochromes − from Informative Model Systems of Phytochrome Function to Powerful Tools in Cell Biology. *Curr. Opin. Struct. Biol.* **2019**, *57*, 72−83.
(141) Claesson, E.; Wahlgren, W. Y.; Takala, H.; Pandey, S.; Castillon, L.; Kuznetsova, V.; Henry, L.; Panman, M.; Carrillo, M.; Kübel, J.; et al. The Primary Structural Photoresponse of Phytochrome Proteins Captured by a Femtosecond X-Ray Laser. *eLife* **2020**, *9*, No. e53514.
(142) Sanchez, J. C.; Carrillo, M.; Pandey, S.; Noda, M.; Aldama, L.; Feliz, D.; Claesson, E.; Wahlgren, W. Y.; Tracy, G.; Duong, P.; et al. High-Resolution Crystal Structures of a Myxobacterial Phytochrome at Cryo and Room Temperatures. *Struct. Dyn.* **2019**, *6*, 054701.
(143) Carrillo, M.; Pandey, S.; Sanchez, J.; Noda, M.; Poudyal, I.; Aldama, L.; Malla, T. N.; Claesson, E.; Wahlgren, W. Y.; Feliz, D.; et al. High-Resolution Crystal Structures of Transient Intermediates in the Phytochrome Photocycle. *Structure* **2021**, *29*, 743−754 (e4)..
(144) Xu, X.; Höppner, A.; Wiebeler, C.; Zhao, K.-H.; Schapiro, I.; Gärtner, W. Structural Elements Regulating the Photochromicity in a Cyanobacteriochrome. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 2432−2440.
(145) Burgie, E. S.; Clinger, J. A.; Miller, M. D.; Brewster, A. S.; Aller, P.; Butryn, A.; Fuller, F. D.; Gul, S.; Young, I. D.; Pham, C. C.; et al. Photoreversible Interconversion of a Phytochrome Photosensory Module in the Crystalline State. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 300−307.
(146) Quail, P. H. Phytochrome Photosensory Signalling Networks. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 85−93.
(147) Hughes, J. Phytochrome Cytoplasmic Signaling. *Annu. Rev. Plant Biol.* **2013**, *64*, 377−402.
(148) Lorrain, S.; Genoud, T.; Fankhauser, C. Let There Be Light in the Nucleus! *Curr. Opin. Plant Biol.* **2006**, *9*, 509−514.
(149) Shimizu-Sato, S.; Huq, E.; Tepperman, J. M.; Quail, P. H. A Light-Switchable Gene Promoter System. *Nat. Biotechnol.* **2002**, *20*, 1041−1044.
(150) Golonka, D.; Gerken, U.; Köhler, J.; Möglich, A. The Association Kinetics Encode the Light Dependence of Arabidopsis Phytochrome B Interactions. *J. Mol. Biol.* **2020**, *432*, 4327−4340.
(151) Golonka, D.; Fischbach, P.; Jena, S. G.; Kleeberg, J. R. W.; Essen, L.-O.; Toettcher, J. E.; Zurbriggen, M. D.; Möglich, A. Deconstructing and Repurposing the Light-Regulated Interplay between Arabidopsis Phytochromes and Interacting Factors. *Commun. Biol.* **2019**, *2*, 448.
(152) Hörner, M.; Eble, J.; Yousefi, O. S.; Schwarz, J.; Warscheid, B.; Weber, W.; Schamel, W. W. A. Light-Controlled Affinity Purification of Protein Complexes Exemplified by the Resting ZAP70 Interactome. *Front. Immunol.* **2019**, *10*, 1−12.
(153) Fankhauser, C.; Yeh, K. C.; Lagarias, J. C.; Zhang, H.; Elich, T. D.; Chory, J. PKS1, a Substrate Phosphorylated by Phytochrome That Modulates Light Signaling in Arabidopsis. *Science* **1999**, *284*, 1539−1541.
(154) Stockhaus, J.; Nagatani, A.; Halfter, U.; Kay, S.; Furuya, M.; Chua, N. H. Serine-to-Alanine Substitutions at the Amino-Terminal Region of Phytochrome A Result in an Increase in Biological Activity. *Genes Dev.* **1992**, *6*, 2364−2372.
(155) McMichael, R. W.; Lagarias, J. C. Phosphopeptide Mapping of Avena Phytochrome Phosphorylated by Protein Kinases in Vitro. *Biochemistry* **1990**, *29*, 3872−3878.
(156) Yeh, K. C.; Lagarias, J. C. Eukaryotic Phytochromes: Light-Regulated Serine/Threonine Protein Kinases with Histidine Kinase Ancestry. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 13976−13981.
(157) Anders, K.; Gutt, A.; Gärtner, W.; Essen, L. O. Phototransformation of the Red Light Sensor Cyanobacterial Phytochrome 2 from Synechocystis Species Depends on Its Tongue Motifs. *J. Biol. Chem.* **2014**, *289*, 25590−25600.
(158) Song, C.; Mroginski, M. A.; Lang, C.; Kopycki, J.; Gärtner, W.; Matysik, J.; Hughes, J. 3D Structures of Plant Phytochrome A as Pr and Pfr from Solid-State NMR: Implications for Molecular Function. *Front. Plant Sci.* **2018**, *9*, 498.
(159) Li, L.; Kehoe, D. M. Abundance Changes of the Response Regulator RcaC Require Specific Aspartate and Histidine Residues and Are Necessary for Normal Light Color Responsiveness. *J. Bacteriol.* **2008**, *190*, 7241−7250.
(160) Wiltbank, L. B.; Kehoe, D. M. Two Cyanobacterial Photoreceptors Regulate Photosynthetic Light Harvesting by Sensing Teal, Green, Yellow, and Red Light. *mBio* **2016**, *7*, No. e02130-15.
(161) Zhao, C.; Gan, F.; Shen, G.; Bryant, D. A. RfpA, RfpB, and RfpC Are the Master Control Elements of Far-Red Light Photoacclimation (FaRLiP). *Front. Microbiol.* **2015**, *6*, 1303.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(162) Ho, M.-Y.; Gan, F.; Shen, G.; Zhao, C.; Bryant, D. A. Far-Red Light Photoacclimation (FaRLiP) in Synechococcus Sp. PCC 7335: I. Regulation of FaRLiP Gene Expression. *Photosynth. Res.* **2017**, *131*, 173−186.
(163) Ricci, A.; Dramis, L.; Shah, R.; Gärtner, W.; Losi, A. Visualizing the Relevance of Bacterial Blue- and Red-Light Receptors during Plant-Pathogen Interaction. *Environ. Microbiol. Rep.* **2015**, *7*, 795−802.
(164) Enomoto, G.; Okuda, Y.; Ikeuchi, M. Tlr1612 Is the Major Repressor of Cell Aggregation in the Light-Color-Dependent c-Di-GMP Signaling Network of Thermosynechococcus Vulcanus. *Sci. Rep.* **2018**, *8*, 5338.
(165) Quail, P.; Boylan, M.; Parks, B.; Short, T.; Xu, Y.; Wagner, D. Phytochromes: Photosensory Perception and Signal Transduction. *Science* **1995**, *268*, 675−680.
(166) Jaedicke, K.; Lichtenthäler, A. L.; Meyberg, R.; Zeidler, M.; Hughes, J. A Phytochrome−Phototropin Light Signaling Complex at the Plasma Membrane. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 12231−12236.
(167) Leopold, A. V.; Chernov, K. G.; Shemetov, A. A.; Verkhusha, V. V. Neurotrophin Receptor Tyrosine Kinases Regulated with Near-Infrared Light. *Nat. Commun.* **2019**, *10*, 1129.
(168) Ochoa-Fernandez, R.; Abel, N. B.; Wieland, F.-G.; Schlegel, J.; Koch, L.-A.; Miller, J. B.; Engesser, R.; Giuriani, G.; Brandl, S. M.; Timmer, J.; et al. Optogenetic Control of Gene Expression in Plants in the Presence of Ambient White Light. *Nat. Methods* **2020**, *17*, 717−725.
(169) Müller, K.; Engesser, R.; Timmer, J.; Zurbriggen, M. D.; Weber, W. Orthogonal Optogenetic Triple-Gene Control in Mammalian Cells. *ACS Synth. Biol.* **2014**, *3*, 796−801.
(170) Okada, K. HO1 and PcyA Proteins Involved in Phycobilin Biosynthesis Form a 1:2 Complex with Ferredoxin-1 Required for Photosynthesis. *FEBS Lett.* **2009**, *583*, 1251−1256.
(171) Wiltbank, L. B.; Kehoe, D. M. Diverse Light Responses of Cyanobacteria Mediated by Phytochrome Superfamily Photoreceptors. *Nat. Rev. Microbiol.* **2019**, *17*, 37−50.
(172) Fushimi, K.; Narikawa, R. Cyanobacteriochromes: Photoreceptors Covering the Entire UV-to-Visible Spectrum. *Curr. Opin. Struct. Biol.* **2019**, *57*, 39−46.
(173) Fushimi, K.; Narikawa, R. Phytochromes and Cyanobacteriochromes: Photoreceptor Molecules Incorporating a Linear Tetrapyrrole Chromophore. *Adv. Exp. Med. Biol.* **2021**, *1293*, 167−187.
(174) Hughes, R. M. A Compendium of Chemical and Genetic Approaches to Light-Regulated Gene Transcription. *Crit. Rev. Biochem. Mol. Biol.* **2018**, *53*, 453−474.
(175) Levskaya, A.; Chevalier, A. A.; Tabor, J. J.; Simpson, Z. B.; Lavery, L. A.; Levy, M.; Davidson, E. A.; Scouras, A.; Ellington, A. D.; Marcotte, E. M.; et al. Engineering Escherichia Coli to See Light. *Nature* **2005**, *438*, 441−442.
(176) Tabor, J. J.; Levskaya, A.; Voigt, C. A. Multichromatic Control of Gene Expression in Escherichia Coli. *J. Mol. Biol.* **2011**, *405*, 315−324.
(177) Tabor, J. J.; Salis, H. M.; Simpson, Z. B.; Chevalier, A. A.; Levskaya, A.; Marcotte, E. M.; Voigt, C. A.; Ellington, A. D. A Synthetic Genetic Edge Detection Program. *Cell* **2009**, *137*, 1272−1281.
(178) Ohlendorf, R.; Vidavski, R. R.; Eldar, A.; Moffat, K.; Möglich, A. From Dusk till Dawn: One-Plasmid Systems for Light-Regulated Gene Expression. *J. Mol. Biol.* **2012**, *416*, 534−542.
(179) Wu, L.; Luo, S.; Ma, S.; Liang, Z.; Wu, J.-R. Construction of Light-Sensing Two-Component Systems in Escherichia Coli. *Sci. Bull.* **2017**, *62*, 813−815.
(180) Ma, S.; Luo, S.; Wu, L.; Liang, Z.; Wu, J. R. Re-Engineering the Two-Component Systems as Light-Regulated in Escherichia Coli. *J. Biosci.* **2017**, *42*, 565−573.
(181) Lee, J. M.; Lee, J.; Kim, T.; Lee, S. K. Switchable Gene Expression in Escherichia Coli Using a Miniaturized Photobioreactor. *PLoS One* **2013**, *8*, 1−8.
(182) Davidson, E. A.; Basu, A. S.; Bayer, T. S. Programming Microbes Using Pulse Width Modulation of Optical Signals. *J. Mol. Biol.* **2013**, *425*, 4161−4166.
(183) Abe, K.; Miyake, K.; Nakamura, M.; Kojima, K.; Ferri, S.; Ikebukuro, K.; Sode, K. Engineering of a Green-Light Inducible Gene Expression System in Synechocystis Sp. PCC6803. *Microb. Biotechnol.* **2014**, *7*, 177−183.
(184) Schmidl, S. R.; Sheth, R. U.; Wu, A.; Tabor, J. J. Refactoring and Optimization of Light-Switchable Escherichia Coli Two-Component Systems. *ACS Synth. Biol.* **2014**, *3*, 820−831.
(185) Schmidl, S. R.; Ekness, F.; Sofjan, K.; Daeffler, K. N.-M.; Brink, K. R.; Landry, B. P.; Gerhardt, K. P.; Dyulgyarov, N.; Sheth, R. U.; Tabor, J. J. Rewiring Bacterial Two-Component Systems by Modular DNA-Binding Domain Swapping. *Nat. Chem. Biol.* **2019**, *15*, 690−698.
(186) Ramakrishnan, P.; Tabor, J. J. Repurposing Synechocystis PCC6803 UirS-UirR as a UV-Violet/Green Photoreversible Transcriptional Regulatory Tool in E. Coli. *ACS Synth. Biol.* **2016**, *5*, 733−740.
(187) Ong, N. T.; Olson, E. J.; Tabor, J. J. Engineering an E. Coli Near-Infrared Light Sensor. *ACS Synth. Biol.* **2018**, *7*, 240−248.
(188) Hughes, R. M.; Bolger, S.; Tapadia, H.; Tucker, C. L. Light-Mediated Control of DNA Transcription in Yeast. *Methods* **2012**, *58*, 1−7.
(189) Sorokina, O.; Kapus, A.; Terecskei, K.; Dixon, L. E.; Kozma-Bognar, L.; Nagy, F.; Millar, A. J. A Switchable Light-Input, Light-Output System Modelled and Constructed in Yeast. *J. Biol. Eng.* **2009**, *3*, 1−16.
(190) Hochrein, L.; Machens, F.; Messerschmidt, K.; Mueller-Roeber, B. PhiReX: A Programmable and Red Light-Regulated Protein Expression Switch for Yeast. *Nucleic Acids Res.* **2017**, *45*, 9193−9205.
(191) Hochrein, L.; Mitchell, L. A.; Schulz, K.; Messerschmidt, K.; Mueller-Roeber, B. L-SCRaMbLE as a Tool for Light-Controlled Cre-Mediated Recombination in Yeast. *Nat. Commun.* **2018**, *9*, 1931.
(192) Müller, K.; Engesser, R.; Metzger, S.; Schulz, S.; Kämpf, M. M.; Busacker, M.; Steinberg, T.; Tomakidi, P.; Ehrbar, M.; Nagy, F.; et al. A Red/Far-Red Light-Responsive Bi-Stable Toggle Switch to Control Gene Expression in Mammalian Cells. *Nucleic Acids Res.* **2013**, *41*, No. e77.
(193) Yen, S.; Trimmer, K. A.; Aboul-Fettouh, N.; Mullen, R. D.; Culver, J. C.; Dickinson, M. E.; Behringer, R. R.; Eisenhoffer, G. T. CreLite: An Optogenetically Controlled Cre/LoxP System Using Red Light. *Dev. Dyn.* **2020**, *249*, 1394−1403.
(194) Schindler, S. E.; McCall, J. G.; Yan, P.; Hyrc, K. L.; Li, M.; Tucker, C. L.; Lee, J.-M.; Bruchas, M. R.; Diamond, M. I. Photo-Activatable Cre Recombinase Regulates Gene Expression in Vivo. *Sci. Rep.* **2015**, *5*, 13627.
(195) Boulina, M.; Samarajeewa, H.; Baker, J. D.; Kim, M. D.; Chiba, A. Live Imaging of Multicolor-Labeled Cells in Drosophila. *Development* **2013**, *140*, 1605−1613.
(196) Kyriakakis, P.; Catanho, M.; Hoffner, N.; Thavarajah, W.; Hu, V. J.; Chao, S. S.; Hsu, A.; Pham, V.; Naghavian, L.; Dozier, L. E.; et al. Biosynthesis of Orthogonal Molecules Using Ferredoxin and Ferredoxin-NADP+ Reductase Systems Enables Genetically Encoded PhyB Optogenetics. *ACS Synth. Biol.* **2018**, *7*, 706−717.
(197) Wu, Y. I.; Frey, D.; Lungu, O. I.; Jaehrig, A.; Schlichting, I.; Kuhlman, B.; Hahn, K. M. A Genetically Encoded Photoactivatable Rac Controls the Motility of Living Cells. *Nature* **2009**, *461*, 104−108.
(198) Müller, K.; Zurbriggen, M. D.; Weber, W. Control of Gene Expression Using a Red- and Far-Red Light-Responsive Bi-Stable Toggle Switch. *Nat. Protoc.* **2014**, *9*, 622−632.
(199) Müller, K.; Siegel, D.; Rodriguez Jahnke, F.; Gerrer, K.; Wend, S.; Decker, E. L.; Reski, R.; Weber, W.; Zurbriggen, M. D. A Red Light-Controlled Synthetic Gene Expression Switch for Plant Systems. *Mol. BioSyst.* **2014**, *10*, 1679−1688.
(200) Yousefi, O. S.; Günther, M.; Hörner, M.; Chalupsky, J.; Wess, M.; Brandl, S. M.; Smith, R. W.; Fleck, C.; Kunkel, T.; Zurbriggen, M. D.; et al. Optogenetic Control Shows That Kinetic Proofreading Regulates the Activity of the T Cell Receptor. *eLife* **2019**, *8*, No. e42475.
(201) Müller, K.; Engesser, R.; Schulz, S.; Steinberg, T.; Tomakidi, P.; Weber, C. C.; Ulm, R.; Timmer, J.; Zurbriggen, M. D.; Weber, W. Multi-Chromatic Control of Mammalian Gene Expression and Signaling. *Nucleic Acids Res.* **2013**, *41*, No. e124.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(202) Olson, E. J.; Hartsough, L. A.; Landry, B. P.; Shroff, R.; Tabor, J. J. Characterizing Bacterial Gene Circuit Dynamics with Optically Programmed Gene Expression Signals. *Nat. Methods* **2014**, *11*, 449−455.
(203) Kaberniuk, A.; Shemetov, A. A.; Verkhusha, V. V. A Bacterial Phytochrome-Based Optogenetic System Controllable with near-Infrared Light. *Nat. Methods* **2016**, *13*, 591−597.
(204) Redchuk, T. A.; Omelina, E. S.; Chernov, K. G.; Verkhusha, V. V. Near-Infrared Optogenetic Pair for Protein Regulation and Spectral Multiplexing. *Nat. Chem. Biol.* **2017**, *13*, 633−639.
(205) Redchuk, T. A.; Karasev, M. M.; Omelina, E. S.; Verkhusha, V. V. Near-Infrared Light-Controlled Gene Expression and Protein Targeting in Neurons and Non-Neuronal Cells. *ChemBioChem* **2018**, *19*, 1334−1340.
(206) Redchuk, T. A.; Kaberniuk, A. A.; Verkhusha, V. V. Near-Infrared Light-Controlled Systems for Gene Transcription Regulation, Protein Targeting and Spectral Multiplexing. *Nat. Protoc.* **2018**, *13*, 1121−1136.
(207) Kaberniuk, A. A.; Baloban, M.; Monakhov, M. V.; Shcherbakova, D. M.; Verkhusha, V. V. Single-Component near-Infrared Optogenetic Systems for Gene Transcription Regulation. *Nat. Commun.* **2021**, *12*, 3859.
(208) Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.; Marraffini, L. A.; et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **2013**, *339*, 819−823.
(209) Mali, P.; Yang, L.; Esvelt, K. M.; Aach, J.; Guell, M.; DiCarlo, J. E.; Norville, J. E.; Church, G. M. RNA-Guided Human Genome Engineering via Cas9. *Science* **2013**, *339*, 823−826.
(210) Bubeck, F.; Hoffmann, M. D.; Harteveld, Z.; Aschenbrenner, S.; Bietz, A.; Waldhauer, M. C.; Börner, K.; Fakhiri, J.; Schmelas, C.; Dietz, L.; et al. Engineered Anti-CRISPR Proteins for Optogenetic Control of CRISPR-Cas9. *Nat. Methods* **2018**, *15*, 924−927.
(211) Gao, Y.; Xiong, X.; Wong, S.; Charles, E. J.; Lim, W. A.; Qi, L. S. Complex Transcriptional Modulation with Orthogonal and Inducible DCas9 Regulators. *Nat. Methods* **2016**, *13*, 1043−1049.
(212) Nihongaki, Y.; Kawano, F.; Nakajima, T.; Sato, M. Photoactivatable CRISPR-Cas9 for Optogenetic Genome Editing. *Nat. Biotechnol.* **2015**, *33*, 755−760.
(213) Polstein, L. R.; Gersbach, C. A. A Light-Inducible CRISPR-Cas9 System for Control of Endogenous Gene Activation. *Nat. Chem. Biol.* **2015**, *11*, 198−200.
(214) Shao, J.; Wang, M.; Yu, G.; Zhu, S.; Yu, Y.; Heng, B. C.; Wu, J.; Ye, H. Synthetic Far-Red Light-Mediated CRISPR-DCas9 Device for Inducing Functional Neuronal Differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, E6722−E6730.
(215) Yu, Y.; Wu, X.; Guan, N.; Shao, J.; Li, H.; Chen, Y.; Ping, Y.; Li, D.; Ye, H. Engineering a Far-Red Light−Activated Split-Cas9 System for Remote-Controlled Genome Editing of Internal Organs and Tumors. *Sci. Adv.* **2020**, *6*, No. eabb1777.
(216) Gomez, E. J.; Gerhardt, K.; Judd, J.; Tabor, J. J.; Suh, J. Light-Activated Nuclear Translocation of Adeno-Associated Virus Nanoparticles Using Phytochrome B for Enhanced, Tunable, and Spatially Programmable Gene Delivery. *ACS Nano* **2016**, *10*, 225−237.
(217) Hörner, M.; Jerez-Longres, C.; Hudek, A.; Hook, S.; Yousefi, O. S.; Schamel, W. W. A.; Hörner, C.; Zurbriggen, M. D.; Ye, H.; Wagner, H. J.; et al. Spatiotemporally Confined Red Light-Controlled Gene Delivery at Single-Cell Resolution Using Adeno-Associated Viral Vectors. *Sci. Adv.* **2021**, *7*, No. eabf0797.
(218) Strickland, D.; Lin, Y.; Wagner, E.; Hope, C. M.; Zayner, J.; Antoniou, C.; Sosnick, T. R.; Weiss, E. L.; Glotzer, M. TULIPs: Tunable, Light-Controlled Interacting Protein Tags for Cell Biology. *Nat. Methods* **2012**, *9*, 379−384.
(219) Kramer, M. M.; Mühlhäuser, W. W. D.; Weber, W.; Radziwill, G. Multichromatic Control of Signaling Pathways in Mammalian Cells. *Adv. Biosyst.* **2021**, *5*, 2000196.
(220) Redchuk, T. A.; Karasev, M. M.; Verkhusha, P. V.; Donnelly, S. K.; Hülsemann, M.; Virtanen, J.; Moore, H. M.; Vartiainen, M. K.; Hodgson, L.; Verkhusha, V. V. Optogenetic Regulation of Endogenous Proteins. *Nat. Commun.* **2020**, *11*, 605.
(221) Winkler, A.; Heintz, U.; Lindner, R.; Reinstein, J.; Shoeman, R. L.; Schlichting, I. A Ternary AppA−PpsR−DNA Complex Mediates Light Regulation of Photosynthesis-Related Gene Expression. *Nat. Struct. Mol. Biol.* **2013**, *20*, 859−867.
(222) Chatelle, C.; Ochoa-Fernandez, R.; Engesser, R.; Schneider, N.; Beyer, H. M.; Jones, A. R.; Timmer, J.; Zurbriggen, M. D.; Weber, W. A Green-Light-Responsive System for the Control of Transgene Expression in Mammalian and Plant Cells. *ACS Synth. Biol.* **2018**, *7*, 1349−1358.
(223) Ochoa-Fernandez, R.; Samodelov, S. L.; Brandl, S. M.; Wehinger, E.; Müller, K.; Weber, W.; Zurbriggen, M. D. Optogenetics in Plants: Red/Far-Red Light Control of Gene Expression. *Methods Mol. Biol.* **2016**, *1408*, 125−139.
(224) Samodelov, S. L.; Zurbriggen, M. D. Quantitatively Understanding Plant Signaling: Novel Theoretical−Experimental Approaches. *Trends Plant Sci.* **2017**, *22*, 685−704.
(225) Nash, A. I.; McNulty, R.; Shillito, M. E.; Swartz, T. E.; Bogomolni, R. A.; Luecke, H.; Gardner, K. H. Structural Basis of Photosensitivity in a Bacterial Light-Oxygen-Voltage/Helix-Turn-Helix (LOV-HTH) DNA-Binding Protein. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 9449−9454.
(226) Ryu, M. H.; Gomelsky, M. Near-Infrared Light Responsive Synthetic c-Di-GMP Module for Optogenetic Applications. *ACS Synth. Biol.* **2014**, *3*, 802−810.
(227) Elbakush, A. M.; Miller, K. W.; Gomelsky, M. CodY-Mediated c-Di-GMP-Dependent Inhibition of Mammalian Cell Invasion in Listeria Monocytogenes. *J. Bacteriol.* **2018**, *200*, No. e00457-17.
(228) Ryu, M. H.; Fomicheva, A.; Moskvin, O. V.; Gomelsky, M. Optogenetic Module for Dichromatic Control of C-Di-GMP Signaling. *J. Bacteriol.* **2017**, *199*, 1−11.
(229) Gomelsky, M. Photoactivated Cells Link Diagnosis and Therapy. *Sci. Transl. Med.* **2017**, *9*, 1−3.
(230) Shao, J.; Xue, S.; Yu, G.; Yu, Y.; Yang, X.; Bai, Y.; Zhu, S.; Yang, L.; Yin, J.; Wang, Y.; et al. Smartphone-Controlled Optogenetically Engineered Cells Enable Semiautomatic Glucose Homeostasis in Diabetic Mice. *Sci. Transl. Med.* **2017**, *9*, No. eaal2298.
(231) Ryu, M. H.; Kang, I. H.; Nelson, M. D.; Jensen, T. M.; Lyuksyutova, A. I.; Siltberg-Liberles, J.; Raizen, D. M.; Gomelsky, M. Engineering Adenylate Cyclases Regulated by Near-Infrared Window Light. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 10167−10172.
(232) Etzl, S.; Lindner, R.; Nelson, M. D.; Winkler, A. Structure-Guided Design and Functional Characterization of an Artificial Red Light−Regulated Guanylate/Adenylate Cyclase for Optogenetic Applications. *J. Biol. Chem.* **2018**, *293*, 9078−9089.
(233) Fomicheva, A.; Zhou, C.; Sun, Q.-Q.; Gomelsky, M. Engineering Adenylate Cyclase Activated by Near-Infrared Window Light for Mammalian Optogenetic Applications. *ACS Synth. Biol.* **2019**, *8*, 1314−1324.
(234) O'Neal, L.; Ryu, M.-H.; Gomelsky, M.; Alexandre, G. Optogenetic Manipulation of Cyclic Di-GMP (c-Di-GMP) Levels Reveals the Role of c-Di-GMP in Regulating Aerotaxis Receptor Activity in Azospirillum Brasilense. *J. Bacteriol.* **2017**, *199*, No. e00020-17.
(235) Bellini, D.; Papiz, M. Z. Structure of a Bacteriophytochrome and Light-Stimulated Protomer Swapping with a Gene Repressor. *Structure* **2012**, *20*, 1436−1446.
(236) Folcher, M.; Oesterle, S.; Zwicky, K.; Thekkottil, T.; Heymoz, J.; Hohmann, M.; Christen, M.; Daoud El-Baba, M.; Buchmann, P.; Fussenegger, M. Mind-Controlled Transgene Expression by a Wireless-Powered Optogenetic Designer Cell Implant. *Nat. Commun.* **2014**, *5*, 5392.
(237) Diensthuber, R. P.; Bommer, M.; Gleichmann, T.; Möglich, A. Full-Length Structure of a Sensor Histidine Kinase Pinpoints Coaxial Coiled Coils as Signal Transducers and Modulators. *Structure* **2013**, *21*, 1127−1136.
(238) Gasser, C.; Taiber, S.; Yeh, C.-M.; Wittig, C. H.; Hegemann, P.; Ryu, S.; Wunder, F.; Möglich, A. Engineering of a Red-Light-Activated Human CAMP/CGMP-Specific Phosphodiesterase. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 8803−8808.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(239) Stabel, R.; Stüven, B.; Hansen, J. N.; Körschen, H. G.; Wachten, D.; Möglich, A. Revisiting and Redesigning Light-Activated Cyclic-Mononucleotide Phosphodiesterases. *J. Mol. Biol.* **2019**, *431*, 3029−3045.
(240) Stierl, M.; Stumpf, P.; Udwari, D.; Gueta, R.; Hagedorn, R.; Losi, A.; Gärtner, W.; Petereit, L.; Efetova, M.; Schwarzel, M.; et al. Light Modulation of Cellular CAMP by a Small Bacterial Photoactivated Adenylyl Cyclase, BPAC, of the Soil Bacterium Beggiatoa. *J. Biol. Chem.* **2011**, *286*, 1181−1188.
(241) Chen, Z.-H.; Raffelberg, S.; Losi, A.; Schaap, P.; Gärtner, W. A Cyanobacterial Light Activated Adenylyl Cyclase Partially Restores Development of a Dictyostelium Discoideum, Adenylyl Cyclase a Null Mutant. *J. Biotechnol.* **2014**, *191*, 246−249.
(242) Möglich, A.; Ayers, R. A.; Moffat, K. Design and Signaling Mechanism of Light-Regulated Histidine Kinases. *J. Mol. Biol.* **2009**, *385*, 1433−1444.
(243) Hu, P. P.; Guo, R.; Zhou, M.; Gärtner, W.; Zhao, K. H. The Red-/Green-Switching GAF3 of Cyanobacteriochrome Slr1393 from Synechocystis Sp. PCC6803 Regulates the Activity of an Adenylyl Cyclase. *ChemBioChem* **2018**, *19*, 1887−1895.
(244) Fushimi, K.; Enomoto, G.; Ikeuchi, M.; Narikawa, R. Distinctive Properties of Dark Reversion Kinetics between Two Red/Green-Type Cyanobacteriochromes and Their Application in the Photoregulation of CAMP Synthesis. *Photochem. Photobiol.* **2017**, *93*, 681−691.
(245) Narikawa, R.; Fukushima, Y.; Ishizuka, T.; Itoh, S.; Ikeuchi, M. A Novel Photoactive GAF Domain of Cyanobacteriochrome AnPixJ That Shows Reversible Green/Red Photoconversion. *J. Mol. Biol.* **2008**, *380*, 844−855.
(246) Blain-Hartung, M.; Rockwell, N. C.; Moreno, M. V.; Martin, S. S.; Gan, F.; Bryant, D. A.; Lagarias, J. C. Cyanobacteriochrome-Based Photoswitchable Adenylyl Cyclases (CPACs) for Broad Spectrum Light Regulation of CAMP Levels in Cells. *J. Biol. Chem.* **2018**, *293*, 8473−8483.
(247) Hirose, Y.; Shimada, T.; Narikawa, R.; Katayama, M.; Ikeuchi, M. Cyanobacteriochrome CcaS Is the Green Light Receptor That Induces the Expression of Phycobilisome Linker Protein. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 9528−9533.
(248) Ong, N. T.; Tabor, J. J. A Miniaturized Escherichia Coli Green Light Sensor with High Dynamic Range. *ChemBioChem* **2018**, *19*, 1255−1258.
(249) Leung, D. W.; Otomo, C.; Chory, J.; Rosen, M. K. Genetically Encoded Photoswitching of Actin Assembly through the Cdc42-WASP-Arp2/3 Complex Pathway. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 12797−12802.
(250) Levskaya, A.; Weiner, O. D.; Lim, W. A.; Voigt, C. A. Spatiotemporal Control of Cell Signalling Using a Light-Switchable Protein Interaction. *Nature* **2009**, *461*, 997−1001.
(251) Shcherbakova, D. M.; Cox Cammer, N.; Huisman, T. M.; Verkhusha, V. V.; Hodgson, L. Direct Multiplex Imaging and Optogenetics of Rho GTPases Enabled by Near-Infrared FRET Article. *Nat. Chem. Biol.* **2018**, *14*, 591−600.
(252) Graziano, B. R.; Gong, D.; Anderson, K. E.; Pipathsouk, A.; Goldberg, A. R.; Weiner, O. D. A Module for Rac Temporal Signal Integration Revealed with Optogenetics. *J. Cell Biol.* **2017**, *216*, 2515−2531.
(253) Toettcher, J. E.; Gong, D.; Lim, W. A.; Weiner, O. D. Light-Based Feedback for Controlling Intracellular Signaling Dynamics. *Nat. Methods* **2011**, *8*, 837−839.
(254) Yousefi, O. S.; Ruggieri, M.; Idstein, V.; von Prillwitz, K. U.; Herr, L. A.; Chalupsky, J.; Köhn, M.; Weber, W.; Timmer, J.; Schamel, W. W. A. Cross-TCR Antagonism Revealed by Optogenetically Tuning the Half-Life of the TCR Ligand Binding. *Int. J. Mol. Sci.* **2021**, *22*, 4920.
(255) Yousefi, O. S.; Hörner, M.; Wess, M.; Idstein, V.; Weber, W.; Schamel, W. Optogenetic Tuning of Ligand Binding to The Human T Cell Receptor Using The Opto-Ligand-TCR System. *BIO-PROTOCOL* **2020**, *10*, e3540.
(256) Yang, X.; Jost, A. P. T.; Weiner, O. D.; Tang, C. A Light-Inducible Organelle-Targeting System for Dynamically Activating and Inactivating Signaling in Budding Yeast. *Mol. Biol. Cell* **2013**, *24*, 2419−2430.
(257) Reichhart, E.; Ingles-Prieto, A.; Tichy, A. M.; McKenzie, C.; Janovjak, H. A Phytochrome Sensory Domain Permits Receptor Activation by Red Light. *Angew. Chem., Int. Ed.* **2016**, *55*, 6339−6342.
(258) Toettcher, J. E.; Gong, D.; Lim, W. A.; Weiner, O. D. Light Control of Plasma Membrane Recruitment Using the Phy−PIF System. *Methods Enzymol.* **2011**, *497*, 409−423.
(259) Wilson, M. Z.; Ravindran, P. T.; Lim, W. A.; Toettcher, J. E. Tracing Information Flow from Erk to Target Gene Induction Reveals Mechanisms of Dynamic and Combinatorial Control. *Mol. Cell* **2017**, *67*, 757−769 (e5)..
(260) Toettcher, J. E.; Weiner, O. D.; Lim, W. a. Using Optogenetics to Interrogate the Dynamic Control of Signal Transmission by the Ras/Erk Module. *Cell* **2013**, *155*, 1422−1434.
(261) Johnson, H. E.; Goyal, Y.; Pannucci, N. L.; Schüpbach, T.; Shvartsman, S. Y.; Toettcher, J. E. The Spatiotemporal Limits of Developmental Erk Signaling. *Dev. Cell* **2017**, *40*, 185−192.
(262) Leopold, A. V.; Chernov, K. G.; Verkhusha, V. V. Optogenetically Controlled Protein Kinases for Regulation of Cellular Signaling. *Chem. Soc. Rev.* **2018**, *47*, 2454−2484.
(263) Leopold, A. V.; Verkhusha, V. V. Light Control of RTK Activity: From Technology Development to Translational Research. *Chem. Sci.* **2020**, *11*, 10019−10034.
(264) Rausenberger, J.; Tscheuschler, A.; Nordmeier, W.; Wüst, F.; Timmer, J.; Schäfer, E.; Fleck, C.; Hiltbrunner, A. Photoconversion and Nuclear Trafficking Cycles Determine Phytochrome A's Response Profile to Far-Red Light. *Cell* **2011**, *146*, 813−825.
(265) Pfeiffer, A.; Nagel, M.-K.; Popp, C.; Wüst, F.; Bindics, J.; Viczián, A.; Hiltbrunner, A.; Nagy, F.; Kunkel, T.; Schäfer, E. Interaction with Plant Transcription Factors Can Mediate Nuclear Import of Phytochrome B. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 5892−5897.
(266) Beyer, H. M.; Juillot, S.; Herbst, K.; Samodelov, S. L.; Müller, K.; Schamel, W. W.; Römer, W.; Schäfer, E.; Nagy, F.; Strähle, U.; et al. Red Light-Regulated Reversible Nuclear Localization of Proteins in Mammalian Cells and Zebrafish. *ACS Synth. Biol.* **2015**, *4*, 951−958.
(267) Juillot, S.; Beyer, H. M.; Madl, J.; Weber, W.; Zurbriggen, M. D.; Römer, W. Signalling to the Nucleus under the Control of Light and Small Molecules. *Mol. BioSyst.* **2016**, *12*, 345−349.
(268) Buckley, C. E.; Moore, R. E.; Reade, A.; Goldberg, A. R.; Weiner, O. D.; Clarke, J. D. W. Reversible Optogenetic Control of Subcellular Protein Localization in a Live Vertebrate Embryo. *Dev. Cell* **2016**, *36*, 117−126.
(269) Buckley, C. E. Optogenetic Control of Subcellular Protein Location and Signaling in Vertebrate Embryos. *Methods Mol. Biol.* **2019**, *1920*, 143−162.
(270) Allard, C. A. H.; Decker, F.; Weiner, O. D.; Toettcher, J. E.; Graziano, B. R. A Size-Invariant Bud-Duration Timer Enables Robustness in Yeast Cell Size Control. *PLoS One* **2018**, *13*, 1−15.
(271) Adrian, M.; Nijenhuis, W.; Hoogstraaten, R. I.; Willems, J.; Kapitein, L. C. A Phytochrome-Derived Photoswitch for Intracellular Transport. *ACS Synth. Biol.* **2017**, *6*, 1248−1256.
(272) Mushnikov, N. V.; Fomicheva, A.; Gomelsky, M.; Bowman, G. R. Inducible Asymmetric Cell Division and Cell Differentiation in a Bacterium. *Nat. Chem. Biol.* **2019**, *15*, 925−931.
(273) Iwai, H.; Züger, S.; Jin, J.; Tam, P.-H. Highly Efficient Protein Trans-Splicing by a Naturally Split DnaE Intein from Nostoc Punctiforme. *FEBS Lett.* **2006**, *580*, 1853−1858.
(274) Shah, N. H.; Eryilmaz, E.; Cowburn, D.; Muir, T. W. Naturally Split Inteins Assemble through a "Capture and Collapse" Mechanism. *J. Am. Chem. Soc.* **2013**, *135*, 18673−18681.
(275) Tyszkiewicz, A. B.; Muir, T. W. Activation of Protein Splicing with Light in Yeast. *Nat. Methods* **2008**, *5*, 303−305.
(276) Losi, A.; Braslavsky, S. E. The Time-Resolved Thermodynamics of the Chromophore−Protein Interactions in Biological Photosensors as Derived from Photothermal Measurements. *Phys. Chem. Chem. Phys.* **2003**, *5*, 2739−2750.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(277) Li, L.; Shemetov, A. A.; Baloban, M.; Hu, P.; Zhu, L.; Shcherbakova, D. M.; Zhang, R.; Shi, J.; Yao, J.; Wang, L. V.; et al. Small Near-Infrared Photochromic Protein for Photoacoustic Multi-Contrast Imaging and Detection of Protein Interactions in Vivo. *Nat. Commun.* **2018**, *9*, 2734.
(278) Yao, J.; Kaberniuk, A. A.; Li, L.; Shcherbakova, D. M.; Zhang, R.; Wang, L.; Li, G.; Verkhusha, V. V.; Wang, L. V. Multiscale Photoacoustic Tomography Using Reversibly Switchable Bacterial Phytochrome as a Near-Infrared Photochromic Probe. *Nat. Methods* **2016**, *13*, 67−73.
(279) Märk, J.; Dortay, H.; Wagener, A.; Zhang, E.; Buchmann, J.; Grötzinger, C.; Friedrich, T.; Laufer, J. Dual-Wavelength 3D Photoacoustic Imaging of Mammalian Cells Using a Photoswitchable Phytochrome Reporter Protein. *Commun. Phys.* **2018**, *1*, 1−10.
(280) Huang, Y.; Xia, A.; Yang, G.; Jin, F. Bioprinting Living Biofilms through Optogenetic Manipulation. *ACS Synth. Biol.* **2018**, *7*, 1195−1200.
(281) Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z. J. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science* **2013**, *339*, 786−791.
(282) Raju, D. N.; Hansen, J. N.; Rassmann, S.; Stüven, B.; Jikeli, J. F.; Strünker, T.; Körschen, H. G.; Möglich, A.; Wachten, D. Cyclic Nucleotide-Specific Optogenetics Highlights Compartmentalization of the Sperm Flagellum into CAMP Microdomains. *Cells* **2019**, *8*, 648.
(283) Jansen, V.; Alvarez, L.; Balbach, M.; Strünker, T.; Hegemann, P.; Kaupp, U. B.; Wachten, D. Controlling Fertilization and CAMP Signaling in Sperm by Optogenetics. *eLife* **2015**, *4*, No. e05161.
(284) Hansen, J. N.; Kaiser, F.; Klausen, C.; Stüven, B.; Chong, R.; Bönigk, W.; Mick, D. U.; Möglich, A.; Jurisch-Yaksi, N.; Schmidt, F. I.; et al. Nanobody-Directed Targeting of Optogenetic Tools to Study Signaling in the Primary Cilium. *eLife* **2020**, *9*, No. e57907.
(285) Hartsough, L. A.; Park, M.; Kotlajich, M. V.; Lazar, J. T.; Han, B.; Lin, C.-C. J.; Musteata, E.; Gambill, L.; Wang, M. C.; Tabor, J. J. Optogenetic Control of Gut Bacterial Metabolism to Promote Longevity. *eLife* **2020**, *9*, No. e56849.
(286) Beyer, H. M.; Thomas, O. S.; Riegel, N.; Zurbriggen, M. D.; Weber, W.; Hörner, M. Generic and Reversible Opto-Trapping of Biomolecules. *Acta Biomater.* **2018**, *79*, 276−282.
(287) Jia, H.; Kai, L.; Heymann, M.; García-Soriano, D. A.; Härtel, T.; Schwille, P. Light-Induced Printing of Protein Structures on Membranes in Vitro. *Nano Lett.* **2018**, *18*, 7133−7140.
(288) Baaske, J.; Mühlhäuser, W. W. D.; Yousefi, O. S.; Zanner, S.; Radziwill, G.; Hörner, M.; Schamel, W. W. A.; Weber, W. Optogenetic Control of Integrin-Matrix Interaction. *Commun. Biol.* **2019**, *2*, 15.
(289) Yüz, S. G.; Ricken, J.; Wegner, S. V. Independent Control over Multiple Cell Types in Space and Time Using Orthogonal Blue and Red Light Switchable Cell Interactions. *Adv. Sci.* **2018**, *5*, 1800446.
(290) Hörner, M.; Raute, K.; Hummel, B.; Madl, J.; Creusen, G.; Thomas, O. S.; Christen, E. H.; Hotz, N.; Gübeli, R. J.; Engesser, R.; et al. Phytochrome-Based Extracellular Matrix with Reversibly Tunable Mechanical Properties. *Adv. Mater.* **2019**, *31*, 1806727.
(291) Beyer, H. M.; Engesser, R.; Hörner, M.; Koschmieder, J.; Beyer, P.; Timmer, J.; Zurbriggen, M. D.; Weber, W. Synthetic Biology Makes Polymer Materials Count. *Adv. Mater.* **2018**, *30*, No. e1800472.
(292) Hörner, M.; Gerhardt, K.; Salavei, P.; Hoess, P.; Härrer, D.; Kaiser, J.; Tabor, J. J.; Weber, W. Production of Phytochromes by High-Cell-Density E. Coli Fermentation. *ACS Synth. Biol.* **2019**, *8*, 2442−2450.
(293) Hörner, M.; Yousefi, O. S.; Schamel, W. W. A.; Weber, W. Production, Purification and Characterization of Recombinant Biotinylated Phytochrome B for Extracellular Optogenetics. *Bio-protocol* **2020**, *10*, No. e3541.
(294) Nakajima, M.; Abe, K.; Ferri, S.; Sode, K. Development of a Light-Regulated Cell-Recovery System for Non-Photosynthetic Bacteria. *Microb. Cell Fact.* **2016**, *15*, 1−8.
(295) Westers, L.; Westers, H.; Quax, W. J. Bacillus Subtilis as Cell Factory for Pharmaceutical Proteins: A Biotechnological Approach to Optimize the Host Organism. *Biochim. Biophys. Acta, Mol. Cell Res.* **2004**, *1694*, 299−310.
(296) Castillo-Hair, S. M.; Baerman, E. A.; Fujita, M.; Igoshin, O. A.; Tabor, J. J. Optogenetic Control of Bacillus Subtilis Gene Expression. *Nat. Commun.* **2019**, *10*, 3099.
(297) Miyake, K.; Abe, K.; Ferri, S.; Nakajima, M.; Nakamura, M.; Yoshida, W.; Kojima, K.; Ikebukuro, K.; Sode, K. A Green-Light Inducible Lytic System for Cyanobacterial Cells. *Biotechnol. Biofuels* **2014**, *7*, 1−8.
(298) Fischer, A. J.; Lagarias, J. C. Harnessing Phytochrome's Glowing Potential. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 17334−17339.
(299) Piatkevich, K. D.; Suk, H.-J.; Kodandaramaiah, S. B.; Yoshida, F.; DeGennaro, E. M.; Drobizhev, M.; Hughes, T. E.; Desimone, R.; Boyden, E. S.; Verkhusha, V. V. Near-Infrared Fluorescent Proteins Engineered from Bacterial Phytochromes in Neuroimaging. *Biophys. J.* **2017**, *113*, 2299−2309.
(300) Cook, Z. T.; Brockway, N. L.; Tobias, Z. J. C.; Pajarla, J.; Boardman, I. S.; Ippolito, H.; Nkombo Nkoula, S.; Weissman, T. A. Combining Near-Infrared Fluorescence with Brainbow to Visualize Expression of Specific Genes within a Multicolor Context. *Mol. Biol. Cell* **2019**, *30*, 491−505.
(301) Stepanenko, O. V.; Stepanenko, O. V.; Shpironok, O. G.; Fonin, A. V.; Kuznetsova, I. M.; Turoverov, K. K. Near-Infrared Markers Based on Bacterial Phytochromes with Phycocyanobilin as a Chromophore. *Int. J. Mol. Sci.* **2019**, *20*, 6067.
(302) Stepanenko, O. V.; Baloban, M.; Bublikov, G. S.; Shcherbakova, D. M.; Stepanenko, O. V.; Turoverov, K. K.; Kuznetsova, I. M.; Verkhusha, V. V. Allosteric Effects of Chromophore Interaction with Dimeric Near-Infrared Fluorescent Proteins Engineered from Bacterial Phytochromes. *Sci. Rep.* **2016**, *6*, 18750.
(303) Shcherbakova, D. M.; Baloban, M.; Emelyanov, A. V.; Brenowitz, M.; Guo, P.; Verkhusha, V. V. Bright Monomeric Near-Infrared Fluorescent Proteins as Tags and Biosensors for Multiscale Imaging. *Nat. Commun.* **2016**, *7*, 12405.
(304) Yu, D.; Dong, Z.; Gustafson, W. C.; Ruiz-González, R.; Signor, L.; Marzocca, F.; Borel, F.; Klassen, M. P.; Makhijani, K.; Royant, A.; et al. Rational Design of a Monomeric and Photostable Far-Red Fluorescent Protein for Fluorescence Imaging in Vivo. *Protein Sci.* **2016**, *25*, 308−315.
(305) Rumyantsev, K. A.; Shcherbakova, D. M.; Zakharova, N. I.; Emelyanov, A. V.; Turoverov, K. K.; Verkhusha, V. V. Minimal Domain of Bacterial Phytochrome Required for Chromophore Binding and Fluorescence. *Sci. Rep.* **2016**, *5*, 1−10.
(306) Subach, O. M.; Barykina, N. V.; Anokhin, K. V.; Piatkevich, K. D.; Subach, F. V. Near-Infrared Genetically Encoded Positive Calcium Indicator Based on GAF-FP Bacterial Phytochrome. *Int. J. Mol. Sci.* **2019**, *20*, 3488.
(307) Shcherbakova, D. M.; Stepanenko, O. V.; Turoverov, K. K.; Verkhusha, V. V. Near-Infrared Fluorescent Proteins: Multiplexing and Optogenetics across Scales. *Trends Biotechnol.* **2018**, *36*, 1230−1243.
(308) Simon, J.; Losi, A.; Zhao, K. H.; Gärtner, W. FRET in a Synthetic Flavin- and Bilin-Binding Protein. *Photochem. Photobiol.* **2017**, *93*, 1057−1062.
(309) Rockwell, N. C.; Martin, S. S.; Lagarias, J. C. Red/Green Cyanobacteriochromes: Sensors of Color and Power. *Biochemistry* **2012**, *51*, 9667−9677.
(310) Fushimi, K.; Miyazaki, T.; Kuwasaki, Y.; Nakajima, T.; Yamamoto, T.; Suzuki, K.; Ueda, Y.; Miyake, K.; Takeda, Y.; Choi, J.-H.; et al. Rational Conversion of Chromophore Selectivity of Cyanobacteriochromes to Accept Mammalian Intrinsic Biliverdin. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 8301−8309.
(311) Oliinyk, O. S.; Shemetov, A. A.; Pletnev, S.; Shcherbakova, D. M.; Verkhusha, V. V. Smallest Near-Infrared Fluorescent Protein Evolved from Cyanobacteriochrome as Versatile Tag for Spectral Multiplexing. *Nat. Commun.* **2019**, *10*, 279.
(312) Wu, X.-J.; Yang, H.; Sheng, Y.; Zhu, Y.-L.; Li, P.-P. Fluorescence Properties of a Novel Cyanobacteriochrome GAF Domain from Spirulina That Exhibits Moderate Dark Reversion. *Int. J. Mol. Sci.* **2018**, *19*, 12−14.

https://doi.org/10.1021/acs.chemrev.1c00194
Chem. Rev. 2021, 121, 14906−14956

(313) Li, L.; Murphy, J. T.; Lagarias, J. C. Continuous Fluorescence Assay of Phytochrome Assembly in Vitro. *Biochemistry* **1995**, *34*, 7923−7930.
(314) Tang, K.; Ding, W. L.; Höppner, A.; Zhao, C.; Zhang, L.; Hontani, Y.; Kennis, J. T. M.; Gärtner, W.; Scheer, H.; Zhou, M.; et al. The Terminal Phycobilisome Emitter, Lcm: A Light-harvesting Pigment with a Phytochrome Chromophore. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 15880−15885.
(315) Wu, X.-J.; Chang, K.; Luo, J.; Zhou, M.; Scheer, H.; Zhao, K.-H. Modular Generation of Fluorescent Phycobiliproteins. *Photochem. Photobiol. Sci.* **2013**, *12*, 1036.
(316) Hou, Y.; Ding, W.; Hu, J.; Jiang, X.; Tan, Z.; Zhao, K.-H. Very Bright Phycoerythrobilin Chromophore for Fluorescence Biolabeling. *ChemBioChem* **2019**, *20*, 2777−2783.
(317) Zhao, B.; Ding, W.; Tan, Z.; Tang, Q.; Zhao, K.-H. A Large Stokes Shift Fluorescent Protein Constructed from the Fusion of Red Fluorescent MCherry and Far-Red Fluorescent BDFP1.6. *ChemBioChem* **2019**, *20*, 1167−1173.
(318) Gan, F.; Shen, G.; Bryant, D. Occurrence of Far-Red Light Photoacclimation (FaRLiP) in Diverse Cyanobacteria. *Life* **2015**, *5*, 4−24.
(319) Gan, F.; Zhang, S.; Rockwell, N. C.; Martin, S. S.; Lagarias, J. C.; Bryant, D. A. Extensive Remodeling of a Cyanobacterial Photosynthetic Apparatus in Far-Red Light. *Science* **2014**, *345*, 1312−1317.
(320) Li, X.-D.; Tan, Z.-Z.; Ding, W.-L.; Hou, Y.-N.; Kong, C.-D.; Zhao, B.-Q.; Zhao, K.-H. Design of Small Monomeric and Highly Bright Near-Infrared Fluorescent Proteins. *Biochim. Biophys. Acta, Mol. Cell Res.* **2019**, *1866*, 1608−1617.
(321) Yu, D.; Gustafson, W. C.; Han, C.; Lafaye, C.; Noirclerc-Savoye, M.; Ge, W.-P.; Thayer, D. A.; Huang, H.; Kornberg, T. B.; Royant, A.; et al. An Improved Monomeric Infrared Fluorescent Protein for Neuronal and Tumour Brain Imaging. *Nat. Commun.* **2014**, *5*, 3626.
(322) Ding, W. L.; Hou, Y. N.; Tan, Z. Z.; Jiang, S. P.; Miao, D.; Losi, A.; Gärtner, W.; Scheer, H.; Zhao, K. H. Far-Red Acclimating Cyanobacterium as Versatile Source for Bright Fluorescent Biomarkers. *Biochim. Biophys. Acta, Mol. Cell Res.* **2018**, *1865*, 1649−1656.
(323) Hou, Y. N.; Ding, W. L.; Jiang, S. P.; Miao, D.; Tan, Z. Z.; Hu, J. L.; Scheer, H.; Zhao, K. H. Bright Near-Infrared Fluorescence Bio-Labeling with a Biliprotein Triad. *Biochim. Biophys. Acta, Mol. Cell Res.* **2019**, *1866*, 277−284.
(324) Pennacchietti, F.; Serebrovskaya, E. O.; Faro, A. R.; Shemyakina, I. I.; Bozhanova, N. G.; Kotlobay, A. A.; Gurskaya, N. G.; Bodén, A.; Dreier, J.; Chudakov, D. M.; et al. Fast Reversibly Photoswitching Red Fluorescent Proteins for Live-Cell RESOLFT Nanoscopy. *Nat. Methods* **2018**, *15*, 601−604.
(325) Montecinos-Franjola, F.; Lin, J. Y.; Rodriguez, E. A. Fluorescent Proteins for in Vivo Imaging, Where's the Biliverdin? *Biochem. Soc. Trans.* **2020**, *48*, 2657−2667.
(326) Gerhardt, K. P.; Olson, E. J.; Castillo-Hair, S. M.; Hartsough, L. A.; Landry, B. P.; Ekness, F.; Yokoo, R.; Gomez, E. J.; Ramakrishnan, P.; Suh, J.; et al. An Open-Hardware Platform for Optogenetics and Photobiology. *Sci. Rep.* **2016**, *6*, 35363.
(327) Richter, F.; Scheib, U. S.; Mehlhorn, J.; Schubert, R.; Wietek, J.; Gernetzki, O.; Hegemann, P.; Mathes, T.; Möglich, A. Upgrading a Microplate Reader for Photobiology and All-Optical Experiments. *Photochem. Photobiol. Sci.* **2015**, *14*, 270−279.
(328) Olson, E. J.; Tzouanas, C. N.; Tabor, J. J. A Photoconversion Model for Full Spectral Programming and Multiplexing of Optogenetic Systems. *Mol. Syst. Biol.* **2017**, *13*, 926.
(329) Dietler, J.; Stabel, R.; Möglich, A. Pulsatile Illumination for Photobiology and Optogenetics. In *Methods in Enzymology*; Deiters, A., Ed.; 2019; pp 227−248.
(330) Stüven, B.; Stabel, R.; Ohlendorf, R.; Beck, J.; Schubert, R.; Möglich, A. Characterization and Engineering of Photoactivated Adenylyl Cyclases. *Biol. Chem.* **2019**, *400*, 429−441.
(331) Bugaj, L. J.; Lim, W. A. High-Throughput Multicolor Optogenetics in Microwell Plates. *Nat. Protoc.* **2019**, *14*, 2205−2228.
(332) Thomas, O. S.; Hörner, M.; Weber, W. A Graphical User Interface to Design High-Throughput Optogenetic Experiments with the OptoPlate-96. *Nat. Protoc.* **2020**, *15*, 2785−2787.
(333) Repina, N. A.; McClave, T.; Johnson, H. J.; Bao, X.; Kane, R. S.; Schaffer, D. V. Engineered Illumination Devices for Optogenetic Control of Cellular Signaling Dynamics. *Cell Rep.* **2020**, *31*, 107737.
(334) Repina, N. A.; Johnson, H. J.; McClave, T.; Kane, R. S.; Schaffer, D. V. Protocol to Fabricate Engineered Illumination Devices for Optogenetic Control of Cellular Signaling Dynamics. *STAR Protoc.* **2020**, *1*, 100141.
(335) Olson, E. J.; Tabor, J. J. Optogenetic Characterization Methods Overcome Key Challenges in Synthetic and Systems Biology. *Nat. Chem. Biol.* **2014**, *10*, 502−511.
(336) Ruan, H.; Brake, J.; Robinson, J. E.; Liu, Y.; Jang, M.; Xiao, C.; Zhou, C.; Gradinaru, V.; Yang, C. Deep Tissue Optical Focusing and Optogenetic Modulation with Time-Reversed Ultrasonically Encoded Light. *Sci. Adv.* **2017**, *3*, No. eaao5520.
(337) Yang, J.; Li, L.; Shemetov, A. A.; Lee, S.; Zhao, Y.; Liu, Y.; Shen, Y.; Li, J.; Oka, Y.; Verkhusha, V. V.; et al. Focusing Light inside Live Tissue Using Reversibly Switchable Bacterial Phytochrome as a Genetically Encoded Photochromic Guide Star. *Sci. Adv.* **2019**, *5*, No. eaay1211.
(338) Quail, P. H. Phytochrome-Interacting Factors. In *Light and Plant Development*; Whitelam, G. C., Halliday, K. J., Eds.; Blackwell Publishing Ltd: Oxford, UK, 2007; pp 81−105.
(339) Losi, A.; Gärtner, W.; Raffelberg, S.; Cella Zanacchi, F.; Bianchini, P.; Diaspro, A.; Mandalari, C.; Abbruzzetti, S.; Viappiani, C. A Photochromic Bacterial Photoreceptor with Potential for Super-Resolution Microscopy. *Photochem. Photobiol. Sci.* **2013**, *12*, 231−235.

NOTE ADDED IN PROOF

Employment of PhyA (PAS-GAF-PHY construct) from *A. thaliana* in red light-dependent complex formation with the far-red elongated hypocotyl 1 protein was recently reported by Zhou et al. to act in a similar manner as the PhyB/PIF couples [Zhou, Y.; Kong, D.; Wang, X.; Yu, G.; Wu, X.; Guan, N.; Weber, W.; Ye, H. A small and highly sensitive red/far-red optogenetic switch for applications in mammals. *Nat. Biotech.* **2021**, Article ASAP].

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Chem. Rev. 2021, 121, 14906−14956