Review

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A guide to designing photocontrol in proteins: methods, strategies and applications

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Abstract: Light is essential for various biochemical processes in all domains of life. In its presence certain proteins inside a cell are excited, which either stimulates or inhibits subsequent cellular processes. The artificial photocontrol of specifically proteins is of growing interest for the investigation of scientific questions on the organismal, cellular and molecular level as well as for the development of medicinal drugs or biocatalytic tools. For the targeted design of photocontrol in proteins, three major methods have been developed over the last decades, which employ either chemical engineering of small-molecule photosensitive effectors (photopharmacology), incorporation of photoactive non-canonical amino acids by genetic code expansion (photoxenoprotein engineering), or fusion with photoreactive biological modules (hybrid protein optogenetics). This review compares the different methods as well as their strategies and current applications for the light-regulation of proteins and provides background information useful for the implementation of each technique.

Keywords: light-regulation; non-canonical amino acids; optogenetics; photopharmacology; photoxenoprotein engineering; proteins.

Introduction

Light plays a decisive role for most forms of life as it stimulates various essential processes in organisms. Phototaxis, the migration of microorganisms towards light, phototropism, the growth of plants towards light, photosynthesis, the turnover of light energy into chemical energy as well as vision in animals and humans are only some examples of light-mediated, biological processes. In particular, light offers dynamic control with a high level of spatiotemporal resolution, which is most paramount for each of these processes. In recent years, this attribute of light was exploited to artificially control diverse biological systems.

One of the most important targets for the implementation of artificial photocontrol are proteins (Gautier et al. 2014), as they are key molecules of life and the object of study in various research areas (Figure 1). The dynamic light-mediated regulation of protein function allows to answer fundamental questions and to develop novel tools in organismal, cellular and molecular research (Figure 1, left box). To this end, light can be applied noninvasively and remotely, as it penetrates tissue in *in vivo* approaches nanometer to centimeter deep and water-based solutions, e.g., for enzymatic studies or industrial purposes, even meter deep depending on the wavelength of irradiation, and the density and absorption properties of the sample (Marblestone et al. 2013).

For the design of photocontrol in proteins, three major methods have been established (Figure 1, right box). Fundamental to each technique are chromophore bearing receiver molecules, which absorb light of specific wavelengths and subsequently undergo different types of reactions. Two of the three methods employ light-sensitive small-molecular synthetic receiver compounds and are thus summarized with the term optochemistry (Ankenbruck et al. 2018). In the first method, dubbed photopharmacology (Velema et al. 2014), the receiver consists of a light-responsive molecular cage (photocage) or switch (photoswitch) and is attached to a biologically active moiety. The term originates from the underlying idea to create light-responsive medical drugs targeting mostly key proteins of disease (Hüll et al. 2018), which are only activated at the site of the malady thereby reducing side effects. While photopharmacology primarily uses small-molecular ligands that are attached (non)covalently post translation of the target protein, the second method incorporates a light-responsive non-canonical amino acid (ncAA) into the target protein during

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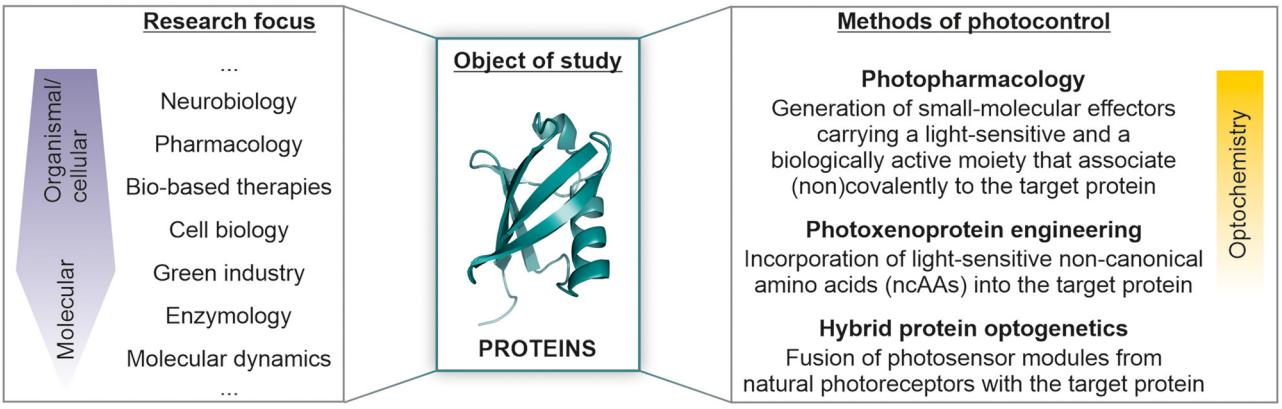

Figure 1: Overview of methods to photocontrol the biological activity of proteins.

translation (Courtney and Deiters 2018). Thus, the receiver, again either a molecular photocage or a photoswitch, is coupled to an amino acid moiety. This method has not yet received an official name; since here a ncAA-containing protein, or xenoprotein (Agostini et al. 2017), that is light-sensitive is generated, I will hence refer to it as "photoxenoprotein engineering". Probably the most widespread photocontrol method is dubbed optogenetics (Deisseroth et al. 2006). It uses natural light-sensitive photoreceptors, often ion channels, to artificially induce cellular processes primarily *in vivo* (Nagel et al. 2002, 2003). Controlling the function of a specific target protein is the goal of a particular sub-category of optogenetics. Here, the photosensor modules of the natural photoreceptors are used as receivers; these proteins carry a light-responsive cofactor, which undergoes a conformational change upon irradiation with light. They are genetically fused to the target protein to create a hybrid protein. Aside from these three methods other diverse strategies to photocontrol proteins exist. These comprise e.g., photosensitizers, which produce radical oxygen species upon irradiation with light, light-responsive matrices or photodegradation and are summarized in detail elsewhere (Claaßen et al. 2019; Szymański et al. 2013).

The focus of this review is a comparison of the three widely used methods photopharmacology, photoxenoprotein engineering, and hybrid protein optogenetics. By this, I aim to give an overview for the interested reader that might help to decide which technique might be best suited to photocontrol their protein of interest. I will thereby concentrate on the method concepts, specific strategies and current applications. Finally, I will provide user-relevant fundamentals of the three techniques regarding their photochemical properties, efficient irradiation, and the engineering process.

Photopharmacology

Method concept

Already at the start of the last century it was discovered that light can be used to cure illness demonstrated by, e.g., the phototherapeutical treatment of lupus vulgaris, a cutaneous form of tuberculosis (Grzybowski and Pietrzak 2012), or the targeted tissue ablation through the local production of radical oxygen species by irradiation of photosensitizers also called photodynamic therapy (Agostinis et al. 2011; Dougherty et al. 1998; Raab 1900; Tappeiner and Jodlbauer 1904).

Decades later in the 1970s (Bieth et al. 1969, 1970; Deal et al. 1969), a new development of light as a therapeutic agent was pioneered, which set out to solve a conventional problem with classical medical drugs as outlined in the following. The effect of therapeutic compounds is based on their interaction with protein-based targets such as receptors, ion channels or enzymes (Ritter et al. 2020). This biological activity evokes a pharmacological response necessary for the treatment of the malady. To prevent potential short-, mid-, and long-term side effects and to keep the dose efficacy high, the drug must inevitably be selective for its target (Edwards and Aronson 2000). Selectivity can be achieved by local administration of the drug (Gaudana et al. 2010), targeting macromolecules that are only present in specific organs or that are overexpressed in certain pathogenic tissue such as tumor cells (Hanahan and Weinberg 2000) or microorganisms (Hutchings et al. 2019). Nevertheless, selectivity is in general difficult to realize since most macromolecular targets are constitutively expressed in healthy and diseased tissue. Therefore, light-responsive drugs that can be activated with spatiotemporal resolution promise a straightforward solution to attain selectivity (Lerch et al. 2016). While traditional drugs

directly demonstrate the required biological activity, light-sensitive drugs are administered in an inactive form and are activated at the target site by local irradiation. For this purpose, effectors of the target molecules are designed that contain a small molecular synthetic chromophore, which changes its structure upon irradiation (Lerch et al. 2016; Hüll et al. 2018); only the isomer obtained after irradiation shows the desired biological activity. As a result, the activity of the drug in healthy tissue is suppressed and side effects can be prevented. From this initial idea, a general method concept has been developed for the dynamic regulation of protein activity (Figure 2). A light-sensitive inhibitor is synthesized, commonly by redesign of an existing inhibitor, which efficiently hampers the function of the protein, e.g., by binding to the active site. Irradiation at a different location (spatial resolution) or time (temporal resolution) leads to the interconversion of the light-sensitive inhibitor to a less active state, e.g., by loosing affinity towards the active site. Hence, while an inhibitory effect is maintained with a traditional, light-insensitive inhibitor (Figure 2, left panels), the protein regains activity in the presence of a light-sensitive inhibitor after irradiation (Figure 2, right panels).

Leaning on optogenetics, which emerged at the exact same time, this alternative photocontrol method has occasionally been named "chemical optogenetics" (Reis et al. 2016). Owing to the misrepresentation that light-sensitivity might be genetically encoded by chemical means, this term was replaced with the name "photopharmacology", which originates from the observation that both isomers of the light-responsive compound can show different pharmacokinetic and pharmacodynamic properties in the body (Broichhagen et al. 2015a; Velema et al. 2014; Hüll et al. 2018). Furthermore, this term clearly distinguishes photopharmacology and optogenetics, which follow different approaches: chemical engineering of ligands (photopharmacology) and repurposing of natural photoreceptors by alteration of the genetic code (optogenetics).

While photopharmaceuticals have not yet been approved as medical drugs, many light-sensitive inhibitors have been developed for a wide range of protein targets (Hüll et al. 2018) and either constitute precursors for future therapeutics or facilitate advanced fundamental studies in a spatiotemporal manner.

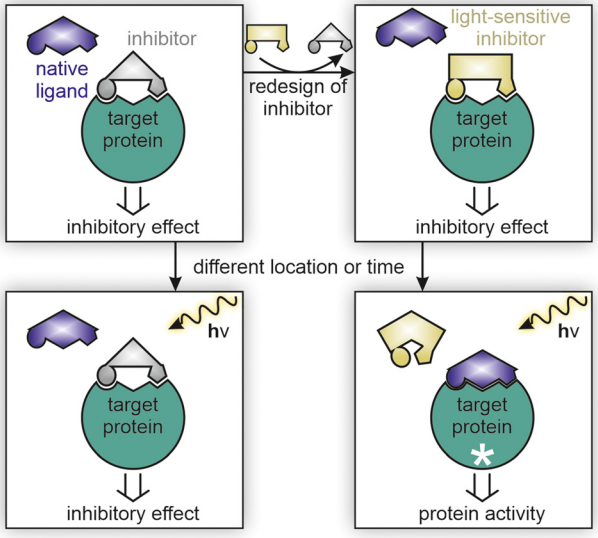

Figure 2: The method concept of photopharmacology for the photocontrol of proteins.
The design of protein inhibitors is commonly based on the imitation and replacement of a native ligand (upper left). The resulting inhibitory effect is insensitive to light (bottom left). In photopharmacology, the inhibitor is redesigned to exhibit two states, which can be interconverted by light. One state can bind to the target protein and induce the desired inhibitory effect (upper right). The other state cannot interact with the target protein and allows for binding of the native ligand (bottom right).

Strategies

The five main strategies of photopharmacology (Figure 3) utilize two different kinds of receiver molecule, i.e. photocages and photoswitches. One of the most widely used strategies is the photocaging of molecular drugs (Ellis-Davies 2007; Klán et al. 2013; Young and Deiters 2007). For this purpose, a light-responsive protecting group is coupled to an inhibitor thus blocking its interaction with the binding site of a target protein through steric hindrance. Exposure to light of a certain wavelength excites the delocalized electron system of the molecular cage, which triggers a decaging reaction allowing the released inhibitor to bind to the target protein. However, molecular photocages are only able to irreversibly activate the protein of interest presenting an one-way strategy. Alternatively, a light-responsive molecular switch can be incorporated into the inhibitor. These so-called photoswitches either undergo an $E \rightarrow Z$ isomerization or a ring-open → ring-closed reaction upon irradiation with light of a certain wavelength ($h\nu_1$) (Szymański et al. 2013). While one isomer demonstrates only a weak affinity to the target protein, the ligands obtain a high affinity after light-induced isomerization, which mediates their efficacy. Notably, these reactions are reversible ($Z \rightarrow E$ or ring-closed → ring-open) either by irradiation with light of a different wavelength ($h\nu_2$) or thermally (ΔT).

While these first two strategies present a non-covalent approach using free ligands, the next three strategies deploy

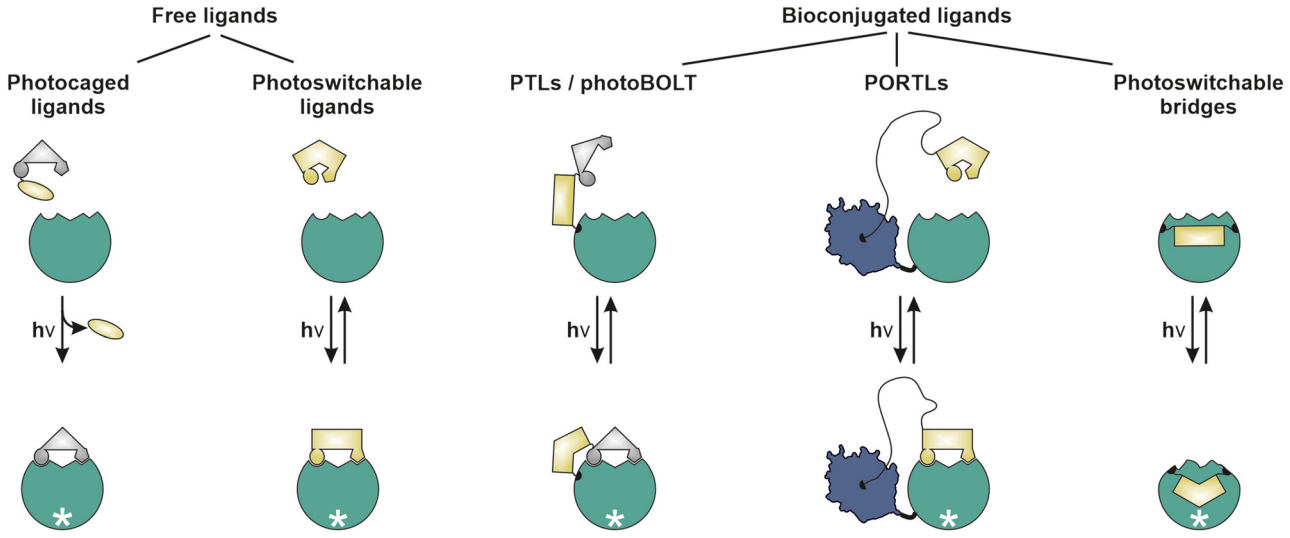

Figure 3: Strategies of photopharmacology.
The five main strategies include photocaged ligands, photoswitchable free ligands, photoswitchable tethered ligands (PTLs) or photoswitchable bioorthogonal ligand tethering (photoBOLT), photoswitchable orthogonal remotely tethered ligands (PORTLs) and photoswitchable bridges. Blue-green: Target protein; white asterisk: Inhibition effect; dark blue: protein tag; grey: inhibitor; gold: photocage or photoswitch with substituted binding motifs or coupled to the inhibitor moiety; black half circles: Site of bioconjugation.

a bioconjugation step to covalently tether the light-responsive inhibitor to the target protein (Hermanson 2013). For this purpose, a small bioconjugation motif can be attached to the ligand, which reacts with a specific residue on the target protein close to the active site; examples are the reactions of maleimide with native cysteine residues (Yamada et al. 2007) or the copper(I)-mediated click reaction of an alkyne group with the azide moiety of the ncAA *p*-azido-L-phenylalanine (pAzF) (Schlesinger et al. 2018). This allows for a site-specific modification of the target protein, which leads to a local enrichment and capturing of the ligand at the site of activity and ultimately increases the efficacy of the inhibitor. The inhibitory effect is then regulated by the conformation of the photoswitch; while one isomer facilitates binding of the moiety with the biological activity to the protein, the other prevents it. For this strategy the expressions "photoswitchable tethered ligands (PTLs)" (Acosta-Ruiz et al. 2020; Donthamsetti et al. 2017; Lin et al. 2018) and "photoswitchable bioorthogonal ligand tethering (photoBOLT)" (Tsai et al. 2015) are commonly used. Moreover, the ligands can be tethered distant to the active site, which reduces the enrichment effect but allows for larger bioconjugation motifs (Krishnamurthy et al. 2007). In many cases, the photoswitchable ligand is bioconjugated to a self-labeling protein tag such as the SNAP tag, which is genetically fused with either the target protein or an auxiliary protein interacting with the target protein (Leippe et al. 2017; Farrants et al. 2018). This strategy was dubbed "photoswitchable orthogonal remotely tethered ligands (PORTLs)" (Broichhagen et al. 2015b). Finally, the photoswitchable ligands can also be tethered to two residues of the target protein generating a light-responsive bridge or crosslink (Bozovic et al. 2020; Schierling et al. 2010; Liu et al. 2017b). Light-induced isomerization then leads to a conformational change of the protein, which consequently alters the accessibility or catalytic capability of the active site.

In general, all light-responsive molecules are based on different types of chromophores. Light-induced decaging or isomerization can occur with UV or visible light, however, the exact wavelength range can be adjusted and tuned by different substitution patterns. The basic scaffolds of photocages include aromatic ketones (Klán et al. 2013), in which the carbonyl group is the key to the photochemical cleavage reaction (Figure 4A). The electronic transitions of these photocages and, hence, their spectral properties and their photoreaction can be modulated by substitution on the phenyl ring and by the polarity of the solvent. Nitroaryl groups constitute a second class of photocages, among them the widely used nitrobenzyl derivatives (Bardhan and Deiters 2019; Klán et al. 2013). Their photo-induced cleavage reaction (Figure 4B) is induced by excitation of the nitro group and strongly depends on the substitution, solvent and pH in aqueous solution. The nitrobenzyl moiety is usually directly attached to carboxylic acids (Patchornik et al. 1970) and thiols (Bayley et al. 1998), whereas a carbonic acid linker is employed to improve the quality of alcohols and amines as leaving

group setting free carbon dioxide in the photo-induced decaging reaction. Since nitrobenzyl groups exhibit limited spectral properties, the aromatic ring is furthermore often substituted with, e.g., methoxy or methylenedioxy groups, which alters the absorption strength, quantum yield and solubility. Unfortunately, the released nitrosoaldehyde can undergo a condensation reaction with amines such as lysine residues on proteins, which makes it potentially toxic *in vivo*. A third class of photocages are coumarin-4-ylmethyl groups (Bardhan and Deiters 2019; Klán et al. 2013). Irradiation of the integrated coumarin thereby results in a heterolysis reaction (Figure 4C), which sets free the attached ligand and coumarinylmethyl cation; the latter subsequently reacts with a nucleophile. Advantages of these photocages include their large molar absorption coefficients at longer wavelengths, the fast release rates and their fluorescent properties, which allow for the monitoring of decaging (Givens et al. 2012; Mayer and Heckel 2006; Schultz 2007). Finally, arylmethyl groups can also be used as photocages (Klán et al. 2013). Here, decaging is realized by a photo-induced hydrolysis reaction (Figure 4D) with benzyl or heterobenzyl esters or ethers as leaving group.

With regard to photoswitches, azobenzenes are the most prominent and studied class (Schmermund et al. 2019). Isomerization (Figure 4E) occurs for $E \rightarrow Z$ with UV light, or with visible light for derivatives that contain a push-pull electron system. Isomerization for $Z \rightarrow E$ can be achieved thermally or by using visible light (>420 nm). Advantages of azobenzenes include their fast isomerization and a low rate of photobleaching; however, quantitative switching is difficult to obtain and the thermal lifetime of the *Z* isomer highly depends on the substituents (Dokić et al. 2009). Modification of established ligands can occur either through the extension of the molecule core with an azobenzene, known as the "azo-extension" approach (Rodríguez-Soacha et al. 2021), or the replacement of moieties to generate an azobenzene, known as the

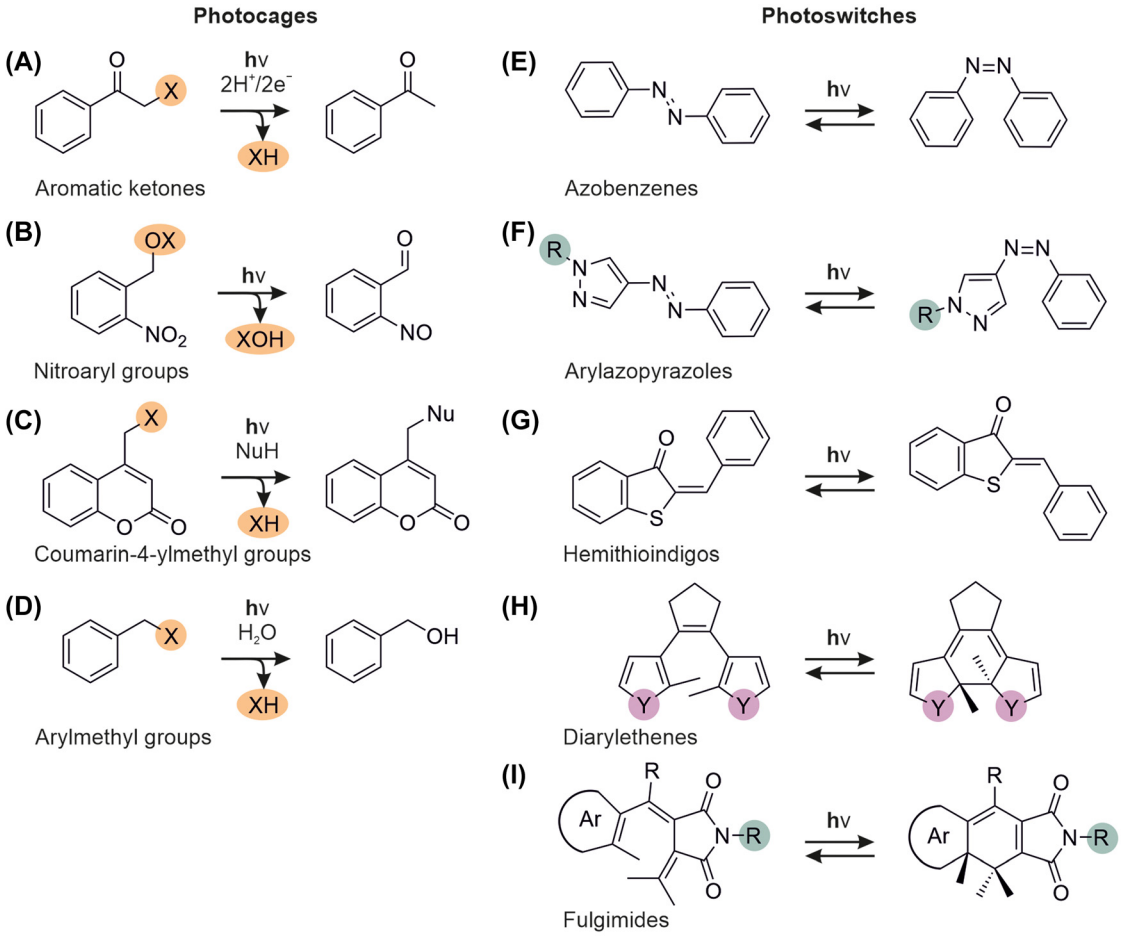

Figure 4: Main examples of photocages (A–D) and photoswitches (E–I).
Only the core scaffold of each chromophore is shown. For more detailed information, in particular on substitution patterns and photochemical properties, see references (Klán et al. 2013; Komarov et al. 2018; Lachmann et al. 2019; Petermayer and Dube 2018; Szymański et al. 2013; Weston et al. 2014). X: Leaving group; R: H and other substituents; Y: S, O, N.

"azologization" approach (Rustler et al. 2019). Arylazopyrazoles are yet another class of photoswitches (Weston et al. 2014). They include a nitrogen-containing five-membered aromatic ring, at which the azo-phenyl group is either substituted at position 4 (4-arylazopyrazoles; Figure 4F) or at position 3, which is equivalent to position 5 taking the tautomerization equilibrium of the heterocycle into account [3(5)-arylazopyrazoles]. Such heterocycles show nearly to completely quantitative conversion between *E* and *Z* isomers, and slower thermal reversion (Weston et al. 2014). Furthermore, hemithioindigos are the most advanced and most used photoswitches from the family of indigoids (Petermayer and Dube 2018). They consist of a thioindigo moiety that is connected to a stilbene by an isomerizable double bond (Figure 4G). Light-induced isomerization in both directions occurs with visible light of different wavelength, which is highly desirable for biological applications. Their advantages also include high photostabilities with little fatigue over many switching cycles, fast isomerization and high thermal stability. Next to $E \rightarrow Z$, certain photoswitches can undergo a light-induced ring-open → ring-closed interconversion. Upon irradiation with light the photoswitch undergoes a conrotatory electrocyclization reaction that causes a rigidity and electronic change instead of a geometrical change. Prominent examples thereof are diarylethenes (Figure 4H) with high thermostability of both isomers and efficient photo-induced interconversion (Komarov et al. 2018), however, they tend to degrade after only a few switching-cycles, which is why fulgimides (Lachmann et al. 2019) emerged as an alternative (Figure 4I). They show excellent photochemical properties, including thermal stability of the isomers and reversible switching, as well as quantitative conversion. Photoswitching occurs upon irradiation between the ring-open *E* isomer and the ring-closed isomer; though, depending on the substitution pattern of the 1,3,5-hexatriene system (*R'*), an $E \rightarrow Z$ isomerization of the ring-open isomer would be possible. It has to be noted that these photoswitches are only some current examples; other examples include stilbenes, spiropyrans and thiophenefulgides (Szymański et al. 2013).

Applications

Drug development

The main goal of photopharmacology is to develop smart drugs that can be switched on and off by irradiation with light to tackle off-target toxicity. This area of application is quite comprehensive as it does include all stages of drug design meaning early precursor development towards a specific target protein, analysis of the mode of action *in vitro* and/or *in vivo*, and refinement of the drug. Moreover, light-responsive compounds are often used to study the molecular basis of a disease.

A prerequisite of photopharmacological drug design is to choose target proteins that are associated with a localized disease where the delivery of light and, hence, the spatiotemporal control by light is feasible. The target proteins must further be specific for the cells that are the cause of the disease, e.g., overproduced proteins in tumor cells or proteins that solely exist in bacteria. While examining the efficacy of the light-responsive drug it became apparent that the difference in activity prior to and after irradiation is more powerful *in vivo* than *in vitro* (Bieth et al. 1969; Wainberg and Erlanger 1971). I will use the term light regulation factor (LRF) to describe this difference in activity from now on. (Other publications use the term dynamic range for this factor.) For example, while an azobenzene-based drug shows only an LRF of 1.5-fold *in vitro*, it might cause a drastic phenotypic effect such as reduction of cell growth *in vivo*. This can be explained by the strong connectivity of proteins in a cell; small *in vitro* effects can be amplified especially when the target protein is part of a signaling cascade. Another important issue is that many synthetic light-responsive compounds are designed to permeate the cell-membrane (Mentel et al. 2011), which results in the local entrapment and increased concentration of the drug inside the cell. Thus, targeting of intracellular proteins is feasible, as well. Before a photopharmacological drug can be tested in a clinical study, however, its behavior *in vivo* requires critical analysis. The drug should neither be metabolized nor rapidly degraded (pharmacokinetics) and it should not demonstrate toxicity (pharmacodynamics).

One of the most pertinent diseases that is addressed by photopharmacological drugs is cancer. Solid tumors are highly localized and constitute an excellent target for the light-responsive compounds. Moreover, current therapies, first and foremost chemotherapy, lead to severe systemic side effects that have an enormous impact on the health and well-being of patients (Edwards and Aronson 2000). Hence, the field of photo-induced anticancer medication targeting, e.g., kinases or microtubule dynamics, is constantly growing (Dunkel and Ilaš 2021). A current example for kinase inhibitors are the azobenzene-based azoaxitinibs designed against the vascular endothelial growth factor receptor (VEGFR)-2 kinase, which show a more than 40-fold difference in their inhibitory strength upon irradiation (Heintze et al. 2020). Furthermore, a study recently showcased the attractiveness of the rarely used

photoswitch styrylbenzothiazole for the photocontrol of the microtubule cytoskeleton as it is metabolically stable compared to previously designed azobenzene-based inhibitors (Gao et al. 2021). Another highly promising approach for photopharmacological cancer treatment is the photocontrol of proteolysis-targeting chimeras (PROTACs), which induce the degradation via the ubiquitin-proteasome pathway of pathogenic proteins by mediating binding of the respective protein of interest with an E3 ligase (Dunkel and Ilaš 2021; Zeng et al. 2021). A very successful example thereof is the development of two approaches, in which PROTACs were coupled with photocaging groups (Naro et al. 2020).

Next to cancer, local bacterial infections are a prominent target of photopharmacology. Here, photocontrol can help to tackle the build-up of antibiotic resistance in the environment and to prevent the attack of symbiotic bacteria in the human microbiome by developing drugs, which only exhibit an antibiotic activity in one of the two isomers. To this end, we recently developed a novel antibiotic precursor against *Salmonella typhimurium* tryptophan synthase, a key enzyme in the metabolism of bacteria. The compound comprises an azobenzene photoswitch, which inhibited tryptophan production reversibly and in an allosteric manner (Simeth et al. 2021). An example of irreversible photoactivation with caged antibiotics is the synthesis of vancomycin and cephalosporin derivatives, which were active against five pathogenic bacteria in a light-dependent manner (Shchelik et al. 2021). Notably, the two isomers not only cause different effects in bacteria, but they also evoke different resistance mechanisms than the initial light-unresponsive drug as could be shown with tetra-ortho-chloroazobenzene-trimethoprim conjugates and the antibiotic trimethoprim in the *Escherichia coli* strain CS1562 (Lauxen et al. 2021).

Other current examples include the study of neural networks and associated disorders by targeting GABA and nicotinic acetylcholine receptors (Bregestovski and Ponomareva 2021), glycine receptors (Maleeva et al. 2021) human cannabinoid receptors (Rodríguez-Soacha et al. 2021), G protein-coupled receptors (Donthamsetti et al. 2021) and muscarinic receptors (Barbero-Castillo et al. 2021). Moreover, azobenzene-based ligands were designed to analyze the distinct functions of the cryptochrome CRY1 in the circadian clock (Kolarski et al. 2021a).

Functional studies on proteins

Synthetic ligands have a long history as tools for the study of the function of proteins, particularly to investigate enzyme catalysis. Hence, it is not surprising that light-responsive ligands can also be used for fundamental research on proteins. Nevertheless, hitherto there are only rare examples, which primarily set out to investigate dynamics and allostery of proteins. It should be noted that I will use the term "dynamics" to summarize all kind of motions spanning from fast, sub-ms fluctuations to slow conformational changes in the ms and s time range. 20 years ago, one of the first examples demonstrated that protein conformation can be modulated with light by conjugating an azobenzene to an α-helix using two cysteine or homocysteine residues at positions i and $i + 7$ and two reactive moieties flanking the azobenzene core (Kumita et al. 2000, 2002). Through computational-based engineering of the ligand they achieved to control the stability of an α-helix in the target peptide; while the Z isomer sustained the α-helical structure, the E isomer led to its destabilization and degradation (Figure 5A). Although they did not apply this technique to a protein determining the effect on ligand binding or enzymatic turnover, they set the ground for further developments. Only recently, this strategy was revived to photocontrol the binding of a peptide ligand to the PDZ3 domain of the postsynaptic density-95 protein (Bozovic et al. 2020). The authors targeted an α-helix, which is localized outside the active site, but which has been shown to affect peptide binding nonetheless (Petit et al. 2009), and accomplished a temperature – as well as ligand-dependent LRF for peptide binding of 120-fold. They further proposed a thermodynamic cycle of ligand binding and photo-induced conformational change (Bozovic et al. 2020) as well as determined the speed of the signal transmittance from the α-helix to the active site (Bozovic et al. 2021) using extensive biophysical studies. Although an effect originating outside the active sites is nowadays often called allosteric, allostery is actually traditionally defined as the control of protein activity in the orthosteric site by binding of a ligand to an allosteric site (Liu and Nussinov 2016). Hence, our groups addressed the photocontrol of true allostery by designing a photoswitchable diarylethene-based inhibitor for imidazole glycerol phosphate synthase (Kneuttinger et al. 2018). In this model enzyme for allostery, ligand binding to one subunit activates the enzymatic activity of the second subunit over a distance of ~25 Å (Douangamath et al. 2002). By mimicking the allosteric ligand using a diarylethene, we could not only competitively inhibit the catalytic turnover of the first subunit (HisF), but also control the activity of the second subunit (HisH) with the same efficiency by light (Figure 5B). Finally, bioconjugation of an azobenzene derivative to a lipase was used to explore the effect of photo-induced isomerization on protein dynamics and ultimately enzyme activity (Agarwal et al. 2012). By covalent

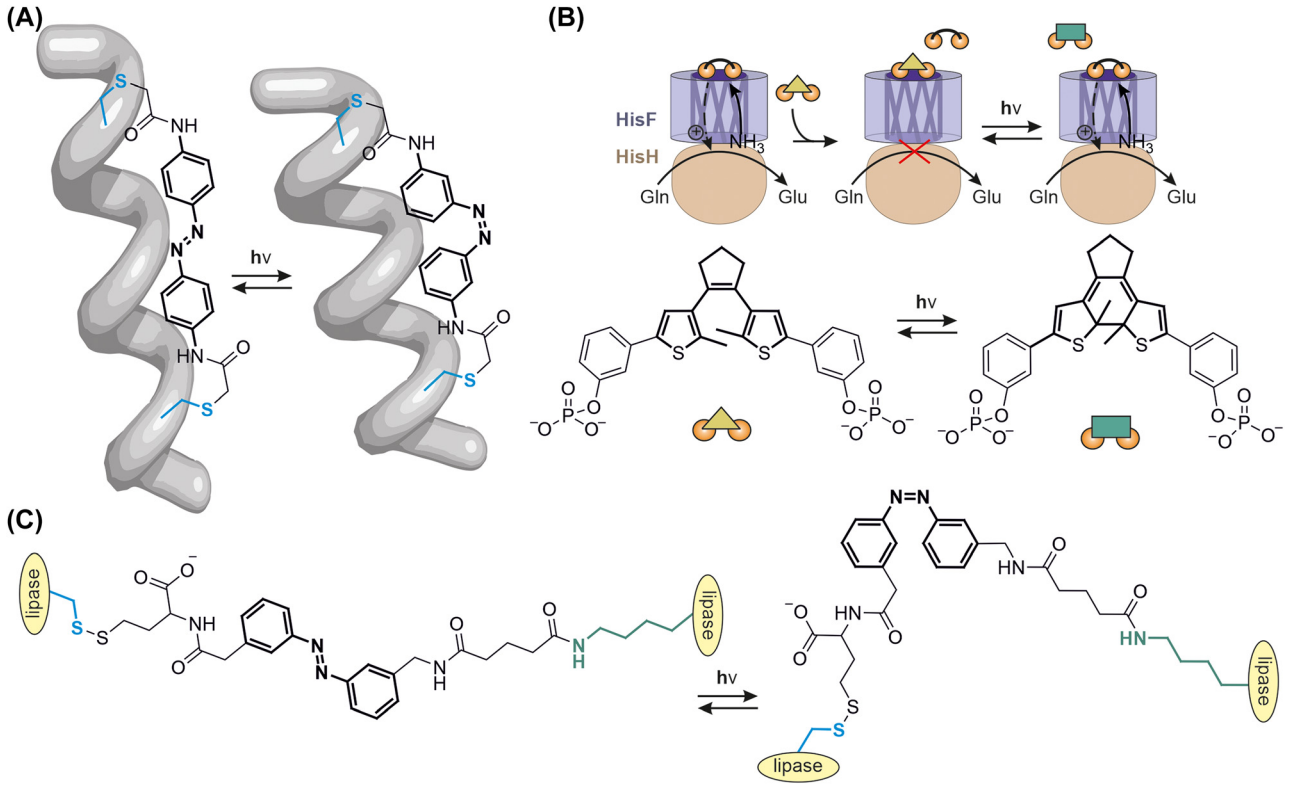

Figure 5: Exemplary photoswitch applications.
(A) Bioconjugation of an azobenzene inhibitor to two cysteine residues (blue) of an alpha helix result in destabilization of the secondary structure in the *E* configuration (left) while maintaining it in the *Z* configuration (right) (Kumita et al. 2000). (B) Photocontrol of allostery in the HisF:HisH bienzyme complex. While the open diarylethene (triangle) prohibits binding of the allosteric ligand (barbell), the closed diarylethene (rectangle) loses affinity towards the HisF active site and facilitates binding of the allosteric ligand. Binding of the allosteric ligand initiates the glutaminase reaction in HisH (Kneuttinger et al. 2018). (C) Bioconjugation of an azobenzene inhibitor to two surface loops via a cysteine (blue) and a lysine (green) residue mediates the photocontrol of lipase dynamics and as a result activity (Agarwal et al. 2012). Bold: Photoswitch cores as represented in Figure 4.

attachment of the photoswitch, they cross-linked two surface loops of the lipase (Figure 5C), which they previously identified computationally to be essential for protein dynamics. While the cross-linking efficiency was unfavorably low, the catalytic activity of the enzyme was significantly increased compared to the wildtype enzyme and could be further enhanced by light. These examples demonstrate that small molecular light-responsive compounds are highly promising for functional studies of proteins. This trend is even more pronounced for the next photocontrol method.

Photoxenoprotein engineering

Method concept

The next main photocontrol method has developed as a spin-off of photopharmacology. For roughly 20 years, photocages and photoswitches have been synthesized with an amino acid moiety and incorporated into proteins through genetic code expansion. Early examples of these light-sensitive ncAAs include photocaged cysteine (Wu et al. 2004), photocaged tyrosine (Deiters et al. 2006) and the photoswitchable phenylalanine-4′-azobenzene (AzoF) (Bose et al. 2006; Nakayama et al. 2004). The genetic insertion of optochemical molecules into a protein rendering it light-responsive is according to Hüll et al. “strictly speaking not photopharmacology” (Hüll et al. 2018). While photopharmacology focuses on the post-translational modification of a protein, the incorporation of ncAAs during the translation process inside a cell creates artificial proteins that can be regulated with light (photoxenoproteins). The strong protein engineering character of this technique will become obvious in the following chapters. The mostly rational design of such photoxenoproteins exploits the complexity of the protein machinery. Protein function depends on sites for ligand or

substrate binding, for catalytic turnover in the case of enzymes, for signal propagation in protein networks and for allosteric control. Besides these often clearly identifiable domains the conformation and dynamics of a protein are crucial for its activity. As a result, a protein possesses multiple residues located in these sites, which are essential for its function by conveying ligand binding, catalysis, allostery, multimerization or flexibility/rigidity. These residues can be found in the orthosteric site or distant thereof in allosteric sites. In photoxenoprotein engineering, the residue at such an important position is replaced with a light-sensitive ncAA, which renders the protein biologically inactive (Figure 6) since the ncAA cannot take over the role of the native residue. However, upon irradiation the ncAA is decaged setting free a native residue or changes its structure, which allows for the required effect and establishes the biological activity of the protein.

The successful implementation of photoxenoprotein engineering strongly relies on the efficient incorporation of the ncAA through genetic code expansion (Furter 1998; Wang et al. 2001), the concept of which is briefly described in the following. Common to all living cells, proteins are encoded in a gene, which is transcribed into a messenger RNA (mRNA) and subsequently decoded at the ribosome. For this translation step, all native amino acids are delivered to the ribosome by specific transfer RNAs (tRNAs), which carry an anticodon matching the triplet codon, in response to which the amino acid is incorporated (Ibba and Söll 2004). The correct selection and aminoacylation onto the tRNA is the task of unique aminoacyl tRNA synthetases (aaRSs) (Patrick and Zaida 2003) (Figure 7A). Each aaRS only recognizes its designated native amino acid and the respective tRNAs and the tRNAs in turn will not bind to aaRSs specific for another native amino acid. Hence, the different aaRS/tRNA pairs constitute modules of a large aaRS/tRNA network, which are orthogonal to each other (Chin 2006). This orthogonality ensures the fidelity and efficiency of the natural translation system. The redesign of the translation system by genetic code expansion has to ensure that both the fidelity and efficiency of the natural protein synthesis are maintained (Hoesl and Budisa 2012). One of the most commonly and most successfully used strategies to facilitate the incorporation of ncAAs is the amber suppression technique, in which the ncAA is inserted by a newly introduced aaRS/tRNA pair in response to an amber codon introduced in-frame at a specific position in the DNA sequence (Figure 7B). Three strict requirements guide this redesign (Liu and Schultz 2010): the biological suitability of the ncAA, the strictness of amber codon recognition solely by the new tRNA, and the orthogonality as well as specificity for the ncAA of the new aaRS/tRNA pair. The first requirement includes that the ncAA is metabolically stable, can enter the cell, and is accepted by the elongation factor Tu (EF-Tu) and the ribosome. This is mostly fulfilled since ncAAs show relatively good cell permeability, and EF-Tu and the ribosome are highly promiscuous (Wang and Schultz 2005). By choosing the amber codon, the second requirement is also usually met, however, the third requirement includes two challenges. A heterologous aaRS/tRNA pair needs to be selected, which is orthogonal in the chosen host system, e.g., *E. coli*, meaning neither the aaRS nor the tRNA recognizes host specific tRNAs or aaRSs, respectively. tRNA recognition by aaRSs is often domain or species specific (Kwok and Wong 1980) and, hence, phylogenetically distant aaRS/tRNA pairs often exhibit orthogonality such as the TyrRS/tRNA pair from *Methanocaldococcus jannaschii* in *E. coli* (Santoro et al. 2002). Notably, the anticodon of the heterologous tRNA is changed to CUA to match the amber codon (UAG). Moreover, to ensure the specificity of the selected aaRS for the ncAA, a two-step evolution of the amino acid binding pocket of the enzyme is applied, which establishes binding of the ncAA by the heterologous aaRS

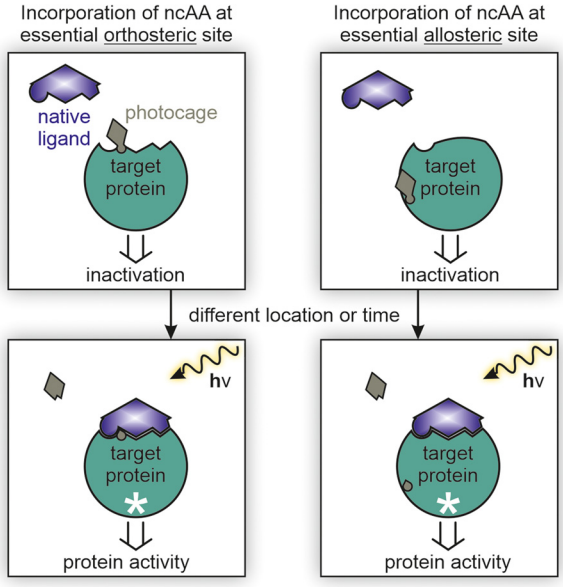

Figure 6: Method concept of photoxenoprotein engineering. In general, proteins carry residues in orthosteric and allosteric sites, which are essential for their biological activity. In photoxenoprotein engineering, incorporation of a light-sensitive ncAA replacing such a residue leads to inactivation of the biological function through various mechanisms of inhibition such as the blockage (left) or conformational change (right) of a ligand binding site. Irradiation triggers a change of the ncAA such as the decaging of a light-sensitive protecting group (shown), which as a result establishes the biological activity of the protein such as binding of a ligand (shown).

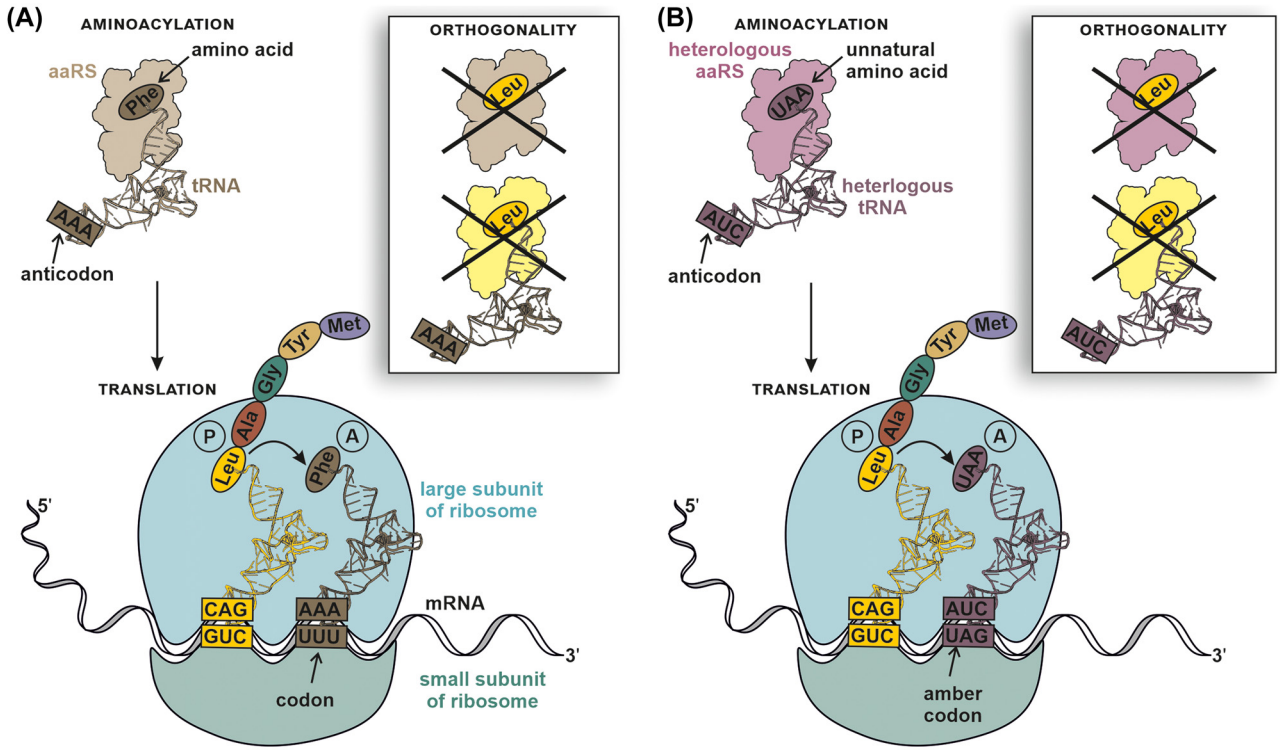

Figure 7: The native and redesigned protein translation machinery for the incorporation of endogenous and non-canonical amino acids. (A) For the incorporation of endogenous amino acids, endogenous tRNAs are aminoacylated with the amino acid by their allocated aaRS. In the A site of the ribosome, the arriving tRNA base pairs with the matching codon of its anticodon. The loaded amino acid can then react with the nascent protein chain waiting in the P site of the ribosome. To ensure high fidelity and efficiency of the system, the aaRS is not allowed to bind another amino acid and the tRNA must not bind to another aaRS. (B) For the incorporation of ncAAs, heterologous tRNAs need to be aminoacylated with the ncAA by a heterologous aaRS. Using the amber suppression technique, the tRNA base pairs with an amber codon at the ribosome so that the ncAA can then react with the nascent protein chain. Similar to the native system, the heterologous aaRS is not allowed to bind an endogenous amino acid and the heterologous tRNA must not bind to an endogenous aaRS, to ensure high fidelity and efficiency.

in positive selection rounds, while making sure that native amino acids are not recognized in negative selection rounds (Liu and Schultz 1999; Melançon and Schultz 2009; Santoro et al. 2002). Similarly, the heterologous tRNA can be evolved to prevent cross-reactivities with native aaRSs (Liu and Schultz 1999; Wang and Schultz 2005).

Although the development of the genetic code expansion technique progresses since the 1980s (Noren et al. 1989), its limitations are still highly discussed. For the non-expert these drawbacks often appear to outweigh the benefits of this approach. In a later chapter on the design process, I will describe the major concerns and what advances have been made in recent years to overcome these issues.

Strategies

Photoxenoprotein engineering basically manipulates the native regulation mechanisms of proteins. Hence, existing strategies can be grouped into three super-categories namely blockage of a ligand binding site, multimerization and conformational change (Figure 8). As for photopharmacology, the strategies are furthermore divided into the type of optochemical receiver that is used. In addition to photocages and photoswitches, photoxenoprotein engineering utilizes photocrosslinks, which form a covalent bond with adjacent atoms after irradiation.

In the first super-category the ncAA is incorporated at any site, which mediates binding of a ligand, substrate or allosteric effector, or substrate turnover in the case of enzymes. Photocaged ncAAs are the most powerful tool to use for this strategy. These ncAAs decage upon irradiation to set free a native amino acid. Thus, the photocaged ncAA can simply replace a residue, which is essential for binding or catalysis preventing either event. After photoinduced cleavage of the light-sensitive protecting group the biological activity of the protein is regained. This photocontrol strategy is very straightforward and has been successfully applied in multiple studies (Baker and Deiters 2014; Courtney and Deiters 2018). The second approach to manipulate a

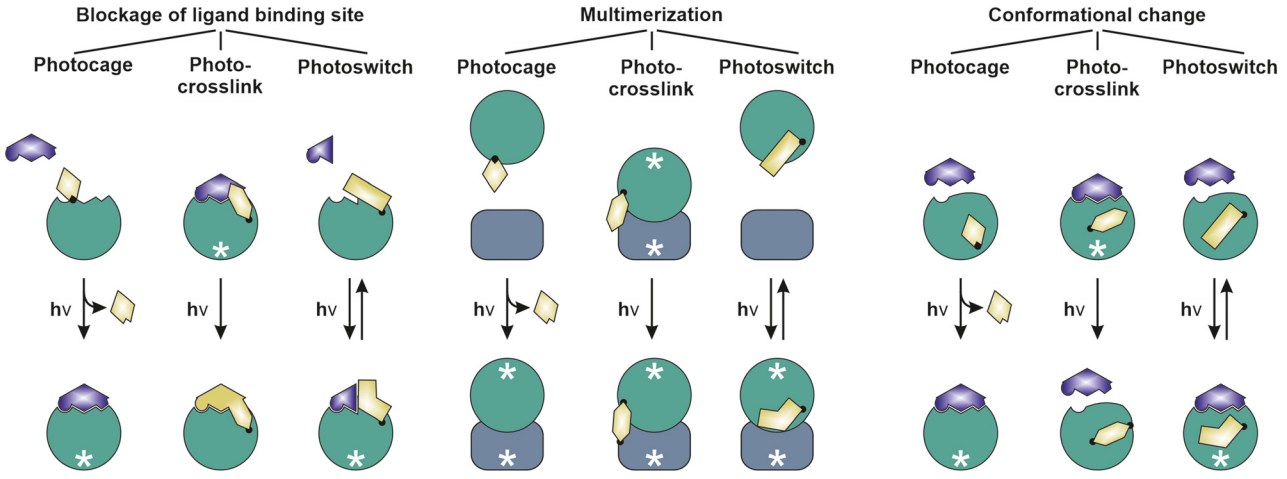

Figure 8: Strategies of photoxenoprotein engineering.
The strategic super-categories include blockage of a ligand binding site, multimerization and conformational change and can be realized by the utilization of photocaged, photocrosslinking or photoswitchable ncAAs. Green circle/dark grey square: Target protein; purple: Ligand; gold: ncAA; white asterisk: Biological activity.

ligand binding site is to incorporate a photocrosslinking ncAA in close proximity. Upon irradiation, the ncAA locks the ligand permanently into position on the protein. To this end, protein–ligand interactions can be analyzed (Rannversson et al. 2016; Wagner et al. 2020). Both approaches are, however, irreversible meaning that photodecaging and photocrosslinking reactions cannot be reversed. To achieve reversible manipulation of the protein function, photoswitchable ncAAs can be used. By insertion close to the entrance of the binding site the ncAA can act as lid blocking the entry of the ligand, substrate or effector in one configuration and allowing it in the other (Bose et al. 2006; Muranaka et al. 2002). However, this strategy is not as easily realizable than blockage through photocaged ncAAs. One has to keep in mind that after isomerization a photoswitch such as azobenzene is still extremely bulky so that the size of the lid effect is only limited. In addition, the conformation of ncAAs strongly depends as for native amino acids on physical constraints. First and foremost, the side chains can only adopt a certain number of energetically preferred conformations called rotamers. For this reason, it is hard to predict whether and if so in which position the ncAA covers the binding site in one configuration while unblocking the site in the other configuration.

Next, the ncAAs can be used to control homo- or hetero-multimerization by light. Insertion of photocaged ncAAs into the protein interface can thereby disturb complex formation (Engelke et al. 2014; Kropp et al. 2022), whereas decaging and reconstitution of the native interface will once again allow it. Similar to the first strategy, photocrosslinking ncAAs are mainly used to analyze the interactome of proteins (Tian and Ye 2016) by covalently linking two proteins or peptides. And finally, reversible association and dissociation can be obtained by incorporating a photoswitchable ncAA into the protein interface (Nakayama et al. 2004, 2005). It has to be noted that, in general, this strategy depends on the binding strength (K_D value) of the protein complex and the size of the contribution to the K_D of the replaced residue. Is the binding strength high or does the ncAA itself contribute to the protein-protein interaction then complex formation may still occur at naturally significant concentrations and instead the conformation of the multimer might change by irradiation with light. This has been the case for the photocontrol of the two multienzyme complexes imidazole glycerol phosphate synthase (Kneuttinger et al. 2020) and tryptophan synthase (Kneuttinger et al. 2019b).

Moreover, recent studies showed that all three types of light-sensitive ncAAs can be incorporated distant to ligand binding sites and to the protein interface and still cause a significant disturbance in biological activity (Kneuttinger et al. 2019a, 2020; Zhu et al. 2014). The reasoning behind this effect are conformational changes that are caused by the ncAA and extend to the site of action. One possible interpretation is shown in the panel "conformational change" in Figure 8, which is based on the induced fit theory (Koshland Jr. 1995). Here, light-induced decaging, crosslinking or isomerization promote a transition from an unproductive protein conformation with low affinity towards the ligand to a productive protein conformation with high affinity towards the ligand, or vice versa. Instead of ligand binding, the induced conformational changes might also affect the proper orientation of catalytic residues and instead of large-

timescale conformational transitions the ncAAs might fine-tune the flexibility/rigidity of the protein. Since the conformational landscape and dynamics of proteins are still a matter of extensive research and possibly individual to each protein, this photocontrol design principle is hard to accomplish. Nevertheless, we and others have particularly shown the potential of photoswitchable ncAAs for the efficient and especially reversible photocontrol of proteins using this strategy (Hoppmann et al. 2014, 2015; Kneuttinger et al. 2019a, 2020). Notably, the line between influencing protein conformation and using photoswitchable ncAAs as active site lid is quite thin; it is likely that instead of reversible steric hindrance photocontrol might be established due to a change in protein conformation.

Figure 9 gives a brief overview of the most commonly used light-sensitive ncAAs in the field of photoxenoprotein engineering. Just as for the ligands in photopharmacology, light-induced decaging, crosslinking or isomerization can occur with UV or visible light depending on the substitution pattern. Most importantly, for each here presented ncAA the respective aaRS/tRNA pair is available from literature either for bacterial, mammalian or both host systems. Especially for photocaged ncAAs, one can pick from a broad repertoire, which the interested reader can look up in detail in more comprehensive reviews (Ankenbruck et al. 2018; Courtney and Deiters 2018; Nödling et al. 2019). The most popularly used photocaged ncAA is *o*-nitrobenzyl-O-tyrosine (NBY) (Arbely et al. 2012; Deiters

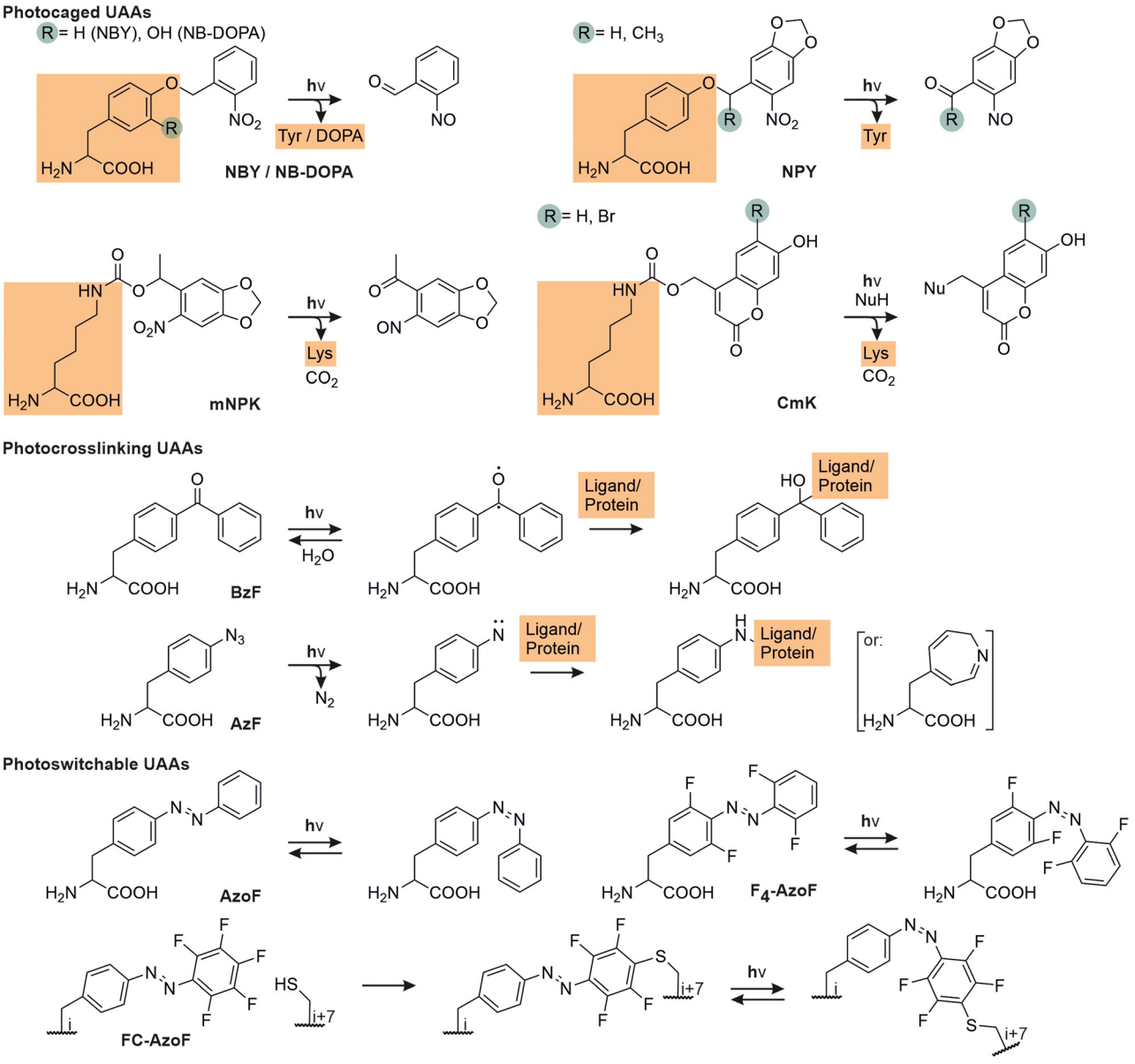

Figure 9: Main examples of photocaged, photocrosslinking and photoswitchable ncAAs.

et al. 2006), which decages upon irradiation with UV-light to set free tyrosine. Photocaged tyrosine has been further developed in terms of its photochemical properties. By choosing nitropiperonyl instead of the nitrobenzyl caging group in NPY they shifted the absorption maximum from 254 to 365 nm and increased the decaging efficiency (Luo et al. 2017a). In addition to photocaged tyrosine, photocaged lysines such as methyl-*o*-nitropiperonyllysine (mNPK) (Gautier et al. 2010) have often been applied for the photocontrol of proteins. Furthermore, to shift the irradiation wavelength towards the visible spectrum of light, photocaged lysine derivatives have been developed that use a coumarin-based caging group (CmK) instead of the nitropiperonyl group used in mNPK (Luo et al. 2014). Moreover, cysteine (Wu et al. 2004; Nguyen et al. 2014; Uprety et al. 2014) and serine (Lemke et al. 2007) have been rendered light-responsive using various photocages for the genetic incorporation in pro- or eukaryotic cells. In addition to caged canonical amino acids, the amino acid 3,4-dihydroxiphenylalanine (DOPA), which is formed as post-translational modification in nature, was also caged recently with an *o*-nitrobenzyl group and incorporated as the ncAA NB-DOPA into proteins (Budisa and Schneider 2019; Hauf et al. 2017).

The two most prominent photocrosslinking ncAAs are 4-benzoylphenylalanine (BzF) and 4-azidophenylalanine (AzF). While BzF might block the binding of a ligand or protein owing to its large size, its crosslinking efficiency is quite high because the intermediate diradical formed upon irradiation is stable and reverses back to BzF through hydration instead of transforming into an unreactive by-product (Nguyen et al. 2018). Moreover, the diradical reacts with adjacent CH bonds, preferably with the γ-CH bond of methionine upon irradiation with UV-light of 350–365 nm (Preston and Wilson 2013; Tanaka et al. 2008). The second photocrosslinking ncAA AzF is small in size similar to phenylalanine or tyrosine; however, its crosslinking efficiency is reduced due to the instability of the nitrene intermediate, which results in an intermolecular ring expansion and unreactive by-product formation (Nguyen et al. 2018). Nevertheless, AzF is frequently used owing to its broad reactivity with neighboring CH and heteroatom-H bonds upon irradiation with UV-light of 250 nm. Similar to photocaged ncAAs more examples for photocrosslinking ncAAs exist (Nödling et al. 2019).

To date only azobenzene-based ncAAs are available from literature derived from the first photoswitchable ncAA AzoF (Bose et al. 2006; Muranaka et al. 2002). AzoF isomerizes upon irradiation with UV light of 365 nm between its *E* and *Z* state. The back-reaction can either be initiated by irradiation with visible light of 420 nm or thermally (ΔT). In order to shift the $E \rightarrow Z$ switch into the visible spectrum of light, various fluorinated AzoFs (e.g., F_4-AzoF) have been developed and incorporated with existing or newly evolved aaRS/tRNA pairs (John et al. 2015; Luo et al. 2018). Notably, fluorination also facilitates thermal stability of the *Z* state. Moreover, additional click functions such as an alkene, keto, chloro or fluoro (FC-ncAA) functional group (Hoppmann et al. 2014, 2015) can extend the scope of effect of AzoF. When incorporated into position *i* in an α-helix, these photoswitchable click ncAAs react with a cysteine in position $i + 7$. While the *E* state maintains the α-helical structure, irradiation with UV (alkene, keto, chloro substituents) or visible (fluorinated) light to the *Z* state shortens the distance between position *i* and $i + 7$ resulting in the disturbance of the α-helix. This structural change was evidenced by changes in the circular dichroism spectrum of calmodulin. Photoswitchable click ncAAs follow the same principle as the photopharmacological strategy in which azobenzene photoswitches were bioconjugated at both ends to an α-helix (Bozovic et al. 2020). However, in this approach both the photoswitch and the reaction partner are genetically encoded, which eases the engineering process and reduces side reactions of the photoswitch.

Applications

Tool development for *in vivo* fundamental studies

Just as for the other two photocontrol methods, the main focus of photoxenoprotein engineering lies currently within the application *in vivo*. For this purpose, many proof-of-principle studies have light-regulated key players of the cell to use as tools in future fundamental studies. While photoreceptors in optogenetics can be encoded relatively straightforward into the genome of organisms, the introduction of the synthetic machinery required for genetic code expansion has faced two major challenges (Chen et al. 2017a). ncAAs are no native substrate and, hence, the delivery to the target tissue in the model organism can be difficult to accomplish. The best strategies so far are direct injection into the tissue (Han et al. 2017; Kang et al. 2013) or supplementation in the drinking water (Chen et al. 2017b; Ernst et al. 2016; Liu et al. 2017a). The second challenge is the transfer of the DNA encoding the aaRS/tRNA pair and the mutated protein of interest into the genetic material of the organism. To this end, various vector systems have been introduced *in utero* by e.g., electroporation, such as plasmids (Kang et al. 2013) and vectors of viral origin, e.g., lentiviral (Chen et al. 2017b; Han et al. 2017). As a result, many model organisms could be modified to express ncAA-bearing proteins including mice (Chen et al. 2017b; Han

et al. 2017), zebrafish (Chen et al. 2017b; Liu et al. 2017a), and fruit fly (Bianco et al. 2012).

Over the years, multiple studies developed and incorporated particularly photocaged ncAAs into the genome of eukaryotic cells or even organisms. In contrast, only one proof-of-principle example exists for the use of photoswitchable ncAAs in eukaryotic cells (Luo et al. 2018). The general success of these studies has been outlined comprehensively in various review articles (Ankenbruck et al. 2018; Baker and Deiters 2014; Bardhan and Deiters 2019; Courtney and Deiters 2018; Deiters 2018). While some studies use standard model proteins to test the ncAAs such as luciferase (Luo et al. 2017a, 2018; Uprety et al. 2014) or tobacco etch virus (TEV) protease (Luo et al. 2017a; Nguyen et al. 2014), multiple studies targeted various types of key proteins starting with enzymes for gene editing. The first example thereof is the spatiotemporal control of DNA recombination in mammalian cells by Cre recombinase, which has been rendered light-sensitive by incorporation of a photocaged tyrosine into the active site (Edwards et al. 2009). A later study exchanged a critical lysine residue with a coumarin-protected lysine that can be decaged with visible light and tested the photocontrolled recombination in the developing zebrafish embryo (Brown et al. 2018). An important success in this direction is the photocontrol of the CRIPR/Cas9 gene editing tool. Incorporation of a caged lysine into key positions for guide RNA and DNA binding rendered the Cas9 enzyme catalytically inactive and allowed for the photocontrolled as well as site-specific gene deletion of a transmembrane receptor (Hemphill et al. 2015). Another example of photocaged DNA/RNA processing enzymes is T7 RNA polymerase, in which NBY (Chou et al. 2010) and mNPK (Hemphill et al. 2013) were incorporated at positions essential for discrimination of ribose and deoxyribose substrates as well as binding of nucleoside triphosphates. Similarly, Taq DNA polymerase was photocontrolled with a photocaged tyrosine (Chou et al. 2009). Moreover, photocontrol of zinc-finger nuclease was achieved by inserting a photocaged tyrosine in the DNA binding site to induce a DNA double-strand break and subsequent homologous recombination upon irradiation (Chou and Deiters 2011). To ease the study of diseases, which are caused by a defect in DNA helicases, another study applied coumarin-caged lysine in the ATP binding site of the DNA helicase UvrD (Luo et al. 2017b). Besides gene editing, photocontrol of proteins involved in cell signaling networks is highly desired. As such, kinases are frequent targets. A prominent example is MAP kinase kinase 1 (MEK1), which has been put under photocontrol by incorporating caged lysine variants into a position essential for ATP binding. Light-dependent activation of the MEK/ERK pathway has then been attained in human embryonic kidney (HEK)293ET cells (Gautier et al. 2011) as well as in zebrafish embryos (Liu et al. 2017a), by which the effects of ERK phosphorylation during different developmental stages could be observed. A recent study has refined this strategy for gain-of-function studies on kinases in cells (Wang et al. 2019a). They introduced an orthogonal MEK1, which they photocontrolled by incorporating NBY in proximity to the active site, and at the same time turned off the endogenous MEK1 counterparts using kinase inhibitors. Since the orthogonal MEK1 was rendered resistant to those inhibitors through the mutation to tyrosine after light-induced decaging, they generated an experimental setup to distinctively switch MEK1 function on and off. Besides MEK1, photocaging has also been successfully applied to Janus kinase (Arbely et al. 2012) and adenylate kinase (Zhou et al. 2020). Further cellular processes have also been made accessible for photocontrol through photocaged ncAAs including nuclear translocation (Gautier et al. 2010), the GTPase activity of KRAS (Wang et al. 2019a) and apoptosis through regulation of caspase-3 activity (Wang et al. 2019a).

The here presented examples and many more compile a large toolbox of especially photocaged key proteins *in vivo*, which is powerful for spatiotemporally resolved studies in various biological fields. Compared to hybrid protein optogenetics and photopharmacology, the method of light-responsive ncAAs appears to be more versatile, as this technique is highly efficient not only for *in vivo* studies but also for diverse other applications, which I will elaborate in the following chapters.

Functional studies on proteins

Over the last decades, mutational analysis of proteins has become routine in biochemistry, in particular, for the analysis of protein function. For this, specific amino acid residues, which are considered to play a certain role, are substituted with one of the other 19 canonical amino acids in site-directed mutagenesis. Moreover, using ncAAs instead of canonical amino acids has widened the scope of functionalities that can be introduced into proteins (Agostini et al. 2017). It is therefore not surprising that site-directed incorporation of light-responsive ncAAs is used to analyze protein function, as their key benefit is the temporal resolution of the introduced effect.

Already in 1998, the photocaged tyrosine NBY was applied in a nicotinic acetylcholine receptor to investigate the influence of multiple highly conserved tyrosine residues on pore opening upon binding of acetylcholine (Miller et al. 1998). Through time-resolved kinetic analyses they

found that each residue affected the electrochemical potential differently, which ultimately resulted in two activation phases of ion channel opening. Another study, almost a decade later, used photocaged serine to analyze the function of the transcription factor Pho4 (Lemke et al. 2007), which is exported from the nucleus in response to increased concentrations of inorganic phosphate in yeast (Komeili and O'Shea 1999). By exchanging the two phosphorylation sites S2 and S3 separately with photocaged serine, they could show that phosphorylation of S2 has slower export kinetics after irradiation than phosphorylation of S3. A good example of how photocrosslinking ncAAs can be utilized to investigate protein:ligand interactions was given by the investigation of serotonin transporters (Rannversson et al. 2016). By incorporating AzF at positions surrounding two distinct binding sites, S1 and S2, they substantiated that inhibitor binding solely occurs at the S1 site. Moreover, AzF has been employed for the investigation of the relationship between dynamics and activity in an N-methyl-D-aspartate (NMDA) receptor (Zhu et al. 2014). The gating mechanism of these receptors relies on conformational transitions at the interface between the N-terminal domains of the GluN1 and GluN2 subunits (Mony et al. 2011; Zhu et al. 2013), which have not been clearly understood at the time of the publication. The authors introduced AzF at this interface into a position, which has been known to influence the sensitivity of the receptor to allosteric effectors. Upon irradiation, the formation of a crosslink between the ncAA and a nearby loop put the receptor into a low-activity mode. This inactivation unambiguously showed that the flexibility of the GluN1 loop is decisive for the gating mechanism. The same group later achieved to use the same strategy to distinguish the mechanism of allosteric regulation by binding of Zn^{2+} and the small molecule ifenprodil (Tian and Ye 2016). They positioned AzF strategically at the interface of GluN1 and GluN2 close to the binding site of ifenprodil. Light-induced crosslinking of the two subunits led to the inactivation of ifenprodil inhibition. However, not ifenprodil binding but instead the transmittance of the allosteric signal from the binding site to the gate was interrupted and caused this inactivation. Since the inhibition by Zn^{2+} was not affected, they concluded that Zn^{2+} and ifenprodil follow differential structural pathways for inhibition. In another follow-up study they chose FC-AzoF to induce conformational changes in each structural domain of the NMDA receptor subunits by light-induced switching of the azobenzene moiety without making use of the click function (Klippenstein et al. 2017). Irradiation and further analysis of receptor function revealed that photoswitching especially influenced the agonist-binding domain leading to decreased gating efficiency and agonist affinity as well as the transmembrane domain hampering the permeation properties of the channel. This study nicely demonstrated that photoswitchable ncAAs can reversibly control protein activity through a light-induced conformational change. Our subsequent studies also follow this strategy aiming to light-regulate allostery in the prominent model enzyme complex imidazole glycerol phosphate synthase, which undergoes large-scale conformational rearrangements upon allosteric ligand binding (Wurm et al. 2021). To this end, AzoF as well as the photocaged ncAAs NPY and mNPK were incorporated at positions close to sites involved in these conformational rearrangements and thus important for allostery (Kneuttinger et al. 2019a). As a result, it was possible to photocontrol the allosteric activation of one subunit by the other. Furthermore, molecular dynamics (MD) simulations demonstrated that the interface configuration as well as the conformational arrangement of a catalytic residue were hampered when the transmittance of the allosteric signal was interrupted by the ncAA. In a second study, we deliberately exchanged a key residue in the subunit interface with AzoF to answer the long-standing question whether a closing of the interface occurs during the allosteric activation process (Kneuttinger et al. 2020). In fact, biochemical and X-ray crystallographic studies showed that AzoF incorporation facilitated the photocontrol of the interface configuration and that a closing increases enzyme activity implying that this conformational change is part of the allosteric signal.

Although the number of publications that use light-responsive ncAAs for functional studies on proteins are currently modest, the great potential of these time-resolved mutational tools promises that more studies might follow.

Therapeutic approaches

Similar to photopharmacology, photoxenoprotein engineering has the potential to broaden the efficacy of therapeutic approaches. To this end, light-responsive ncAAs can be used to add spatiotemporal control to a protein-based drug such as monoclonal antibodies (Raquel et al. 2021) or therapeutic enzymes (Yari et al. 2017). Similar to the fundamental idea of photopharmacology, photocontrol of these so-called biotherapeutics allows to activate them only at the target site and prevents harmful side effects.

Two recent studies have set out to apply this principle to antibodies. The therapeutic effect of antibodies relies on the blockage of cell surface receptors or the delivery of cytotoxic drugs to target cells. Since often the targeted receptors e.g., the epidermal growth factor receptor (EGFR) in cancer cells (Normanno et al. 2006), are also present on the surface of

healthy cells, the therapy with antibodies can cause severe side effects (Hansel et al. 2010). Thus, the first study redesigned a so-called nanobody, which is a class of single domain antibody fragments (Ingram et al. 2018), to photocontrol its binding to EGFR (Bridge et al. 2019). To this end, they incorporated NBY into the antigen binding region of the 7D12 nanobody. This resulted in a largely reduced binding strength of the nanobody-EGFR interaction, which could be restored to its usually high affinity upon irradiation. The second study further promoted the success of this approach. They established efficient photocontrol in an anti-GFP nanobody, an EgA1 nanobody targeting EGFR, and a 2Rs15d nanobody targeting the human epidermal growth factor receptor 2 (HER2) with photocaged tyrosines (Jedlitzke et al. 2020). Another recent study tested the potential of photocontrolled cytotoxic proteins for the treatment of cancer. They applied NBY to inactivate lethal factor LF, which can enter cells with the help of the protein-protective antigen PA. Both proteins are components of anthrax toxin. In high dosage, LF is toxic to cancer and healthy cells as it breaks down MAP kinases. However, photocaging of LF allowed to activate LF in a controlled manner by irradiation resulting in significant reduction of tumor growth and tumor size (Wang et al. 2019a).

Production of challenging peptides and proteins

Protein biochemists face various difficulties in the production of proteins through heterologous gene expression. The most prominent example is that the protein or peptide in question is toxic to the host cell. Since the production using bacterial or yeast strains are still often preferred as standard technique over e.g., cell free systems, these problems need to be circumvented. While it often helps to use specialized strains or optimized expression conditions (Rosano et al. 2019), the production of some proteins remains challenging. Isolated cases have successfully applied photocaged ncAAs to tackle exactly this issue. To this effect the activity of the proapoptotic cysteine protease caspase 3 was switched off by incorporation of a photocaged cysteine in the active site to allow for the expression in yeast host cells (Wu et al. 2004). Similarly, the expression of *Staphylococcus aureus* toxins YoeBSa1/2 in *E. coli* host cells turned out to be extremely difficult with traditional approaches e.g., coexpression of the antitoxin YefM (Larson and Hergenrother 2014). However, when the RNase activity was reduced by incorporation of NBY into a tyrosine position crucial for toxicity, the toxins could be produced and reactivated by irradiation after cell lysis. The third example is the spatiotemporal control of inteins, which are protein domains able to autocatalytically excise themselves from the exteins of a precursor protein in a splicing process (Di Ventura and Mootz 2019). Inteins are valuable tools in synthetic biology because they allow for the reconstitution of split proteins, the post-translational attachment of tags or the generation of therapeutically significant cyclic peptides or proteins. Since the production of such cyclic peptides entails challenging purification steps, a recent publication rendered the M86 intein light-sensitive by incorporation of NBY into an essential position close to the active site (Böcker et al. 2015, 2019). Photocontrol of the splicing reaction thus promises to ease the purification and to facilitate high-throughput directed evolution screenings. Moreover, challenges in the production of mussel-derived adhesive proteins (MAPs) were addressed using photoxenoprotein engineering. The adhesive properties of MAPs are highly beneficial for various applications, however, the proteins are difficult to produce recombinantly, as they comprise DOPA as key amino acid. While neither post-translational hydroxylation (Hwang et al. 2007) nor other production strategies obtained sufficient yields, incorporation and post-production irradiation of NB-DOPA turned out to successfully obtain reasonable yields of MAPs (Budisa and Schneider 2019; Hauf et al. 2017).

From biotechnology to industry

The spatiotemporal control of protein function is not only highly desirable for fundamental or medicinal research studies. Many biotechnological and industrial processes would benefit strongly from an orthogonal and precise reaction initiation system. This is particularly true for the field of synthetic biology, which strives to equip, reengineer or rebuild biological modules with unnatural, artificial functions (Ausländer et al. 2017) for various applications. One example is the bottom-up reconstitution of a synthetic cell. To this end, spatiotemporal control over cell division is regarded as very important. A key player for this goal is the filamenting temperature-sensitive mutant Z (FtsZ), which polymerizes into a division ring, similar to tubulin in eukaryotes, for the initiation of bacterial cell division (Romberg and Levin 2003). To control FtsZ ring formation by light, ONBY was recently introduced into a key tyrosine position that is essential for FtsZ assembly (Sun et al. 2021). As a result, the GTPase activity of FtsZ, which is required for polymerization, and, hence, ring formation was regulated with an LRF of 22-fold. Another recent biotechnological study has demonstrated photocontrol of elastin-like polypeptides (ELPs) (Israeli et al. 2021), which constitute a family of artificial protein-based biomaterials. The most crucial feature of ELPs is their lower critical transition temperature, which triggers a reversible soluble-to-insoluble phase transition valuable for various

applications such as drug delivery (Bellucci et al. 2013) and tissue engineering (Christensen et al. 2009). Incorporation of AzoF into these biomaterials facilitated reversible external control of the transition temperature and, hence, of the phase transition itself.

Moreover, one of the currently most important goals that biological, biochemical and chemical research areas pursue is the development of more sustainable industrial processes. Already in 1998, a list of 12 principles of green chemistry was put together specifically for this endeavor (Anastas and Warner 1998). Within the plethora of research fields that strive to accomplish this goal, many use isolated proteins and especially enzymes for biocatalytic purposes. Owing to the rapid and vigorous development of bio-based strategies, more and more situations emerge, which require the spatial, temporal or spatiotemporal control of protein and especially enzyme function (Schmidt-Dannert and Lopez-Gallego 2016). A few first publications pave the way for the application of specifically photoxenoprotein engineering to fine-tune green chemistry approaches. One goal of biocatalysis is to achieve temporal control over the flux and dynamics of synthetic enzyme cascades (Schrittwieser et al. 2018), which resemble synthetic metabolic pathways. Thus, one of the first attempts to implement photocontrol in biocatalysis was the computationally guided creation of an artificial multienzyme complex from the enzymes of the synthetic pathway for the degradation of the toxic groundwater pollutant 1,2,3-trichloropropane that associates only upon phosphorylation and irradiation (Yang et al. 2017). For the light-induced complexation, the authors incorporated BzF into a redesigned Src homology 3 domain coupled to one enzyme subunit. Binding of this domain to its corresponding peptide fused with a second enzyme resulted in the conjugation of BzF with a methionine and, hence, covalent complex formation.

These and other publications (Claaßen et al. 2019; Schmermund et al. 2019) emphasize the potential of photocontrol in the fields of biotechnology, in particular synthetic biology, and industrial biocatalysis. Nevertheless, it remains to be seen whether photocontrol of proteins will prove to have high impact and which specific advantages photoxenoprotein engineering possesses for industrial applications.

Hybrid protein optogenetics

Method concept

The cornerstone of optogenetics was laid at the end of the twentieth century with the discovery of the light-sensitive rhodopsin in microbes (Oesterhelt and Stoeckenius 1971) and rhodopsin-regulated currents in *Chlamydomonas* (Harz and Hegemann 1991). Rhodopsin belongs to the family of opsins, a class of seven-pass transmembrane proteins (Zhang et al. 2011), and is light-sensitive through incorporation of the vitamin A derived cofactor retinal (Hegemann et al. 1991). In 2002 and 2003, two groups simultaneously reported on the successful application of rhodopsin to render usually light-insensitive cells light-sensitive (Nagel et al. 2002, 2003; Zemelman et al. 2002, 2003). Three years later, this novel method of photocontrol was named "optogenetics" (Deisseroth et al. 2006). Since then rhodopsins have been widely used as light-driven ion pumps and light-gated ion channels in the neurosciences (Deisseroth 2015). Yet, rhodopsins are only one type of a plethora of proteins that stimulate biological processes in the presence of light, the so-called photoreceptors (Hegemann 2008; Möglich et al. 2010). Several other types have been identified and deployed in optogenetics over the last two decades.

Members of the photoreceptor family consist of two modules: i) an N-terminal photosensor ("receiver") module, which contains a light-responsive cofactor, and ii) a C-terminal effector ("output") module, which conveys the biological response (Figure 10, left panels). Both modules can either be fused as domains or interact noncovalently as subunits of a protein complex. Common to all photoreceptors is the conformational transition of the photosensor module induced by the excitation of the cofactor by light of a certain wavelength, and a subsequent signal transmittance to the effector module. As a result, the effector changes its output signal meaning while some effectors gain activity others are inactivated. After (in)activation, the excited state of the photoreceptor thermally reverts back to the inactive dark state within time constants ranging from sub-seconds to hours (Losi et al. 2018; Möglich and Moffat 2010). Light-gated ion channels (Figure 11A) such as channelrhodopsin (Nagel et al. 2002) are most widely used in optogenetics. These proteins consist of seven transmembrane α-helices, which bind the retinal cofactor via a lysine-mediated Schiff base (Oesterhelt and Stoeckenius 1971). The cofactor pocket is located in the core of the protein and isomerization of the bound retinal upon irradiation with light induces rearrangements of some of the α-helices, which line the core (Lórenz-Fonfría and Heberle 2014; Volkov et al. 2017). This conformational transition allows for the flow of ions through the plasma membrane, why light-gated ion channels are applied to control action potentials in neurons at will (Deisseroth 2015). However, for the photocontrol of specific proteins of interest, rhodopsins are not well-suited, because the photosensor and

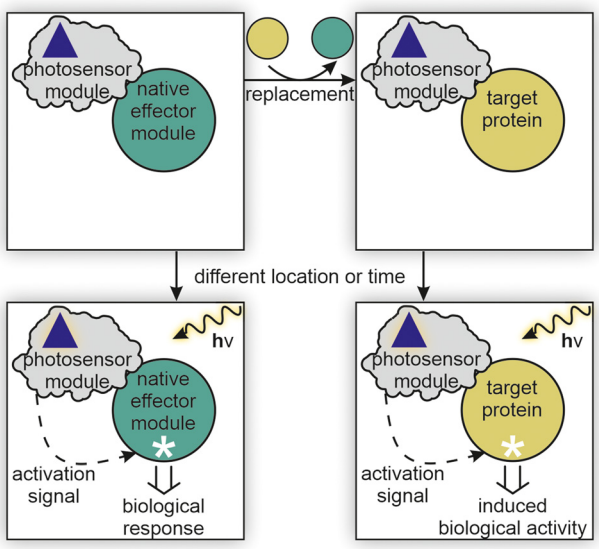

Figure 10: The method concept of hybrid protein optogenetics. A photoreceptor consists of a photosensor module and an effector module (upper left). After irradiation and excitation of the light-responsive cofactor the photosensor module transmits an (in) activation signal, e.g., in form of a conformational change, to the effector module, which as a result is (in)activated (bottom left; activation shown). In hybrid protein optogenetics, the effector module is replaced by a target protein (upper right) resulting in the induction of the desired biological activity (bottom right).

effector module are tightly connected within the transmembrane region. Hence, other classes of photoreceptors are employed. To this end, the photosensor module of such a natural photoreceptor is genetically fused to the target protein creating a hybrid protein (Figure 10, right panels). I will refer to this particular sub-category as hybrid protein optogenetics to clearly distinguish it from light-gated ion channels. While the genetic engineering with rhodopsins has become a powerful tool in biology accounting for probably ~90% of optogenetic applications, the design of artificial hybrid proteins is still in its learning phase. Nevertheless, with the choice of several versatile natural photoreceptors it is a highly promising and already frequently used tool for the control of diverse biologically relevant processes with light.

Strategies

The hybrid protein optogenetic approach can be categorized into four main strategies (Figure 11B). The first two strategies are summarized as non-associating techniques. In strategy (1), the photosensor module partly undergoes a conformational transition in response to light, which is transmitted to the target protein influencing its activity. This effect has also been described as an allosteric switch (Liu and Tucker 2017; Mathony and Niopek 2021). Strategy (2) regulates protein accessibility through steric decaging. In the dark state, the photosensor module blocks the active site or the binding interface of the effector module constituted by a large target protein or a small target peptide. Upon irradiation, the domain linking the photosensor and effector module rearranges, and the protein or peptide is set free.

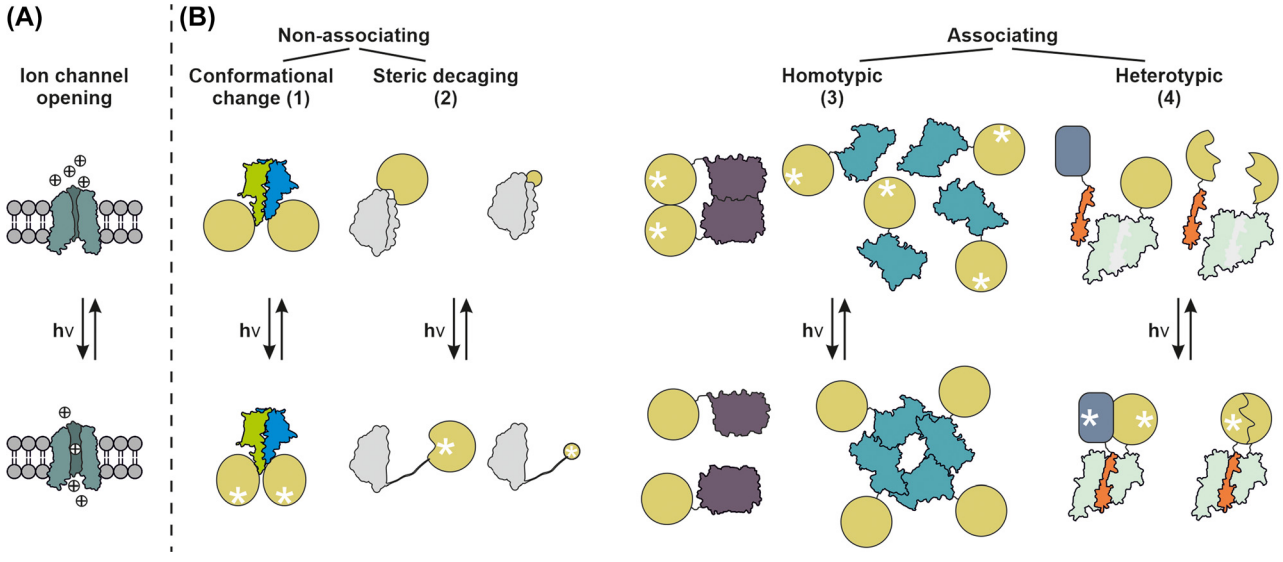

Figure 11: Optogenetic strategies.
(A) Light-gated ion channels. (B) The four main strategies of hybrid protein optogenetics including transduction of conformational changes, steric decaging of proteins or peptides, homotypic association/dissociation and heterotypic association/dissociation of heterocomplexes or split proteins. Yellow circle/dark grey square: Target protein or peptide; white asterisk: Activated state.

A prominent example of a photosensor that can be used for both non-associating strategies is the light-oxygen-voltage (LOV) domain from plant and algal phototropins (Christie et al. 1998; Huala et al. 1997), a class of photoreceptors containing a kinase-based effector module involved in phototropism. Homologs of these initially found photoreceptors have also been identified in fungal and bacterial systems. The photosensor LOV domains harbor a flavin chromophore (Krauss et al. 2010), which reacts with a conserved neighboring cysteine residue to form a covalent thioether bond upon irradiation with blue light (Salomon et al. 2001). A C-terminal α-helix named Jα constitutes a linker to the target protein. Depending on the type of LOV domain this helix can undergo two different conformational transitions. For example, in *Bacillus subtilis* the YtvA LOV photosensor forms a stable dimer in both dark and excited state, while Jα extends from its core and rotates 40°–60° upon irradiation (Möglich and Moffat 2007). In the monomeric LOV2 domain from *Avena sativa*, however, Jα is folded and attached to the core of the photosensor module in the dark state; in the excited state it unfolds and dissociates from the rest of the protein (Harper et al. 2003). The former conformational transition can be used to induce an allosteric switch in the target protein, while the latter is applied for steric decaging.

Finally, light-induced multimerization delineates two associating optogenetic strategies. Multiple proteins are activated in the cell by homotypic or heterotypic multimerization (Goodsell and Olson 2000). Such targets can be photocontrolled by attaching them to photosensor modules, which themselves associate and dissociate light-dependently in a homotypic or heterotypic manner as illustrated in strategy (3). This general approach can be executed in a number of ways. Light-induced association can occur through dimerization or oligomerization. Furthermore, multimerization can lead either to activation of the multimeric target protein or to deactivation of the monomeric target protein through sequestering into clusters. Notably, monomeric target proteins can also be activated through heterotypic dimerization by using the split protein technique, which constitutes strategy (4). For this, the protein of interest is split into two inactive halves, which are coupled to the effector domains of a heterodimeric photoreceptor pair. Upon irradiation the photoreceptors dimerize, and the split fragments approach each other to reconstitute the active protein. While creating split proteins involves some intricate engineering steps, e.g., screening multiple split sites in solvent- exposed loops, light-induced multimerization is in general the most powerful strategy in hybrid protein optogenetics. Its advantages include fast binding kinetics, reversibility and relatively straightforward engineering (Repina et al. 2017).

Cryptochromes are photosensor modules, which can be used for both homotypic or heterotypic association strategies since they either oligomerize or bind an effector protein upon irradiation with blue light. The actual photoactivation mechanism of this class of flavin-containing photoreceptors is still under investigation, however, the use as photosensor module in hybrid protein optogenetics is well-established (Losi et al. 2018; Repina et al. 2017). The structure of cryptochromes consists of an N-terminal α/β domain, a C-terminal all-helical domain and diverse C-terminal extensions (CCTs). Homotypic multimerization is facilitated by interactions of the CCTs (Duan et al. 2017), while heterotypic multimerization involves the N-terminal domain and cryptochrome-interacting basic helix-loop-helix proteins (CIBs) (Taslimi et al. 2016). Apart from cryptochromes, LOV domains can also dimerize (Wang et al. 2012) and form heterodimers with e.g., engineered affibodies called Zdark (Wang et al. 2016) upon irradiation.

Apart from rhodopsins, LOV domains and cryptochromes several other photosensor modules are utilized in hybrid protein optogenetics. Figure 12 provides a brief overview of the most prominent photosensor modules employed, the approximate wavelength they respond to, the strategies they can be used for, the chromophore they contain, and the recovery timescale of the dark state. For detailed information on each system and new photosensory domains, I refer to more extensive reviews specified on hybrid protein optogenetics (Jiang et al. 2017; Losi et al. 2018; Möglich and Moffat 2010; Oh et al. 2021; Repina et al. 2017; Tang et al. 2021; Ziegler and Möglich 2015).

Applications

In vivo fundamental studies

Optogenetics has been developed to regulate biological reactions *in vivo*. Specifically in the context of hybrid protein optogenetics, dynamic control of proteins of interest with light offers a convincing advantage: the effect of a modification can be observed in a spatially and timely resolved manner within the living system. This spatiotemporal control allows to better analyze and understand the effect of protein function and, hence, the dynamic character of cells, which comprises an intricate coordination of various input and output signals as well as actions and reactions. In comparison, static control approaches such as mutagenesis of a critical amino acid, overexpression or knockdown and knockout techniques provide comparatively little information on this dynamic environment. In this regard, the photoreceptor-based strategies offer many advantages.

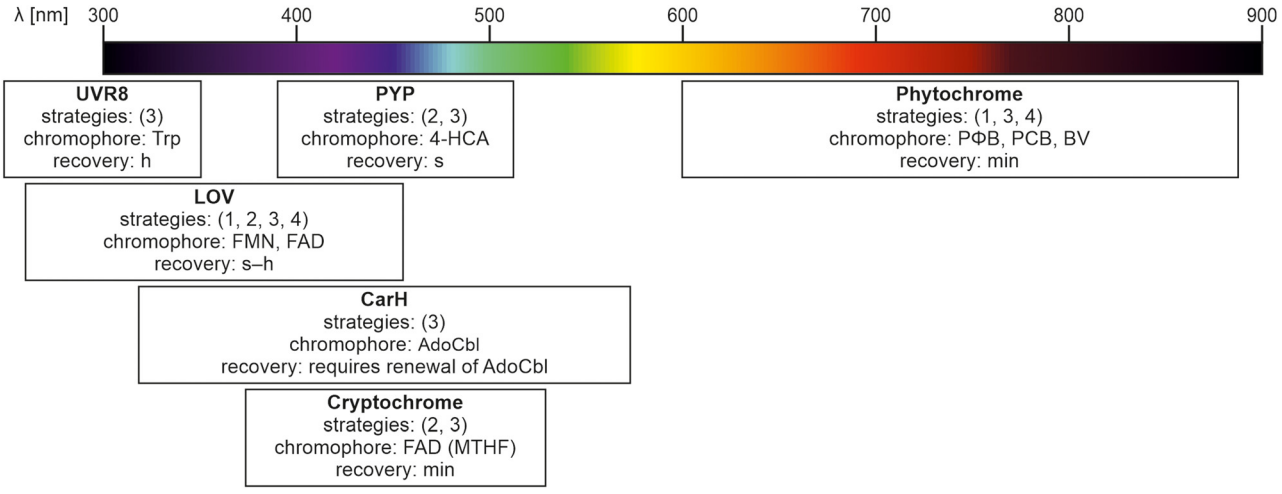

Figure 12: Most important examples of photosensory domains used to create hybrid proteins in optogenetics.
Strategies 1–4 refer to the ones shown in Figure 11. For more detailed information see references (Losi et al. 2018; Padmanabhan et al. 2019; Ziegler and Möglich 2015). UVR, UV-B sensitive photoreceptor; PYP, photoactive yellow protein; 4-HCA, *p*-coumaric acid; PΦB, phytochromobilin; PCB, phycocyanobilin; BV, biliverdin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; AdoCbl, 5′-deoxyadenosylcobalamin; MTHF, methenyltetrahydrofolate.

Primarily, the photosensor-tags are genetically encoded together with the target proteins, and they use light-sensitive cofactors, which are often naturally available within the cell, e.g., flavins for LOV domains. Moreover, some studies already accomplished the production of chromophores not inherent to the target system, e.g., PCB for phytochromes in animal cells, *in vivo* (Müller et al. 2013; Uda et al. 2017). Hence, hybrid protein optogenetics is widely developed and applied for the purpose of fundamental cellular and organismal studies. Even though engineering of hybrid proteins constitutes only a fraction of the massive publication activity in the field of optogenetics (>600 publications alone in 2021 according to Web of Science), the plethora of studies exceeds the limits of this general overview on photocontrol in proteins. Since other reviews comprehensively cover the many *in vivo* applications for fundamental studies (Kramer et al. 2021b; Losi et al. 2018; Oh et al. 2021; Repina et al. 2017; Tang et al. 2021), I herein concentrate on a brief overview on publications within the last year.

Hybrid protein optogenetic studies foremost concentrate on three cellular targets for the artificial control with light: signaling cascades, (translocation between) organelles and gene expression (Oh et al. 2021). Some of the most important players of cell signaling that constitute a target for photocontrol are phosphatases. To facilitate the regulation of tyrosine phosphatase 1B with light, three different strategies using LOV2 were compared in a very recent study, i.e., steric decaging, conformational change and heterotypic split protein association (cf. Figure 11B) (Hongdusit et al. 2022). The authors show that the conformational change strategy is particularly successful for this type of phosphatase allowing to analyze various tyrosine phosphatase related physiological processes in the cell. Another study focused on the intricately interconnected pathways of the two receptor tyrosine kinases ERK and AKT. To resolve the complexity of this signaling network they developed a two-way photocontrol system, which responds differently to blue and red/far-red light. While homotypic clustering of cryptochrome 2 (CRY2) activated the AKT pathway, ERK signaling could be controlled with light through heterotypic association of phytochrome PhyB with its phytochrome interaction factor (PIF) (Kramer et al. 2021a). CRY2 was further used to assess the role of talin in the activation of integrins at the cell membrane. Talin was thereby recruited as a CRY2 hybrid protein to the plasma membrane through light-induced heterotypic interaction of CRY2 with a constitutively membrane-associated CIB protein (Liao et al. 2021). Their findings showed that binding of talin to the cell membrane additionally required the association of Ras-related protein 1 (Rap1)-GTP for the activation of integrines. Similarly, localization of the small GTPase RhoA, a key actuator in cytoskeletal function, was photocontrolled using the LOV domain from *Botrytis cinerea* (BcLOV4), which directly associates with lipids in the cell membrane (Berlew et al. 2021). Moreover, optogenetic systems have been established for the translocation of proteins to other organelles than the cell membrane. To shuttle proteins from the cytoplasm to the nucleus for example, LOV2 has been coupled to a nuclear export sequence, which is sterically decaged upon irradiation (Niopek et al. 2016). This so-called LEXY module was deployed in a recent study to investigate the function of the myocardin-related transcription factor A, which regulates actin-associated gene expression and promotes invasion and

metastasis of tumors (Zhao and Grosse 2021). Besides such one-component systems, two-component systems can also be used for the analysis of nuclear import and export. To investigate the translocation rates of the transcriptional regulators YAP1 and TAZ, the authors of a recent publication tagged these proteins of interest with a fluorescent label as well as the synthetic peptide Zdk98 (Dowbaj et al. 2021). Zdk98 mediates light-dependent binding to LOV2, which was immobilized on the surface of mitochondria. Dissociation of the target proteins and, hence, nuclear import was then initiated by irradiation of this two-component system. Regarding the photocontrol of gene expression, the LOV domain from *Rhodobacter sphaeroides* was currently employed to inactivate the widely used Tet repressor by photoinduced dissociation of homodimers resulting in gene transcription (Dietler et al. 2021). Homotypic association/dissociation is a commonly used optogenetic strategy for the light-induced regulation of transcription. To facilitate this type of photocontrol with near-infrared light, bacterial phytochrome based photosensor modules were currently re-engineered to undergo homotypic oligomerization instead of heterotypic oligomerization. The resulting optogenetic transcription regulation system was called iLight (Kaberniuk et al. 2021).

Therapeutic approaches

Besides the fundamental research sector, optogenetics is also a promising therapeutic tool despite some limitations (Wichert et al. 2021). Ion channel based optogenetics pioneers this field; The most advanced example is the treatment of retinopathy by delivering a viral plasmid containing a microbial opsin directly to the eye in order to restore vision (Duebel et al. 2015). Nevertheless, optogenetic hybrid proteins are likewise developed for diverse therapeutic approaches. Current reviews give a comprehensive overview over present preclinical studies (Tang et al. 2021; Wichert et al. 2021) including the use of the cryptochrome system in Alzheimer's disease (Lim et al. 2020), phytochrome as well as LOV based targeted bone regeneration (Hörner et al. 2019b; Wang et al. 2019b), and advancement of photoacoustic tomography using phytochromes for the improved identification of e.g., metastases (Li et al. 2018). These examples as well as two further topical approaches, described in the following, illustrate the wide range of medicinal applications for which photocontrol of proteins can be employed.

The first of these two approaches is the photocontrol of the smaller sized and disulfide-bond lacking single-domain counterparts of antibodies, nanobodies and monobodies. Using either the split protein (Yu et al. 2019) or the LOV2 based conformational change technique (Carrasco-López et al. 2020; Gil et al. 2020; He et al. 2021), nano- and monobodies targeting different fluorescent proteins, the Src Homology 2 domain of Abelson tyrosine kinase or actin, respectively, were engineered in the last years. The authors primarily show the advantage of their light-induced nano- and monobodies as so-called intrabodies for intracellular studies; however, they also point out the potential use for medicinal purposes, e.g., for anticancer treatment as outlined earlier for the light-sensitive ncAA-containing nanobodies in the photoxenoprotein engineering section.

Most remarkably, two optogenetic approaches have also been combined with smart electronic devices to facilitate a photocontrolled drug supply in mice (Mansouri et al. 2021; Shao et al. 2017). For this purpose, the production of human glucagon-like peptide-1 (hGLP-1), which is clinically licensed for the treatment of type-2 diabetes (Müller et al. 2019), was put under the control of a light-regulated transcription factor. While the first study uses the far-red light activated cyclic diguanylate monophosphate synthase BphS coupled to an endogenous signaling pathway (Shao et al. 2017), the second study has optimized a CarH (e.g., Figure 12) based two-component system that releases the transcription factor from the plasma membrane upon irradiation with green light (Mansouri et al. 2021). Moreover, while the first study requires a hydrogel implant containing the modified cells and a light-emitting diode (LED) array, the second study merely subcutaneously transplants the factory cells and then takes advantage of the green light functionality in smart wearable electronic devices, used to monitor health parameters, such as the Apple Watch (Raja et al. 2019). The authors of the combined hybrid protein optogenetics and smart watch study could further prove that their concept successfully treats diabetes and associated symptoms in mice (Mansouri et al. 2021).

Biotechnology

As in the case of photoxenoprotein engineering, some rare examples for the application of optogenetic systems outside fundamental or medicinal research exist, which focus on the advancement of biotechnological methods. A widespread application is to control the properties of diverse biomaterials with light (Beyer et al. 2018; Hörner et al. 2019b). A very recent publication, for example, engineered a light-responsive silk-elastin-like protein biopolymer with the help of CarH (Narayan et al. 2021). CarH tetramerization in the dark thereby mediated hydrogel formation that is reversible through light-induced monomerization of the photosensory domain. Another current study developed an innovative pluripotent stem

cell culture system, which can be maintained by blue light-dependent stimulation of the fibroblast growth factor (FGF) signaling pathway (Choi et al. 2021). This obviates the need to supplement stem cell cultures with expensive FGF 2. For this purpose, the authors used the LOV domain of Vaucheria fridida to artificially dimerize the FGF receptor upon irradiation. Other examples include the improvement of *in vitro* mass-spectroscopic workflows (Hörner et al. 2019a) and the development of optogenetic cell-sorting strategies (Yüz et al. 2018) as described elsewhere (Tang et al. 2021).

User-relevant fundamentals of the three photocontrol methods

Photochemical properties

Expectations on light-triggered on/off switches

Controlling the biological activity of a protein with light is sometimes compared with a physical light switch, which turns light on and off. As a consequence, we might be misled to envision photocontrol processes to render a protein completely inactive in the dark state while fully active in the light state. However, just as the efficiency of turning light on and off with a physical switch depends on the initial darkness of the room and the power of the bulb, the effect of a photocontrol strategy on the biological activity depends on the photochemical properties of the chromophore and on the response of the protein to those molecular changes on the structural level. A detailed description of these properties is provided in the following to facilitate a better understanding of what can be expected of each photocontrol strategy.

On the molecular level of the chromophore

In general, the reaction of a chromophore in response to light depends on two essential factors. The first is the probability that a photon is absorbed at a specific wavelength λ given commonly by the extinction coefficient ε_λ [L mol^{-1} cm^{-1}] or the absorption cross section σ_λ [cm^2]. Both constants are interrelated and can be converted into each other by the formula $\varepsilon_\lambda = 2.62{\cdot}10^{20}\ \sigma_\lambda$ (Lenci et al. 2012). The second factor is the quantum efficiency Φ, which describes the probability that once the chromophore has been excited by the absorption of a photon it falls back to the ground state through e.g., decaging or isomerization. The product of these two factors $\Phi{\cdot}\varepsilon_\lambda$ [L mol^{-1} cm^{-1}] is proportional to the concentration of the product molecules formed by these light-induced reactions. Hence, the chromophores used as receiver should exhibit high extinction coefficients and high quantum yields to increase the absolute sensitivity to light in all photocontrol methods.

Light-induced decaging and photocrosslinking reactions exhibit several reaction steps after initial excitation of the chromophore. This is illustrated by the exemplary decaging reaction of NBY in Figure 13A (Il'ichev and Wirz 2000; Klán et al. 2013). The total rate k of this multi-step process is inversely proportional to the lifetime τ of the intermediate of the rate-determining step ($k = 1/\tau$). Hence, (de)stabilization of the intermediates by solvent or adjacent acids and bases can significantly influence the total rate, even though the initial photoinduced step might be faster in comparison. In proteins such interferences appeared to result in the incomplete decaging of ncAAs incorporated at positions close to acidic or basic side chains (Kneuttinger et al. 2019a), although the exact reason of this effect is not entirely clear. In case of nitrobenzyl caging groups the decaging reaction can even last significantly longer than the microsecond time scale (Klán et al. 2013). In addition to disturbances in the reaction rate, the photoreactions can suffer from side reactions as outlined for the photocrosslinking ncAA AzF in Figure 9.

Reversible, light-induced switching reactions of chromophores occur in photosensory domains and synthetic photoswitches. The complex photochemical properties of these chromophores are best explained by means of the well-studied azobenzenes (Hoorens and Szymanski 2018). The foundation for the switching reaction is that the two configurations of the photoswitch exhibit different absorption characteristics in the UV/visible spectrum (Figure 13B). The planar structure with C_{2h} symmetry of the *E*-azobenzene (Brown 1966; Crecca and Roitberg 2006) prohibits a $n \rightarrow \pi^*$ transition resulting in a weak absorption band in the visible region at ~450 nm (Cusati et al. 2008). The allowed $\pi \rightarrow \pi^*$ transition, however, is much stronger and causes the main absorption maximum in the UV range of ~320 nm of the *E* isomer (Forber et al. 1985). On the other hand, the *Z* isomer adopts a non-planar conformation with C2 symmetry (Gagliardi et al. 2004; Mostad et al. 1971), which allows the $n \rightarrow \pi^*$ transition causing a stronger absorption at ~450 nm and weakens the $\pi \rightarrow \pi^*$ transition leading to a red-shift and reduction in absorption strength (Forber et al. 1985). Isomerization in the directions $E \rightarrow Z$ and $Z \rightarrow E$ can be controlled by irradiation at either transition. Thus, overlapping of the characteristic absorption bands for each transition in the *E* and *Z* spectra makes quantitative switching from 100% *E* to 100% *Z* impossible. For most switchable chromophores this is the case. The exemplary scenario in Figure 13C illustrates the

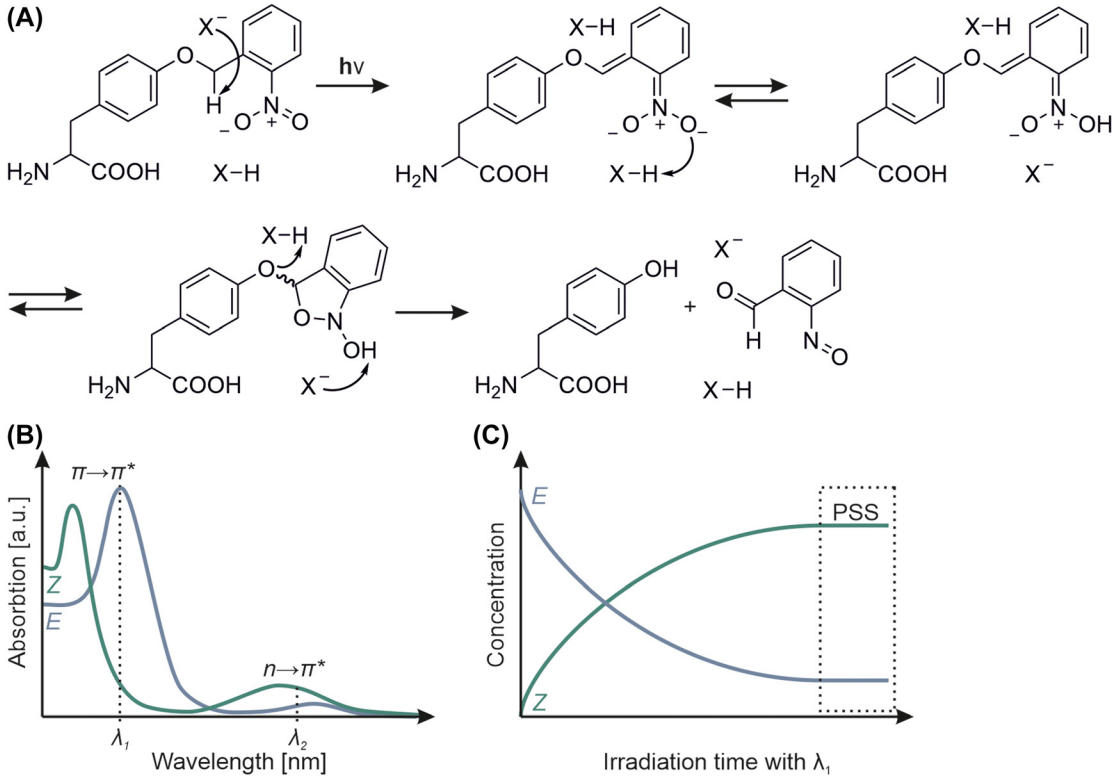

Figure 13: Photochemical properties of synthetic chromophores.
(A) Decaging reaction of NBY (Il'ichev and Wirz 2000; Klán et al. 2013). Irradiation with light excites the nitro group and triggers the acid-base (X) catalyzed formation of a five-membered ring, which requires conformational freedom. The subsequent fragmentation to tyrosin and *o*-nitrosobenzylaldehyde is irreversible. (B) Representative absorption spectra of *E* and *Z* azobenzenes. (C) Isomerization of an azobenzene induced by light of λ_1 shifts the equilibrium of *E* and *Z* until a photostationary state (PSS) is reached.

consequences of overlapping absorptions bands. Irradiation of the *E* isomer with light of the wavelength λ_1 excites the azobenzene in the $\pi \rightarrow \pi^*$ transition band. Relaxation to the ground state then results in the formation of the *Z* isomer dependent on $\Phi^{E\rightarrow Z}\cdot\varepsilon_{\lambda 1}{}^{E}$. The kinetics of this isomerization process occur in the picosecond timescale (Bandara and Burdette 2012; Chang et al. 2004; Satzger et al. 2003). While λ_1 excites the *E* isomer, it also excites the newly formed *Z* isomer, which isomerizes back as a function of $\Phi^{Z\rightarrow E}\cdot\varepsilon_{\lambda 1}{}^{Z}$. This back-and-forth reaction continues until a thermodynamic equilibrium is reached, which is known as the photostationary state (PSS). The ratio between *E* and *Z* in this equilibrium is called the photostationary state distribution (PSD). Intentional, light-induced isomerization of the *Z* isomer back to the *E* isomer is achieved in the exemplary scenario with the wavelength λ_2 and follows the same principle. For photoswitches with well separated absorption bands of each isomer, the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions can be addressed selectively obtaining quantitative PSDs of up to 99:1. In most cases, the absorption bands are reasonably well separated resulting in a sufficiently strong isomeric shift. However, for less well separated absorption bands the PSDs can be much lower than 85:15. Importantly, the time needed to reach the PSS can be regulated by adjusting the intensity of the applied light and the PSD can be controlled by changing the irradiation wavelength. In practice, this means that different light sources vary in their switching efficiency. Conventional light-bulbs emit light in an extremely broad wavelength range and additionally radiate a lot of heat during irradiation, which can negatively affect the biological reaction. Modern LEDs have narrower wavelength ranges and barely give off energy in form of heat. Hence, these light sources are much better suited for the photocontrol of proteins. Lasers are without a doubt the best but also the most expensive light source one can use for photocontrol experiments since the light beam is monochromatic and of high intensity.

Another factor that needs to be considered with photoswitches is the thermal stability of the two isomers. As we have seen in previous chapters most photosensory domains and most *Z* isomers thermally relax back to the thermodynamic more stable form after initial irradiation. These instable isomers exhibit half-lives of microseconds

to minutes (Garcia-Amorós et al. 2016), whereas stable isomers last for hours to even years (Knie et al. 2014; Weston et al. 2014).

On the conformational level of the protein

The response of the protein to light-induced changes of the chromophore on the molecular level is best studied for photoreceptors (Möglich and Moffat 2010; Ziegler and Möglich 2015). The theory draws upon the fundamental functionality of allosteric proteins, which undergo a conformational transition resulting in (de)activation after binding of a ligand at a site distant from the orthosteric center. One way to visualize this is the energy landscape of the proteins (Hans et al. 1991). To this end, the conformational transition induced by ligand binding (allostery) or light (photoreceptors) and thus the change in biological activity can be described as a shift in conformational populations (Kar et al. 2010). Greatly simplified, the photoreceptor is assumed to exist in an equilibrium between a standby conformation (*S*) of low activity and a working conformation (*W*) of high activity. In the absence of light, the chromophore in the protein adopts the thermally equilibrated dark-adapted state, which is defined by the equilibrium constant K_{dark} (Figure 14A). For light-activated photoreceptors, the standby conformation *S* is energetically favored over the working mode *W*; for light-inactivated photoreceptors, *W* is more stable than *S*. Moreover, K_{dark} is proportional to the difference in free energy ΔG_{dark} of both conformations in the energy landscape of the protein (Figure 14B, slate blue). Isomerization of the chromophore upon irradiation introduces a free energy perturbation $\Delta\Delta G$, which triggers a shift in the equilibrium to reach the lit state described by K_{lit} and ΔG_{lit} (Figure 14A). This results in the stabilization of *W* to *W** and destabilization of *S* to *S** in light-activated photoreceptors or vice versa in light-inactivated photoreceptors (Figure 14B, green). The rates of the forward (k_1) and reverse (k_{-1}) reaction between the dark and lit state are highly simplified in this principle assuming a unimolecular, single reaction step. The rate constant k_1 is very fast and depends on the product $\Phi{\cdot}\varepsilon_\lambda$ controlled by the wavelength as well as formation of the PSS regulated by the intensity of light, as we have seen in the earlier chapter. The back reaction in this principle is defined by the thermal reversion of the chromophore to the dark-adapted state and k_{-1} therefore depends on its half-live. This thermodynamic cycle of photoreceptors is called the "photocycle" (Mowery and Stoeckenius 1981; Polland et al. 1986).

The response of the protein to the different isomers of the chromophore is thus relatively clear in the case of hybrid protein optogenetics. In contrast, the effect of the photocaged or photoswitchable ligands in photopharmacology is based on the change in their affinity towards the binding site of a protein. This determines the strength of competition with the native ligand. Hence,

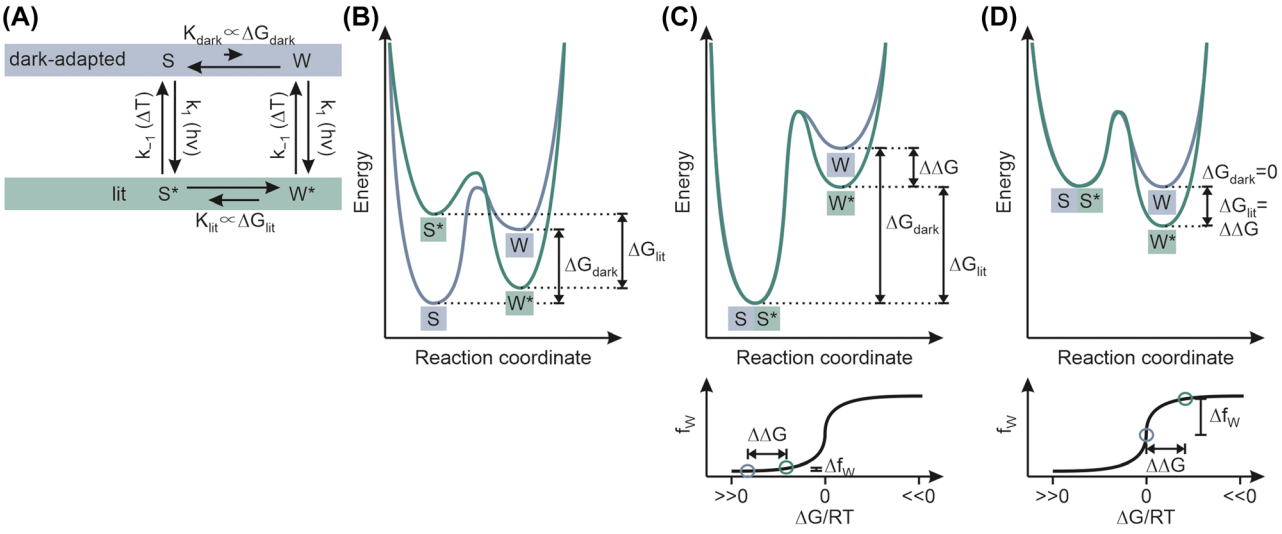

Figure 14: The response of the protein to light-induced changes of the chromophore.
(A) Photocycle of photoreceptors explaining the thermodynamic equilibria in the dark-adapted (slate blue) and lit state (green). The difference between activities results from a shift in S and W conformational populations as demonstrated in panel (B). The size of the activity difference, which correlates with the fraction of the working mode (f_W), depends on the initial position of the equilibrium ΔG_{dark} and the size of the shift $\Delta\Delta G$. While only small changes in f_W are achieved when ΔG_{dark} is >>0 (or <<0, respectively) as shown in panel (C), large differences can be obtained when ΔG_{dark} approximates zero as illustrated in panel (D).

conformational transitions induced by the light-sensitive ligands mostly do not play a role. This is different for photoswitchable bridges in photopharmacology, in which the photoswitchable ligand is bioconjugated at two sites in the protein (Figure 3), and for light-sensitive ncAAs in photoxenoprotein engineering (Figure 8). Although the photocycle for these two photocontrol techniques has hitherto not been investigated it can be assumed that the photocages, photocrosslinkers and photoswitches influence the protein conformation both in the dark-adapted as well as the lit state. The population shift principle as described for photoreceptors above could therefore also be relevant for these photocontrol strategies.

On the activity level

The fundamentals from the previous two chapters allow us to better understand the effects of the on/off switch on the activity of the protein. As was mentioned earlier, the activity difference in proteins controlled by light-sensitive ligands in photopharmacology is primarily determined by the difference in binding strength of the caged and decaged state or the two isomers of the ligand, respectively. In most cases these differences are not particularly high resulting in LRFs of ~2-fold in *in vitro* experiments. However, the same ligands have been shown to mediate a much more impressive effect *in cellulo* or even *in vivo* attributed to various factors (Hüll et al. 2018). First, the ligands usually have a good cell permeability increasing their local concentration through compartmentalization (Velema et al. 2014). More importantly, light-sensitive drugs are often designed to target essential parts of a signaling network (Hüll et al. 2018); consequently, the signaling effect multiplies the initially low LRF making the drug highly effective. Finally, the two states of the ligand can provoke different pharmacodynamic and pharmacokinetic effects, which again might influence the LRF *in vivo*.

For the implementation of photocontrol using strategies from photopharmacology it is important to distinguish the photochemical properties of ligands from those of photosensory domains in hybrid protein optogenetics. Photocaged ligands do not follow a thermodynamic cycle as the total reaction is irreversible. Hence, irradiation is only required until the decaging reaction is complete. As the decaging efficiency correlates with the product $\Phi{\cdot}\varepsilon_\lambda$ this process depends on the wavelength spectrum of the light source and takes microseconds to several minutes (Klán et al. 2013). Photocontrol using photoswitchable ligands with a thermally instable state requires constant irradiation to maintain the PSS. However, the goal of photopharmacology is to produce thermally stable photoswitches and as a result most synthetic ligands exhibit thermal half-lives of hours to years. In such cases, a brief irradiation to obtain the PSS is sufficient. Fine-tuning of the LRF is best achieved by adjusting the wavelength of irradiation as the PSS is strongly dependent on $\Phi{\cdot}\varepsilon_\lambda$ of the two isomers. In contrast, changing the light intensity is not as efficient since it only affects the duration until equilibration, which usually takes sub-seconds to minutes depending on the wavelength of irradiation.

The principles, which determine the LRF in strategies of photoxenoprotein engineering, are hitherto not entirely clarified. The effects are not as simple as for light-sensitive ligands in photopharmacology; differences in binding strength do not matter since the photocages, photocrosslinks and photoswitches are introduced translationally into the protein. As indicated in the previous chapter, the population shift principle as described for photoreceptors may apply to the artificial proteins created in photoxenoprotein engineering, as well. Hence, the following underlying concept of activity tuning in photoreceptors is fundamental to further analyze the possible effects of irradiation on the activity level in ncAA-containing proteins.

In hybrid protein optogenetics, regulation of the activity strongly depends on the photocycle (Figure 14) and its diverse factors (Möglich and Moffat 2010; Ziegler and Möglich 2015). To simplify, we first assume that the standby mode is 0% and the working mode is 100% active, and that the photoreceptor is activated. In this case, the thermodynamic principle demands a large shift of the equilibrium between S and W and hence a large increase in the fraction of W (f_W) to maximize the LRF. The shift depends on two factors, the initial position of the equilibrium in the dark-adapted state ΔG_{dark} and the size of the free energy perturbation $\Delta\Delta G$ induced by irradiation. One might assume that in order to obtain the largest activity difference the shift needs to start at $\Delta G_{\mathrm{dark}} >> 0$ approximating 0% W. However, large $\Delta\Delta G$s would be required to achieve a significant difference in f_W with this starting point (Figure 14C). In comparison, a much bigger f_W is obtained with the same $\Delta\Delta G$ when starting from ΔG_{dark}~0 at an equilibrium of ~50% S and ~50% W (Figure 14D). In addition to ΔG_{dark} and $\Delta\Delta G$ the activity of both conformations S and W is decisive. Contrary to what we assumed at the beginning the activity of proteins is rarely zero; instead, it is regulated down by factors ranging from below 10-fold to more than 1000-fold depending on the strength of the interference. The larger the difference in activity of S and W is, the higher the LRF. Remarkably, the interplay between ΔG_{dark}, $\Delta\Delta G$, and the difference in activity of S and W has been optimally adjusted by nature in photoreceptors.

Engineering of artificial photoreceptors can achieve high LRFs of >100-fold (Carrasco-López et al. 2020); however, often photoreceptor variants only exhibit a modest LRF of 2- to 10-fold (Gasser et al. 2014; Hongdusit et al. 2020; Ryu et al. 2014), which can be further improved to >10-fold by optimization screenings by which primarily ΔG_{dark} is shifted (Strickland et al. 2010), whereas $\Delta\Delta G$ is assumed to be challenging to alter (Ziegler and Möglich 2015). Nevertheless, it has been shown that even LRFs of ≤10-fold can cause significant phenotypical changes *in vivo* (Barends et al. 2009; Wu et al. 2009).

To fine-tune the LRF in an optogenetic experiment it must be considered that most photosensory domains have only short thermal half-lives of tens to 10 thousands of seconds corresponding to merely a few hours (Möglich and Moffat 2010). This means the photosensory domain reverts back from the lit state to the dark-adapted state with the rate constant k_{-1}. A brief light pulse to reach the PSS is therefore not sufficient to evoke a long-lasting effect. The samples thus need to be constantly irradiated. Depending on the rate constant k_1, which can be adjusted by the intensity of light, different equilibria between the lit and the dark-adapted state can be established facilitating a fine-tuning of the LRF.

Coming back to photoxenoprotein engineering, the activity of the dark-adapted state and the activity of the lit state are decisive for the LRF just as in hybrid protein optogenetics. This factor is exploited for the photocontrol with photocaged ncAAs by targeting essential residues in the orthosteric site for replacement. High LRFs of >100-fold can be achieved by this strategy since the photocage efficiently blocks the function of the residue (Jedlitzke et al. 2020). While this factor is just as important for the other strategies in Figure 8, an equilibrium between *S* and *W* conformations, and hence ΔG_{dark} and $\Delta\Delta G$, will play an increasingly pronounced role as well. An easy example to illustrate this is the photocontrol of multimerization using light-sensitive ncAAs. In the dark-adapted state, complex formation of two subunits might be hampered by the presence of the ncAA; however, the monomers (equivalent to the standby mode *S*) and the complex (equivalent to the working mode *W*) will still exist in equilibrium described by the dissociation constant K_D. Light-induced changes of the ncAA might then facilitate multimerization, which translates into a shift in equilibrium and an improvement of K_D comparable to the cycle in Figure 14A. Moreover, it is highly likely that the third photoxenoprotein engineering strategy, the photocontrol of a conformational change, follows the same basic principles as the photocycle of photoreceptors. Notably, the term photocycle would only fit for photoswitchable ncAAs as the change in equilibrium is irreversible for photocaged and photocrosslinking ncAAs. Recent findings proved that introduction of the light-sensitive moiety into strategically chosen positions close to existing conformational transitions can result in high LRFs of >10- (Kneuttinger et al. 2019a, 2020) and >100-fold (Kneuttinger et al. 2019b). Even the photoswitchable bridge strategy of photopharmacology, for which these principles most likely apply as well, achieved LRFs of substrate binding of >100-fold (Bozovic et al. 2020, 2021).

Despite the analogy to hybrid protein optogenetics regarding the photocycle, the strategies of photoxenoprotein engineering differ in terms of implementation. For the same reasons as for the photocages and photoswitches in photopharmacology, no constant irradiation is required for light-sensitive ncAAs. Likewise, fine-tuning of the LRF is more efficient by changing the wavelength of irradiation than light intensity.

Efficient irradiation

In the first chapters, a broad spectrum of applications for the photocontrol of proteins using photopharmacology, photoxenoprotein engineering and hybrid protein optogenetics was introduced. Clearly, all these potential implementations raise demands regarding the translatability of photocontrol. In fact, one should consider these factors before choosing a photocontrol design technique. The currently most central questions are the following: 1) Does light have a toxic effect on the sample? 2) Can light penetrate the sample completely e.g., tissue in *in vivo* applications or the full capacity of reactors in biocatalytic applications? 3) Which technological progresses have been made for the delivery of light in such applications?

The first of these questions depends on the wavelength of the light and the type of sample. UV and blue light are rich in energy and can cause severe damage to cells and tissues with high mutagenic and other nonspecific effects (Brash et al. 1991). Irradiation induces for example the formation of pyrimidine dimers in the DNA (Kneuttinger et al. 2014) and the generation of reactive oxygen species, which in turn destroy the genetic material (Meyskens Jr. et al. 2001). Moreover, UV light can cause an inflammatory response (Sarasin 1999) as well as apoptosis-controlled cell death (Luca et al. 2011). Besides this commonly known effect of UV light most researchers are not aware that visible light can have a detrimental effect as well. Recently, analysis of multiple pyridoxal 5′-phosphate (PLP) dependent enzymes revealed that illumination with visible light can drastically decrease enzymatic activity *in vitro* (Gerlach

et al. 2021). On that account, target enzymes, especially containing light-sensitive cofactors (besides PLP, e.g., flavins), should be tested for inactivating effects by light prior to the design of photocontrol. Nevertheless, photocontrol of such light-sensitive enzymes can still be regarded profitable, if the artificially installed light-activation effect outweighs the natural light-inactivation effect.

The next issue is the penetration depth of light. While irradiation of samples in the analytic scale e.g., proof-of-concept studies in monolayer cell culture or functional studies on isolated proteins, is easy and effective, applications in larger sample sizes can become quite difficult to realize. Especially *in vivo* applications are challenging since tissue is either opaque e.g., bone marrow, or has evolved to protect sensitive regions from harmful UV irradiation by providing multiple endogenous chromophores that absorb the light (Kalka et al. 2000) e.g., melanin in the skin. Hence, the penetration depth of UV light is strongly limited to only a few millimeters in soft tissue (Ash et al. 2017; Weissleder 2001). Light in the red and near infrared range has a much higher penetration depth of up to centimeters (Weissleder 2001; Weissleder and Ntziachristos 2003) and is therefore preferred for therapeutic applications of photocontrol. For this purpose, phytochromes are currently the best choice for the engineering of artificial proteins with red or near infrared light (Figure 12). However, as shown by a recent calculation even the penetration strength of light in the near infrared window of 600–1300 nm is not efficient enough to activate significant amounts of a therapeutic light-sensitive drug in a 5 cm deep tissue (Sharma and Friedman 2021). Another matter is large scale *in vitro* applications such as high volume biocatalytic reactions. Clearly, this kind of sample is not as dense as tissue, nevertheless the depth of light penetration depends on the composition of the reaction. High absorbing reactants can limit the penetration strength in a specific wavelength range but should still allow a penetration depth of up to meters. In comparison, the euphotic zone of oceans, which is suffused by sunlight in the UV/visible range giving shelter to all photosynthesizing organisms, reaches 10–80 m depending on the clarity of the water.

The mentioned limitations of the penetration depth of light make it necessary to develop irradiation processes that facilitate therapeutic or biocatalytic approaches. Light delivery systems for medicinal therapies have primarily been devised by the field of photodynamic therapy (Algorri et al. 2021). Advanced technology is especially required for subdermal applications since irradiation on the skin, in the eye or in tissues reachable by endoscopy e.g., gut or cervix, are usually no problem. At the same time, the irradiation procedure should not be invasive. The current development status of noninvasive light delivery systems is extremely impressive (Morstein and Trauner 2019). One of the most important advances are wireless implants of millimeter small size (Montgomery et al. 2015; Park et al. 2015; Qazi et al. 2021; Veiseh et al. 2015). However, other properties are improved as well. Some devices can be injected with a syringe (Liu et al. 2015) and are connected to subdermal magnetic coil antennas (Shin et al. 2017), whereas others show enhanced *in vivo* stability (Gutruf and Rogers 2018). Furthermore, light delivery systems for biocatalytic applications are under development in the field of photobiocatalysis (Heining and Buchholz 2015); recent examples thereof include photoreactors (Bonfield et al. 2018) and internal wireless light emitters (Heining et al. 2015).

The design process

Photopharmacology

The design of photocontrol in photopharmacological strategies first and foremost requires chemical engineering of the light-sensitive ligand. In principle, the design needs to include moieties that mediate high affinity binding, which are often copied from native ligands, and photocaged or photoswitchable components with beneficial photochemical properties. Moreover, the ligands should be synthetically accessible and rapidly producible. This, however, means that new ligands have to be designed for each new target protein making chemical engineering quite challenging. Customizing the photochemical properties such as the thermal stability of the *Z* isomer (Kolarski et al. 2021b) or the shift to longer wavelengths (Konrad et al. 2016) can be realized by adding substituents to the initial scaffold, but is quite tedious since these substitutions might have adverse effects and make synthesis difficult. Nevertheless, many advances have been made towards for example red-shifted light-sensitive ligands in the last couple of years (Leistner et al. 2021; Morville et al. 2021).

While for the engineering of free ligands only the active site of the target protein needs to be considered, bioconjugated PTLs and PORTLs further require difficile design of the spacer between the protein anchor site and the active site. The design of photoswitchable bridges is even more complex since here the photoswitch is attached to two sites in the protein and must induce significant conformational changes to light-regulate protein activity. Both attachment sites and spacer size need to be precisely adjusted. Thus, this protein-based design component is often supported by computational approaches. Using

photoswitchable bridges to control the α-helical content of proteins is a strategy, which reaches back two decades. One of the earliest publications (Kumita et al. 2000) clicked an azobenzene-based photoswitch to two cysteine residues positioned seven residues apart. Molecular modelling revealed that only the *Z* configuration facilitated the secondary structure, whereas the *E* configuration of the photoswitch was predicted to disturb the α-helix because it was too long. In a follow-up study, the authors again used computational methods to analyze whether steric interactions of residues in between the two cysteines influence the photoswitch, which does not appear to be the case (Kumita et al. 2002). Another approach identified dynamical surface regions in lipase B from *Candida antarctica* that promote catalysis by computational modelling (Agarwal et al. 2012). The authors conjugated an azobenzene photoswitch to these regions. The rationale behind this strategy is the anticipation that the conformational switch of the azobenzene upon irradiation with light might introduce energy into the dynamical regions and hence result in activation of catalysis. In fact, lipase B activity could be light-dependently increased by 24%; after taking the high percentage of non-crosslinked enzymes into account the LRF increased from ~8- to 52-fold. While this approach reflects the power of inducing conformational changes in proteins by light, it also demonstrated that low crosslinking efficiencies are most critical for bioconjugation-based photocontrol strategies. Yet another study made use of a computational approach to calculate crosslinking sites for an azobenzene-based photoswitch in cytosine deaminase (Blacklock et al. 2018). The Rosetta-based simulations successfully predicted a site at which the photoswitchable bridge mediated photocontrol with an LRF of ~3-fold and a site at which it did not have an effect. In the former design the azobenzene ligand crosslinked two subunits of the enzyme dimer, whereas in the latter design it was placed in an α-helix. Additional mutagenesis is rarely used to improve the LRF of the ligand-protein-pair in photopharmacology; however, one study proved that this kind of optimization is possible (Schierling et al. 2010). The authors installed a photoswitchable bridge in the homodimeric restriction enzyme PvuII and initially achieved an LRF of ~7-fold. Additional amino acid exchanges could further increase the LRF to ~16-fold.

Photoxenoprotein engineering

The design of ncAA-based photocontrol in proteins shares some processes with photopharmacology and some with hybrid protein optogenetics. In a first step, a suitable ncAA has to be chosen for the desired strategy and application. Similarly to hybrid protein optogenetics, a library is then created, in which the chosen ncAA(s) is/are incorporated at various sites in the target protein. The selection of positions is primarily based on prior knowledge of the target system including e.g., residues involved in catalysis, ligand binding or allostery, for which the three-dimensional structure of the protein may be beneficial. In a subsequent low-throughput screening variants are identified, which exhibit light-dependent behavior with activities of the lit state comparable to wildtype. Since the effect of the light-sensitive ncAAs is often difficult to anticipate, some computationally based protocols have been developed to ease the choice of positions for ncAA incorporation. Comparable to examples in photopharmacology, a computational approach was recently used to detect regions of high conformational flexibility in firefly luciferase, in which incorporation of AzoF could result in efficient photocontrol (Luo et al. 2018). As a result, the authors obtained two out of seven initial AzoF-variants with which the chemiluminescence of firefly luciferase could be repeatedly switched on and off in live mammalian cells. A more comprehensive study devised a Rosetta-based strategy to determine sites close to the active site, in which the caged tyrosine NBY achieves high LRFs (Wang et al. 2019a). The authors initially screened 60 sites in firefly luciferase feeding the results into the algorithm of Rosetta (Park et al. 2016) to create a powerful prediction tool. They further accomplished to photocontrol multiple cellular processes by photocontrolling various target proteins thereby showing the general applicability of their approach, which they dubbed "computationally aided and genetically encoded proximal decaging (CAGE-prox)". Apart from these studies for improving the protein engineering component of photoxenoprotein engineering, plenty of new ncAAs are designed e.g., with optimized photochemical properties, comparable to the design of ligands in photopharmacology. Nevertheless, chemical engineering of ncAAs is limited by the incorporation capability of the heterologous aaRS/tRNA pairs. Evolved aaRSs may tolerate minimal changes of an ncAA; however, larger changes usually require a new evolution experiment.

In general, the design process of ncAA-based photocontrol largely depends on the incorporation efficiency of the amber suppression technique. Several concerns regarding this method exist. A major drawback is the low ratio of full-length versus truncated protein, which is caused by the competition of the aminoacylated heterologous tRNA with release factor 1 for binding to the amber codon. Another matter is the potentially low expression yields of the full-length protein; a common rule of thumb

approximates the yields to 10% compared to wildtype. Usually, a wide yield range of 1–100 mg of protein per liter expression medium is common (Liu and Schultz 2010). Moreover, the fidelity of incorporation, meaning that canonical amino acids are incorporated in addition to the ncAA, is also a highly discussed issue. Besides these major concerns, practical limitations exist such as high wastage of ncAA due to poor influx rates of the ncAA into the host cells (Chin 2017), varying incorporation efficiencies in different positions of a protein (Young et al. 2010) and the need of optimizing expression conditions anew for each target protein. Nevertheless, various advancements have been made in recent years of which I describe some in the following.

The ratio of full-length versus truncated protein was maximized by the engineered *E. coli* strain C321.ΔA, in which release factor 1 is knocked out and all chromosomally encoded TAG codons are replaced by the other amber codons (Kuznetsov et al. 2017; Lajoie et al. 2013). A significant drawback of this strain is its slow growth rate, which makes the expression of proteins especially in large scale quite difficult reducing the overall yields. A recent study found that primarily the carbohydrate and energy metabolisms are downregulated in this strain (Yi et al. 2021). Hence, other studies have tried to improve the system. One example is the strain B-95.ΔA, which is based on the quite common expression host strain *E. coli* BL21(DE3) and which also lacks release factor 1 (Mukai et al. 2015). Since a large amount of TAG codons in the genome of *E. coli* does not only serve as a stop codon in one open reading frame, but also encode amino acids in an alternative open reading frame, the authors only deleted 95 of the 273 TAG codons present. As a result, the strain retained the good growth properties of the BL21(DE3). Besides elimination of truncated proteins, increased efficiency of multi-site incorporation is a major benefit of the C321.ΔA strain (Zheng et al. 2016). Further advancements in this direction are engineered strains (Robertson et al. 2021) or cell-free protein synthesis systems (Cui et al. 2020), which allow to incorporate different ncAAs in response to sense codons instead or in addition to the amber codon. A second challenge needed to overcome for multi-site incorporation are low affinities of the evolved aaRSs for the targeted ncAA (O'Donoghue et al. 2013; Umehara et al. 2012). These low affinities might partly be owed to the high copy number of plasmids encoding the aaRS in evolution experiments. Two studies circumvented this issue by developing an *in vivo* evolution approach, in which libraries of chromosomally integrated aaRSs were used (Amiram et al. 2015; Israeli et al. 2021). In fact, the engineered aaRSs showed increased aminoacylation efficiencies for diverse ncAAs including AzF and AzoF. In an alternative approach, the affinity of the aaRS for NBY was increased by evolving up to 17 residues—significantly more compared to the six residues in the traditional approach (Deiters et al. 2006)—in the computationally-guided redesign including some that are able to form specific interactions with the bound ncAA (Baumann et al. 2019). As a result, the yields for single-site incorporation were improved to 100% compared to wildtype and the yields for multi-site incorporation were markedly increased as well. In addition to the aaRS, expression yields highly depend on the tRNA and the expression conditions. Thus, an early publication developed an enhanced plasmid system, pEVOL, which encodes two copies of the evolved aaRS, coupled to a constitutive and an arabinose-induced promoter, respectively, and a suppressor tRNA that was optimized in directed evolution experiments (Guo et al. 2009; Young et al. 2010). This plasmid system has become the golden standard for ncAA incorporation experiments. Expression yields could also be significantly improved by choosing the *Vibrio natriegens* based Vmax X2 expression host (González et al. 2021). This strain exhibits rapid growth rates allowing to increase expression yields, is fully compatible with plasmids designed for expression in *E. coli* and additionally shows reduced misincorporation of endogenous amino acids into the amber codon. Finally, wastage of ncAA was reduced by an engineered leucine-binding protein that actively imports a broad repertoire of ncAAs into the bacterial cell and hence significantly reduces the ncAA concentration needed for the expression of the target protein (Ko et al. 2019).

Hybrid protein optogenetics

In optogenetics, it is not uncommon that a natural photoreceptor exists with the exact requirements needed for the desired application, e.g., certain ion channels, and which is then used in divergent tissues. However, most researchers interested in photocontrol of proteins aim to put a specific target protein under the control of light. For this purpose, hybrid proteins are designed in optogenetics by fusion of a photosensory domain with the protein of interest. Although this appears easily achievable, this approach requires cumbersome and elaborate engineering in multiple steps. A generally applicable protocol has hitherto not been established due to the fact that each protein is individual; a strategy, which facilitates a high LRF for one protein, might not be able to attain photocontrol of another protein at all.

In a first step, an appropriate photosensory module needs to be chosen. Attention especially needs to be paid to

whether irradiation should occur with low-energy red or infra-red light; if this is the case, phytochromes are the photosensor of choice (Figure 12). In the next step, a library is created comprising various protein fusion constructs of photosensor and target protein (Möglich and Moffat 2010; Mathony and Niopek 2021). The different constructs may vary in the linker sequence and length or the position in the target protein to which the linker is attached. For the identification of initial light-regulatable constructs a small library and a low-throughput screening usually suffices. Nevertheless, some design rules should be kept in mind when starting to engineer an artificial photoreceptor. For non-associating strategies (Figure 11) to work, the target protein needs to be structurally related to the native effector protein (Mathony and Niopek 2021). Moreover, since the interaction between photosensor and effector is critical for these strategies, the properties of the linker, such as its length, sequence, and structure, are decisive for the design (Möglich et al. 2009; Ryu et al. 2014; Ziegler and Möglich 2015). Associating strategies (Figure 11), on the other hand, are not critically limited by structural homology of the target protein to the native effector or the linker identity. For both strategies, however, the fusion site is of crucial importance. The photosensor can either be attached at the termini or within the target protein, which opens plenty of possibilities for photocontrol but unfortunately also for failures. Many successful studies have therefore relied on prior knowledge of the target protein to choose appropriate sites; this knowledge might include allosteric sites (Gehrig et al. 2017) or other activation mechanisms (Smart et al. 2017) as well as previous protein engineering studies (Fukuda et al. 2014; Hattori et al. 2013).

Ideally, initial screening has identified one or more variants, which allow the photocontrol of the chosen target protein. In the next step, the variants are typically further optimized by introducing point mutations, deletions or insertions in directed evolution experiments (Mathony and Niopek 2021; Möglich and Moffat 2010). These aim at either optimizing the linker length (Bubeck et al. 2018; Dagliyan et al. 2019) or directly improve the photochemical properties of the photoreceptor by modulating the recovery time (Pudasaini et al. 2015; Ziegler and Möglich 2015) or increasing the LRF (Strickland et al. 2010). Most studies thereby shift the equilibrium between the *S* and *W* conformation (Strickland et al. 2010; Yao et al. 2008; Ziegler and Möglich 2015).

The most critical step in this outlined design process is probably the initial search for a light-regulatable photoreceptor, which is not very intuitive. Hence, multiple studies tried to ease this first step by computational design approaches (Dagliyan et al. 2019) or by a switch to high-throughput screenings. Coevolution studies using statistical coupling analysis identified allosteric regulation hotspots on the surface of *E. coli* dihydrofolate reductase. Fusion of these hotspots to the LOV2 domain from *A. sativa* resulted in a two-fold light-induced regulation of enzyme activity and the authors speculated that this approach might be used for the design of other photoreceptors (Lee et al. 2008; Reynolds et al. 2011). In a similar approach the LOV2 domain was inserted into non-conserved surface loops, which were identified to be allosterically coupled to the active site of the target protein by molecular dynamics simulations (Dagliyan et al. 2016). Various proteins could be photocontrolled by this strategy in living cells. Still, these computational approaches are far from generalizable. While the latter study suggested to place the photosensor module into surface loops, authors from another study could not accomplish photocontrol this way; instead, the photosensor needed to be inserted into a β-sheet (Hoffmann et al. 2021). Thus, high-throughput screening of fusion construct libraries designed by the Mu-transposon strategy (Edwards et al. 2008; Nadler et al. 2016) or gene library synthesis (Coyote-Maestas et al. 2020) constitutes an alternative approach. To this end, the photosensor module is inserted at random positions in the target protein. This approach is only feasible for proteins, which exhibit easily detectable activities for high-throughput *in vivo* selection or microtiter-plate-based screening processes. In the course of the experiment, the photoreceptors are sieved to eliminate constitutively active or inactive variants and to enrich constructs that show a significant LRF between activities in the dark and lit state.

Summary and outlook

The regulation of the biological activity of proteins is useful in a number of ways and for a diverse set of purposes. While some consider photocontrol as the method of choice for advanced fundamental studies *in vivo* or even *in vitro*, others aspire to employ photocontrol for the development of innovative therapeutic or industrial strategies. Over the last decades, enormous accomplishments in the field of photocontrol have been made. The two methods photopharmacology and hybrid protein optogenetics have contemporaneously progressed to powerful techniques offering different approaches to the establishment of photocontrol. Photoxenoprotein engineering has emerged as a versatile youngster, which demonstrates some overlaps with photopharmacology and hybrid protein optogenetics but has developed into an independent method with substantial differences to the other two. There is still plenty of room for

Table 1: Main properties of the three photocontrol methods.

	Photopharmacology	Photoxenoprotein engineering	Hybrid protein optogenetics
Method concepts	Genetical fusion of natural photosensor modules with target proteins	Free or post-translationally bioconjugated ligands	Genetically encoded light-sensitive ncAAs
Tools	Light-sensitive cofactors often naturally available	Artificially synthesized light-sensitive compounds	Artificially synthesized light-sensitive amino acids
Applications	*In vivo* fundamental studies, therapeutic approaches, biotechnology	Drug development, functional studies on proteins	*In vivo* fundamental studies, functional studies on proteins, therapeutic approaches, production of challenging proteins, biotechnology, industry
Photocontrol	Reversible	Irreversible (photocages), reversible (photoswitches)	Irreversible (photocaged and photocrosslinking ncAAs), reversible (photoswitchable ncAAs)
	Chromophores responsive to wavelengths of the complete electromagnetic spectrum	Chromophores responsive primarily to wavelengths in the UV to green light range	Chromophores responsive primarily to wavelengths in the UV to green light range
	Lit state stable within seconds to hours	Lit state of photoswitches stable within minutes to years	Lit state of photoswitches (currently) stable within hours to years
	LRFs depend on ΔG_{dark}, $\Delta\Delta G$, and the difference in activity of *S* and *W*	LRFs depend on binding strengths in dark and lit state	LRFs depend on the difference in activity of *S* and *W* (and possibly ΔG_{dark}, $\Delta\Delta G$)
	LRFs of 2 to >100-fold *in vitro*	LRFs of 2 to >10-fold *in vitro*	LRFs of 2 to >100-fold (caged) or ~10-fold (switch) *in vitro*
Design process	Advanced protein engineering with low-throughput screening of initial libraries and subsequent optimization screenings	Chemical engineering and in part protein engineering for bioconjugation strategies	Protein engineering with low-throughput screening of different incorporation positions

S, standby conformation of low activity; *W*, working conformation of high activity.

development of photoxenoprotein engineering and some advances can probably be expected considering the incorporation strategy of ncAAs, the design of novel ncAAs particularly in the visible light spectrum, computational approaches to identify appropriate residue positions for photocontrol, and further optimization screenings similar to hybrid protein optogenetics.

Each of the three techniques shows distinct characteristics (Table 1). Some major points of difference are found regarding *in vivo* applications, for which hybrid protein optogenetics exhibits clear advantages over the other two methods. It facilitates reversible photocontrol strategies, provides chromophores that are found or can be generated in the cell and primarily uses non-toxic, visible light with high penetration depth for photocontrol. In contrast, photopharmacology as well as photoxenoprotein engineering frequently use irreversible strategies, require an exogenous supply, e.g., by injection, of the active compound, and still struggle to provide chromophores responsive to longer wavelengths. While these properties are disadvantageous for *in vivo* applications, they might, however, be favorable for others. Other properties with arguable (dis)advantages are the size of the receiver (large molecular photoreceptors versus small molecular compounds), genetic encoding and the stability of the lit state. Particularly, bistability might seem the better choice for various uses, however, thermally instable switches have the advantage of automatic deactivation. Hence, depending on the application, and whether it is anticipated *in vivo* or *in vitro*, these factors might be strengths or limitations of the individual strategy.

One of the most confusing misconceptions for all three techniques is thereby that photocontrol is no absolute on/off activity switch. Especially important in this context is that the off state shows in most cases at least some background activity. Only the application decides whether this leakage is negligible or unfavorable for the system. In this respect, while photopharmacology and hybrid protein optogenetics remained rather limited in their spectrum of uses, photoxenoprotein engineering has explored various applications, which promise to develop even further in the near future. However, each of the three techniques can in principle be used for this diverse set of applications. In this regard, novel opportunities for photocontrol constantly open up such as in green industry or in time-resolved crystallography. Moreover, the more traditional therapeutic application will face one of the biggest steps yet, the advance of clinical studies. The next decade might hence bring exciting new developments in all three photocontrol techniques that will highlight the power of this set of strategies to implement

dynamic control in biological systems. Finally, the field of photocontrol will altogether strive towards a highly ambitious goal: the development of general reversible regulation strategies, which are easily applicable without complex engineering procedures to any target protein. Until this is accomplished, researchers can choose the technique, which is best suited for their endeavors.

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References

Acosta-Ruiz, A., Gutzeit, V.A., Skelly, M.J., Meadows, S., Lee, J., Parekh, P., Orr, A.G., Liston, C., Pleil, K.E., Broichhagen, J., et al. (2020). Branched photoswitchable tethered ligands enable ultra-efficient optical control and detection of G protein-coupled receptors *in vivo*. Neuron 105: 446–463.

Agarwal, P.K., Schultz, C., Kalivretenos, A., Ghosh, B., and Broedel, S.E. (2012). Engineering a hyper-catalytic enzyme by photoactivated conformation modulation. J. Phys. Chem. Lett. 3: 1142–1146.

Agostini, F., Völler, J.-S., Koksch, B., Acevedo-Rocha, C.G., Kubyshkin, V., and Budisa, N. (2017). Biocatalysis with unnatural amino acids. Enzymology meets xenobiology. Angew. Chem. Int. Ed. 56: 9680–9703.

Agostinis, P., Berg, K., Cengel, K.A., Foster, T.H., Girotti, A.W., Gollnick, S.O., Hahn, S.M., Hamblin, M.R., Juzeniene, A., Kessel, D., et al. (2011). Photodynamic therapy of cancer. An update. CA-Cancer J. Clin. 61: 250–281.

Algorri, J.F., Ochoa, M., Roldán-Varona, P., Rodríguez-Cobo, L., and López-Higuera, J.M. (2021). Photodynamic therapy: a compendium of latest reviews. Cancers 13: 4447.

Amiram, M., Haimovich, A.D., Fan, C., Wang, Y.-S., Aerni, H.-R., Ntai, I., Moonan, D.W., Ma, N.J., Rovner, A.J., Hong, S.H., et al. (2015). Evolution of translation machinery in recoded bacteria enables multi-site incorporation of nonstandard amino acids. Nat. Biotechnol. 33: 1272–1279.

Anastas, P.T. and Warner, J.C. (1998). *Green chemistry. Theory and practice*. Oxford: Oxford University Press.

Ankenbruck, N., Courtney, T., Naro, Y., and Deiters, A. (2018). Optochemical control of biological processes in cells and animals. Angew. Chem. Int. Ed. 57: 2768–2798.

Arbely, E., Torres-Kolbus, J., Deiters, A., and Chin, J.W. (2012). Photocontrol of tyrosine phosphorylation in mammalian cells via genetic encoding of photocaged tyrosine. J. Am. Chem. Soc. 134: 11912–11915.

Ash, C., Dubec, M., Donne, K., and Bashford, T. (2017). Effect of wavelength and beam width on penetration in light-tissue interaction using computational methods. Laser Med. Sci. 32: 1909–1918.

Ausländer, S., Ausländer, D., and Fussenegger, M. (2017). Synthetic biology—the synthesis of biology. Angew. Chem. Int. Ed. 56: 6396–6419.

Baker, A.S. and Deiters, A. (2014). Optical control of protein function through unnatural amino acid mutagenesis and other optogenetic approaches. ACS Chem. Biol. 9: 1398–1407.

Bandara, H.M.D. and Burdette, S.C. (2012). Photoisomerization in different classes of azobenzene. Chem. Soc. Rev. 41: 1809–1825.

Barbero-Castillo, A., Riefolo, F., Matera, C., Caldas-Martínez, S., Mateos-Aparicio, P., Weinert, J.F., Garrido-Charles, A., Claro, E., Sanchez-Vives, M.V., and Gorostiza, P. (2021). Control of brain state transitions with a photoswitchable muscarinic agonist. Adv. Sci. 8: 2005027.

Bardhan, A. and Deiters, A. (2019). Development of photolabile protecting groups and their application to the optochemical control of cell signaling. Curr. Opin. Struct. Biol. 57: 164–175.

Barends, T.R.M., Hartmann, E., Griese, J.J., Beitlich, T., Kirienko, N.V., Ryjenkov, D.A., Reinstein, J., Shoeman, R.L., Gomelsky, M., and Schlichting, I. (2009). Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. Nature 459: 1015–1018.

Baumann, T., Hauf, M., Richter, F., Albers, S., Möglich, A., Ignatova, Z., and Budisa, N. (2019). Computational aminoacyl-tRNA synthetase library design for photocaged tyrosine. Int. J. Mol. Sci. 20: 2343.

Bayley, H., Chang, C.-Y., Todd Miller, W., Niblack, B., and Pan, P. (1998). [7] Caged peptides and proteins by targeted chemical modification. In: *Methods in enzymology: caged compounds*. Academic Press, Cambridge, Massachusetts, USA, pp. 117–135.

Bellucci, J.J., Amiram, M., Bhattacharyya, J., McCafferty, D., and Chilkoti, A. (2013). Three-in-one chromatography-free purification, tag removal, and site-specific modification of recombinant fusion proteins using sortase A and elastin-like polypeptides. Angew. Chem. Int. Ed. 52: 3703–3708.

Berlew, E.E., Kuznetsov, I.A., Yamada, K., Bugaj, L.J., Boerckel, J.D., and Chow, B.Y. (2021). Single-component optogenetic tools for inducible RhoA GTPase signaling. Adv. Biol. 5: 2100810.

Beyer, H.M., Thomas, O.S., Riegel, N., Zurbriggen, M.D., Weber, W., and Hörner, M. (2018). Generic and reversible opto-trapping of biomolecules. Acta Biomater 79: 276–282.

Bianco, A., Townsley, F.M., Greiss, S., Lang, K., and Chin, J.W. (2012). Expanding the genetic code of *Drosophila melanogaster*. Nat. Chem. Biol. 8: 748–750.

Bieth, J., Vratsanos, S.M., Wassermann, N., and Erlanger, B.F. (1969). Photoregulation of biological activity by photocromic reagents. II. Inhibitors of acetylcholinesterase. Proc. Natl. Acad. Sci. U.S.A. 64: 1103–1106.

Bieth, J., Wassermann, N., Vratsanos, S.M., and Erlanger, B.F. (1970). Photoregulation of biological activity by photochromic reagents. IV. A model for diurnal variation of enzymic activity. Proc. Natl. Acad. Sci. U.S.A. 66: 850–854.

Blacklock, K.M., Yachnin, B.J., Woolley, G.A., and Khare, S.D. (2018). Computational design of a photocontrolled cytosine deaminase. J. Am. Chem. Soc. 140: 14–17.

Böcker, J.K., Friedel, K., Matern, J.C.J., Bachmann, A.-L., and Mootz, H.D. (2015). Generation of a genetically encoded, photoactivatable intein for the controlled production of cyclic peptides. Angew. Chem. Int. Ed. 54: 2116–2120.

Böcker, J.K., Dörner, W., and Mootz, H.D. (2019). Rational design of an improved photo-activatable intein for the production of head-to-tail cyclized peptides. Biol. Chem. 400: 417–427.

Bonfield, H.E., Williams, J.D., Ooi, W.X., Leach, S.G., Kerr, W.J., and Edwards, L.J. (2018). A detailed study of irradiation requirements

towards an efficient photochemical Wohl–Ziegler procedure in flow. ChemPhotoChem 2: 938–944.

Bose, M., Groff, D., Xie, J., Brustad, E., and Schultz, P.G. (2006). The incorporation of a photoisomerizable amino acid into proteins in *E. coli*. J. Am. Chem. Soc. 128: 388–389.

Bozovic, O., Jankovic, B., and Hamm, P. (2020). Sensing the allosteric force. Nat. Commun. 11: 5841.

Bozovic, O., Ruf, J., Zanobini, C., Jankovic, B., Buhrke, D., Johnson, P.J.M., and Hamm, P. (2021). The speed of allosteric signaling within a single-domain protein. J. Phys. Chem. Lett. 12: 4262–4267.

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J., and Pontén, J. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc. Natl. Acad. Sci. U.S.A. 88: 10124–10128.

Bregestovski, P.D. and Ponomareva, D.N. (2021). Photochromic modulation of Cys-loop ligand-gated ion channels. J. Evol. Biochem. Physiol. 57: 354–371.

Bridge, T., Shaikh, S.A., Thomas, P., Botta, J., McCormick, P.J., and Sachdeva, A. (2019). Site-specific encoding of photoactivity in antibodies enables light-mediated antibody–antigen binding on live cells. Angew. Chem. Int. Ed. 58: 17986–17993.

Broichhagen, J., Frank, J.A., and Trauner, D. (2015a). A roadmap to success in photopharmacology. Acc. Chem. Res. 48: 1947–1960.

Broichhagen, J., Damijonaitis, A., Levitz, J., Sokol, K.R., Leippe, P., Konrad, D., Isacoff, E.Y., and Trauner, D. (2015b). Orthogonal optical control of a G protein-coupled receptor with a SNAP-tethered photochromic ligand. ACS Cent. Sci. 1: 383–393.

Brown, C.J. (1966). A refinement of the crystal structure of azobenzene. Acta Crystallogr. 21: 146–152.

Brown, W., Liu, J., Tsang, M., and Deiters, A. (2018). Cell-lineage tracing in zebrafish embryos with an expanded genetic code. Chembiochem 19: 1244–1249.

Bubeck, F., Hoffmann, M.D., Harteveld, Z., Aschenbrenner, S., Bietz, A., Waldhauer, M.C., Börner, K., Fakhiri, J., Schmelas, C., Dietz, L., et al. (2018). Engineered anti-CRISPR proteins for optogenetic control of CRISPR–Cas9. Nat. Methods 15: 924–927.

Budisa, N. and Schneider, T. (2019). Expanding the DOPA universe with genetically encoded, mussel-inspired bioadhesives for material sciences and medicine. Chembiochem 20: 2163–2190.

Carrasco-López, C., Zhao, E.M., Gil, A.A., Alam, N., Toettcher, J.E., and Avalos, J.L. (2020). Development of light-responsive protein binding in the monobody non-immunoglobulin scaffold. Nat. Commun. 11: 4045.

Chang, C.-W., Lu, Y.-C., Wang, T.-T., and Diau, E.W.-G. (2004). Photoisomerization dynamics of azobenzene in solution with S_1 excitation. A femtosecond fluorescence anisotropy study. J. Am. Chem. Soc. 126: 10109–10118.

Chen, Y., Lu, L., and Ye, S. (2017a). Genetic code expansion and optoproteomics. Yale J. Biol. Med. 90: 599–610.

Chen, Y., Ma, J., Lu, W., Tian, M., Thauvin, M., Yuan, C., Volovitch, M., Wang, Q., Holst, J., Liu, M., et al. (2017b). Heritable expansion of the genetic code in mouse and zebrafish. Cell Res. 27: 294–297.

Chin, J.W. (2006). Modular approaches to expanding the functions of living matter. Nat. Chem. Biol. 2: 304–311.

Chin, J.W. (2017). Expanding and reprogramming the genetic code. Nature 550: 53–60.

Choi, I.Y., Lim, H., Huynh, A., Schofield, J., Cho, H.J., Lee, H., Andersen, P., Shin, J.H., Heo, W.D., Hyun, S.-H., et al. (2021). Novel culture system via wirelessly controllable optical stimulation of the FGF signaling pathway for human and pig pluripotency. Biomaterials 269: 120222.

Chou, C., Young, D.D., and Deiters, A. (2009). A light-activated DNA polymerase. Angew. Chem. Int. Ed. 48: 5950–5953.

Chou, C., Young, D.D., and Deiters, A. (2010). Photocaged T7 RNA polymerase for the light activation of transcription and gene function in pro- and eukaryotic cells. Chembiochem 11: 972–977.

Chou, C. and Deiters, A. (2011). Light-activated gene editing with a photocaged zinc-finger nuclease. Angew. Chem. Int. Ed. 50: 6839–6842.

Christensen, T., Amiram, M., Dagher, S., Trabbic-Carlson, K., Shamji, M.F., Setton, L.A., and Chilkoti, A. (2009). Fusion order controls expression level and activity of elastin-like polypeptide fusion proteins. Protein Sci. 18: 1377–1387.

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E., and Briggs, W.R. (1998). Arabidopsis NPH1. A flavoprotein with the properties of a photoreceptor for phototropism. Science 282: 1698–1701.

Claaßen, C., Gerlach, T., and Rother, D. (2019). Stimulus-responsive regulation of enzyme activity for one-step and multi-step syntheses. Adv. Synth. Catal. 361: 2387–2401.

Courtney, T. and Deiters, A. (2018). Recent advances in the optical control of protein function through genetic code expansion. Curr. Opin. Chem. Biol. 46: 99–107.

Coyote-Maestas, W., Nedrud, D., Okorafor, S., He, Y., and Schmidt, D. (2020). Targeted insertional mutagenesis libraries for deep domain insertion profiling. Nucleic Acids Res. 48: e11.

Crecca, C.R. and Roitberg, A.E. (2006). Theoretical study of the isomerization mechanism of azobenzene and disubstituted azobenzene derivatives. J. Phys. Chem. A 110: 8188–8203.

Cui, Z., Johnston, W.A., and Alexandrov, K. (2020). Cell-free approach for non-canonical amino acids incorporation into polypeptides. Front. Bioeng. Biotechnol. 8: 1031.

Cusati, T., Granucci, G., Persico, M., and Spighi, G. (2008). Oscillator strength and polarization of the forbidden $n\rightarrow\pi^*$ band of *trans*-azobenzene. A computational study. J. Chem. Phys. 128: 194312.

Dagliyan, O., Tarnawski, M., Chu, P.-H., Shirvanyants, D., Schlichting, I., Dokholyan, N.V., and Hahn, K.M. (2016). Engineering extrinsic disorder to control protein activity in living cells. Science 354: 1441–1444.

Dagliyan, O., Dokholyan, N.V., and Hahn, K.M. (2019). Engineering proteins for allosteric control by light or ligands. Nat. Protoc. 14: 1863–1883.

Deal, W.J., Erlanger, B.F., and Nachmansohn, D. (1969). Photoregulation of biological activity by photochromic reagents. III. Photoregulation of bioelectricity by acetylcholine receptor inhibitors. Proc. Natl. Acad. Sci. U.S.A. 64: 1230–1234.

Deisseroth, K., Feng, G., Majewska, A.K., Miesenböck, G., Ting, A., and Schnitzer, M.J. (2006). Next-generation optical technologies for illuminating genetically targeted brain circuits. J. Neurosci. 26: 10380–10386.

Deisseroth, K. (2015). Optogenetics: 10 years of microbial opsins in neuroscience. Nat. Neurosci. 18: 1213–1225.

Deiters, A., Groff, D., Ryu, Y., Xie, J., and Schultz, P.G. (2006). A genetically encoded photocaged tyrosine. Angew. Chem. Int. Ed. 45: 2728–2731.

Deiters, A. (2018). Special issue on optochemical and optogenetic control of cellular processes. Chembiochem 19: 1198–1200.

Dietler, J., Schubert, R., Krafft, T.G., Meiler, S., Kainrath, S., Richter, F., Schweimer, K., Weyand, M., Janovjak, H., and Moglich, A. (2021).

A light-oxygen-voltage receptor integrates light and temperature. J. Mol. Biol. 433, https://doi.org/10.1016/j.jmb.2021.167107.

Di Ventura, B. and Mootz, H.D. (2019). Switchable inteins for conditional protein splicing. Biol. Chem. 400: 467–475.

Dokić, J., Gothe, M., Wirth, J., Peters, M.V., Schwarz, J., Hecht, S., and Saalfrank, P. (2009). Quantum chemical investigation of thermal cis-to-trans isomerization of azobenzene derivatives: substituent effects, solvent effects, and comparison to experimental data. J. Phys. Chem. A 113: 6763–6773.

Donthamsetti, P., Konrad, D.B., Hetzler, B., Fu, Z., Trauner, D., and Isacoff, E.Y. (2021). Selective photoswitchable allosteric agonist of a G protein-coupled receptor. J. Am. Chem. Soc. 143: 8951–8956.

Donthamsetti, P.C., Winter, N., Schönberger, M., Levitz, J., Stanley, C., Javitch, J.A., Isacoff, E.Y., and Trauner, D. (2017). Optical control of dopamine receptors using a photoswitchable tethered inverse agonist. J. Am. Chem. Soc. 139: 18522–18535.

Douangamath, A., Walker, M., Beismann-Driemeyer, S., Vega-Fernandez, M., Sterner, R., and Wilmanns, M. (2002). Structural evidence for ammonia tunneling across the $(\beta\alpha)_8$ barrel of the imidazole glycerol phosphate synthase bienzyme complex. Structure 10: 185–193.

Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbelik, M., Moan, J., and Peng, Q. (1998). Photodynamic therapy. J. Natl. Cancer Inst. 90: 889–905.

Dowbaj, A.M., Jenkins, R.P., Williamson, D., Heddleston, J.M., Ciccarelli, A., Fallesen, T., Hahn, K.M., O'Dea, R.D., King, J.R., Montagner, M., et al. (2021). An optogenetic method for interrogating YAP1 and TAZ nuclear–cytoplasmic shuttling. J. Cell Sci. 134: jcs253484.

Duan, L., Hope, J., Ong, Q., Lou, H.-Y., Kim, N., McCarthy, C., Acero, V., Lin, M.Z., and Cui, B. (2017). Understanding CRY2 interactions for optical control of intracellular signaling. Nat. Commun. 8: 547.

Duebel, J., Marazova, K., and Sahel, J.-A. (2015). Optogenetics. Curr. Opin. Ophthalmol. 26: 226–232.

Dunkel, P. and Ilaš, J. (2021). Targeted cancer therapy using compounds activated by light. Cancers 13: 3237.

Edwards, I.R. and Aronson, J.K. (2000). Adverse drug reactions. Definitions, diagnosis, and management. Lancet 356: 1255–1259.

Edwards, W.F., Young, D.D., and Deiters, A. (2009). Light-activated Cre recombinase as a tool for the spatial and temporal control of gene function in mammalian cells. ACS Chem. Biol. 4: 441–445.

Edwards, W.R., Busse, K., Allemann, R.K., and Jones, D.D. (2008). Linking the functions of unrelated proteins using a novel directed evolution domain insertion method. Nucleic Acids Res. 36: e78.

Ellis-Davies, G.C.R. (2007). Caged compounds: photorelease technology for control of cellular chemistry and physiology. Nat. Methods 4: 619–628.

Engelke, H., Chou, C., Uprety, R., Jess, P., and Deiters, A. (2014). Control of protein function through optochemical translocation. ACS Synth. Biol. 3: 731–736.

Ernst, R.J., Krogager, T.P., Maywood, E.S., Zanchi, R., Beránek, V., Elliott, T.S., Barry, N.P., Hastings, M.H., and Chin, J.W. (2016). Genetic code expansion in the mouse brain. Nat. Chem. Biol. 12: 776–778.

Farrants, H., Gutzeit, V.A., Acosta-Ruiz, A., Trauner, D., Johnsson, K., Levitz, J., and Broichhagen, J. (2018). SNAP-tagged nanobodies enable reversible optical control of a G protein-coupled receptor via a remotely tethered photoswitchable ligand. ACS Chem. Biol. 13: 2682–2688.

Forber, C.L., Kelusky, E.C., Bunce, N.J., and Zerner, M.C. (1985). Electronic spectra of *cis*- and *trans*-azobenzenes: consequences of *ortho* substitution. J. Am. Chem. Soc. 107: 5884–5890.

Fukuda, N., Matsuda, T., and Nagai, T. (2014). Optical control of the Ca^{2+} concentration in a live specimen with a genetically encoded Ca^{2+}-releasing molecular tool. ACS Chem. Biol. 9: 1197–1203.

Furter, R. (1998). Expansion of the genetic code. Site-directed *p*-fluoro-phenylalanine incorporation in *Escherichia coli*. Protein Sci. 7: 419–426.

Gagliardi, L., Orlandi, G., Bernardi, F., Cembran, A., and Garavelli, M. (2004). A theoretical study of the lowest electronic states of azobenzene. The role of torsion coordinate in the *cis-trans* photoisomerization. Theor. Chem. Acc. 111: 363–372.

Gao, L., Meiring, J.C., Kraus, Y., Wranik, M., Weinert, T., Pritzl, S.D., Bingham, R., Ntouliou, E., Jansen, K.I., Olieric, N., et al. (2021). A robust, GFP-orthogonal photoswitchable inhibitor sacaffold extends optical control over the microtubule cytoskeleton. Cell Chem. Biol. 28: 228–241.e6.

Garcia-Amorós, J., Castro, R., M.C., Coelho, P., Raposo, M., M.M., and Velasco, D. (2016). Fastest non-ionic azo dyes and transfer of their thermal isomerisation kinetics into liquid-crystalline materials. Chem. Commun. 52: 5132–5135.

Gasser, C., Taiber, S., Yeh, C.-M., Wittig, C.H., Hegemann, P., Ryu, S., Wunder, F., and Möglich, A. (2014). Engineering of a red-light-activated human cAMP/cGMP-specific phosphodiesterase. Proc. Natl. Acad. Sci. U.S.A. 111: 8803–8808.

Gaudana, R., Ananthula, H.K., Parenky, A., and Mitra, A.K. (2010). Ocular drug delivery. AAPS J. 12: 348–360.

Gautier, A., Nguyen, D.P., Lusic, H., An, W., Deiters, A., and Chin, J.W. (2010). Genetically encoded photocontrol of protein localization in mammalian cells. J. Am. Chem. Soc. 132: 4086–4088.

Gautier, A., Deiters, A., and Chin, J.W. (2011). Light-activated kinases enable temporal dissection of signaling networks in living cells. J. Am. Chem. Soc. 133: 2124–2127.

Gautier, A., Gauron, C., Volovitch, M., Bensimon, D., Jullien, L., and Vriz, S. (2014). How to control proteins with light in living systems. Nat. Chem. Biol. 10: 533–541.

Gehrig, S., Macpherson, J.A., Driscoll, P.C., Symon, A., Martin, S.R., MacRae, J.I., Kleinjung, J., Fraternali, F., and Anastasiou, D. (2017). An engineered photoswitchable mammalian pyruvate kinase. FEBS J. 284: 2955–2980.

Gerlach, T., Nugroho, D.L., and Rother, D. (2021). The effect of visible light on the catalytic activity of PLP-dependent enzymes. ChemCatChem 13: 2398–2406.

Gil, A.A., Carrasco-López, C., Zhu, L., Zhao, E.M., Ravindran, P.T., Wilson, M.Z., Goglia, A.G., Avalos, J.L., and Toettcher, J.E. (2020). Optogenetic control of protein binding using light-switchable nanobodies. Nat. Commun. 11: 4044.

Givens, R.S., Rubina, M., and Wirz, J. (2012). Applications of p-hydroxyphenacyl (*p*HP) and coumarin-4-ylmethyl photoremovable protecting groups. Photochem. Photobiol. Sci. 11: 472–488.

González, S.S., Ad, O., Shah, B., Zhang, Z., Zhang, X., Chatterjee, A., and Schepartz, A. (2021). Genetic code expansion in the engineered organism Vmax X2. High yield and exceptional fidelity. ACS Cent. Sci. 7: 1500–1507.

Goodsell, D.S. and Olson, A.J. (2000). Structural symmetry and protein function. Annu. Rev. Biophys. Biomol. Struct. 29: 105–153.

Grzybowski, A. and Pietrzak, K. (2012). From patient to discoverer—Niels Ryberg Finsen (1860–1904)—the founder of phototherapy in dermatology. Clin. Dermatol. 30: 451–455.

Guo, J., Melançon, C.E., III, Lee, H.S., Groff, D., and Schultz, P.G. (2009). Evolution of amber suppressor tRNAs for efficient bacterial production of proteins containing nonnatural amino acids. Angew. Chem. Int. Ed. 48: 9148–9151.

Gutruf, P. and Rogers, J.A. (2018). Implantable, wireless device platforms for neuroscience research. Curr. Opin. Neurobiol. 50: 42–49.

Hans, F., Sligar Stephen, G., and Wolynes Peter, G. (1991). The energy landscapes and motions of proteins. Science 254: 1598–1603.

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. Cell 100: 57–70.

Han, S., Yang, A., Lee, S., Lee, H.-W., Park, C.B., and Park, H.-S. (2017). Expanding the genetic code of Mus musculus. Nat. Commun. 8: 14568.

Hansel, T.T., Kropshofer, H., Singer, T., Mitchell, J.A., and George, A.J.T. (2010). The safety and side effects of monoclonal antibodies. Nat. Rev. Drug Discov. 9: 325–338.

Harper, S.M., Neil, L.C., and Gardner, K.H. (2003). Structural basis of a phototropin light switch. Science 301: 1541–1544.

Harz, H. and Hegemann, P. (1991). Rhodopsin-regulated calcium currents in *Chlamydomonas*. Nature 351: 489–491.

Hattori, M., Haga, S., Takakura, H., Ozaki, M., and Ozawa, T. (2013). Sustained accurate recording of intracellular acidification in living tissues with a photo-controllable bioluminescent protein. Proc. Natl. Acad. Sci. U.S.A. 110: 9332–9337.

Hauf, M., Richter, F., Schneider, T., Faidt, T., Martins, B.M., Baumann, T., Durkin, P., Dobbek, H., Jacobs, K., Möglich, A., et al. (2017). Photoactivatable mussel-based underwater adhesive proteins by an expanded genetic code. Chembiochem 18: 1819–1823.

Hegemann, P., Gärtner, W., and Uhl, R. (1991). All-*trans* retinal constitutes the functional chromophore in *Chlamydomonas* rhodopsin. Biophys. J. 60: 1477–1489.

Hegemann, P. (2008). Algal sensory photoreceptors. Annu. Rev. Plant Biol. 59: 167–189.

Heining, M., Sutor, A., Stute, S.C., Lindenberger, C.P., and Buchholz, R. (2015). Internal illumination of photobioreactors via wireless light emitters. A proof of concept. J. Appl. Phycol. 27: 59–66.

Heining, M. and Buchholz, R. (2015). Photobioreactors with internal illumination – a survey and comparison. Biotechnol. J. 10: 1131–1137.

Heintze, L., Schmidt, D., Rodat, T., Witt, L., Ewert, J., Kriegs, M., Herges, R., and Peifer, C. (2020). Photoswitchable azo- and diazocine-functionalized derivatives of the VEGFR-2 inhibitor axitinib. Int. J. Mol. Sci. 21: 8961.

He, L., Tan, P., Huang, Y., and Zhou, Y. (2021). Design of smart antibody mimetics with photosensitive switches. Adv. Biol. 5: 2000541.

Hemphill, J., Chou, C., Chin, J.W., and Deiters, A. (2013). Genetically encoded light-activated transcription for spatiotemporal control of gene expression and gene silencing in mammalian cells. J. Am. Chem. Soc. 135: 13433–13439.

Hemphill, J., Borchardt, E.K., Brown, K., Asokan, A., and Deiters, A. (2015). Optical control of CRISPR/Cas9 gene editing. J. Am. Chem. Soc. 137: 5642–5645.

Hermanson, G.T. (Ed.). (2013). *Bioconjugate techniques*, 3rd ed. Boston: Academic Press.

Hoesl, M.G. and Budisa, N. (2012). Recent advances in genetic code engineering in *Escherichia coli*. Curr. Opin. Biotechnol. 23: 751–757.

Hoffmann, M.D., Mathony, J., Upmeier zu Belzen, J., Harteveld, Z., Aschenbrenner, S., Stengl, C., Grimm, D., Correia, B.E., Eils, R., and Niopek, D. (2021). Optogenetic control of *Neisseria meningitidis* Cas9 genome editing using an engineered, light-switchable anti-CRISPR protein. Nucleic Acids Res. 49: e29.

Hongdusit, A., Zwart, P.H., Sankaran, B., and Fox, J.M. (2020). Minimally disruptive optical control of protein tyrosine phosphatase 1B. Nat. Commun. 11: 788.

Hongdusit, A., Liechty, E.T., and Fox, J.M. (2022). Analysis of three architectures for controlling PTP1B with light. ACS Synth. Biol. 11: 61–68.

Hoorens, M.W. and Szymanski, W. (2018). Reversible, spatial and temporal control over protein activity using light. Trends Biochem. Sci. 43: 567–575.

Hoppmann, C., Lacey, V.K., Louie, G.V., Wei, J., Noel, J.P., and Wang, L. (2014). Genetically encoding photoswitchable click amino acids in *Escherichia coli* and mammalian cells. Angew. Chem. Int. Ed. 53: 3932–3936.

Hoppmann, C., Maslennikov, I., Choe, S., and Wang, L. (2015). In situ formation of an azo bridge on proteins controllable by visible light. J. Am. Chem. Soc. 137: 11218–11221.

Hörner, M., Eble, J., Yousefi, O.S., Schwarz, J., Warscheid, B., Weber, W., and Schamel, W.W.A. (2019a). Light-controlled affinity purification of protein complexes exemplified by the resting ZAP70 interactome. Front. Immunol. 10: 226.

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. (2019b). Phytochrome-based extracellular matrix with reversibly tunable mechanical properties. Adv. Mater. 31: 1806727.

Huala, E., Oeller, P.W., Liscum, E., Han, I.-S., Larsen, E., and Briggs, W.R. (1997). Arabidopsis NPH1. A protein kinase with a putative redox-sensing domain. Science 278: 2120–2123.

Hüll, K., Morstein, J., and Trauner, D. (2018). *In vivo* photopharmacology. Chem. Rev. 118: 10710–10747.

Hutchings, M.I., Truman, A.W., and Wilkinson, B. (2019). Antibiotics: past, present and future. Curr. Opin. Microbiol. 51: 72–80.

Hwang, D.S., Gim, Y., Yoo, H.J., and Cha, H.J. (2007). Practical recombinant hybrid mussel bioadhesive fp-151. Biomaterials 28: 3560–3568.

Ibba, M. and Söll, D. (2004). Aminoacyl-tRNAs. Setting the limits of the genetic code. Genes Dev. 18: 731–738.

Il'ichev, Y.V. and Wirz, J. (2000). Rearrangements of 2-nitrobenzyl compounds. 1. Potential energy surface of 2-nitrotoluene and its isomers explored with ab initio and density functional theory methods. J. Phys. Chem. A 104: 7856–7870.

Ingram, J.R., Schmidt, F.I., and Ploegh, H.L. (2018). Exploiting nanobodies' singular traits. Annu. Rev. Immunol. 36: 695–715.

Israeli, B., Strugach, D.S., Gelkop, S., Weber, S., Gozlan, D.S., and Amiram, M. (2021). Genetically encoding light-responsive protein-polymers using translation machinery for the multi-site incorporation of photo-switchable unnatural amino acids. Adv. Funct. Mater. 31: 2011276.

Jedlitzke, B., Yilmaz, Z., Dörner, W., and Mootz, H.D. (2020). Photobodies: light-activatable single-domain antibody fragments. Angew. Chem. Int. Ed. 59: 1506–1510.

Jiang, J., Cui, H., and Rahmouni, K. (2017). Optogenetics and pharmacogenetics: principles and applications. Am. J. Physiol. 313: R633–R645.

John, A.A., Ramil, C.P., Tian, Y., Cheng, G., and Lin, Q. (2015). Synthesis and site-specific incorporation of red-shifted azobenzene amino acids into proteins. Org. Lett. 17: 6258–6261.

Kaberniuk, A.A., Baloban, M., Monakhov, M.V., Shcherbakova, D.M., and Verkhusha, V.V. (2021). Single-component near-infrared optogenetic systems for gene transcription regulation. Nat. Commun. 12: 3859.

Kalka, K., Merk, H., and Mukhtar, H. (2000). Photodynamic therapy in dermatology. J. Am. Acad. Dermatol. 42: 389–413.

Kang, J.-Y., Kawaguchi, D., Coin, I., Xiang, Z., O'Leary, D.D., Slesinger, P.A., and Wang, L. (2013). *In vivo* expression of a light-activatable potassium channel using unnatural amino acids. Neuron 80: 358–370.

Kar, G., Keskin, O., Gursoy, A., and Nussinov, R. (2010). Allostery and population shift in drug discovery. Curr. Opin. Pharmacol. 10: 715–722.

Klán, P., Šolomek, T., Bochet, C.G., Blanc, A., Givens, R., Rubina, M., Popik, V., Kostikov, A., and Wirz, J. (2013). Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy. Chem. Rev. 113: 119–191.

Klippenstein, V., Hoppmann, C., Ye, S., Wang, L., and Paoletti, P. (2017). Optocontrol of glutamate receptor activity by single side-chain photoisomerization. eLife 6: e25808.

Kneuttinger, A.C., Kashiwazaki, G., Prill, S., Heil, K., Müller, M., and Carell, T. (2014). Formation and direct repair of UV-induced dimeric DNA pyrimidine lesions. Photochem. Photobiol. 90: 1–14.

Kneuttinger, A.C., Winter, M., Simeth, N.A., Heyn, K., Merkl, R., König, B., and Sterner, R. (2018). Artificial light regulation of an allosteric bienzyme complex by a photosensitive ligand. Chembiochem 19: 1750–1757.

Kneuttinger, A.C., Straub, K., Bittner, P., Simeth, N.A., Bruckmann, A., Busch, F., Rajendran, C., Hupfeld, E., Wysocki, V.H., Horinek, D., et al. (2019a). Light regulation of enzyme allostery through photo-responsive unnatural amino acids. Cell Chem. Biol. 26: 1501–1514.e9.

Kneuttinger, A.C., Zwisele, S., Straub, K., Bruckmann, A., Busch, F., Kinateder, T., Gaim, B., Wysocki, V.H., Merkl, R., and Sterner, R. (2019b). Light-regulation of tryptophan synthase by combining protein design and enzymology. Int. J. Mol. Sci. 20: 5106.

Kneuttinger, A.C., Rajendran, C., Simeth, N.A., Bruckmann, A., König, B., and Sterner, R. (2020). Significance of the protein interface configuration for allostery in imidazole glycerol phosphate synthase. Biochemistry 59: 2729–2742.

Knie, C., Utecht, M., Zhao, F., Kulla, H., Kovalenko, S., Brouwer, A.M., Saalfrank, P., Hecht, S., and Bléger, D. (2014). *Ortho*-fluoroazobenzenes. Visible light switches with very long-lived *Z* isomers. Chem. Eur. J. 20: 16492–16501.

Kolarski, D., Miller, S., Oshima, T., Nagai, Y., Aoki, Y., Kobauri, P., Srivastava, A., Sugiyama, A., Amaike, K., Sato, A., et al. (2021a). Photopharmacological manipulation of mammalian CRY1 for regulation of the circadian clock. J. Am. Chem. Soc. 143: 2078–2087.

Kolarski, D., Sugiyama, A., Rodat, T., Schulte, A., Peifer, C., Itami, K., Hirota, T., Feringa, B.L., and Szymanski, W. (2021b). Reductive stability evaluation of 6-azopurine photoswitches for the regulation of CKIα activity and circadian rhythms. Org. Biomol. Chem. 19: 2312–2321.

Komarov, I.V., Afonin, S., Babii, O., Schober, T., and Ulrich, A.S. (2018). Efficiently photocontrollable or not? Biological activity of photoisomerizable diarylethenes. Chem. Eur J. 24: 11245–11254.

Komeili, A. and O'Shea, E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science 284: 977–980.

Konrad, D.B., Frank, J.A., and Trauner, D. (2016). Synthesis of redshifted azobenzene photoswitches by late-stage functionalization. Chem. Eur. J. 22: 4364–4368.

Koshland, D.E., Jr. (1995). The key-lock theory and the induced fit theory. Angew. Chem. Int. Ed. 33: 2375–2378.

Ko, W., Kumar, R., Kim, S., and Lee, H.S. (2019). Construction of bacterial cells with an active transport system for unnatural amino acids. ACS Synth. Biol. 8: 1195–1203.

Kramer, M.M., Mühlhäuser, W.W.D., Weber, W., and Radziwill, G. (2021a). Multichromatic control of signaling pathways in mammalian cells. Adv. Biol. 5: 2000196.

Kramer, M.M., Lataster, L., Weber, W., and Radziwill, G. (2021b). Optogenetic approaches for the spatiotemporal control of signal transduction pathways. Int. J. Mol. Sci. 22: 5300.

Krauss, U., Lee, J., Benkovic, S.J., and Jaeger, K.-E. (2010). LOVely enzymes – towards engineering light-controllable biocatalysts. Microb. Biotechnol. 3: 15–23.

Krishnamurthy, V.M., Semetey, V., Bracher, P.J., Shen, N., and Whitesides, G.M. (2007). Dependence of effective molarity on linker length for an intramolecular protein–ligand system. J. Am. Chem. Soc. 129: 1312–1320.

Kumita, J.R., Smart, O.S., and Woolley, G.A. (2000). Photo-control of helix content in a short peptide. Proc. Natl. Acad. Sci. U.S.A. 97: 3803–3808.

Kropp, C., Bruckmann, A., and Babinger, P. (2022). Controlling enzymatic activity by modulating the oligomerization state via chemical rescue and optical control. ChemBioChem, 23: e202100490.

Kumita, J.R., Flint, D.G., Smart, O.S., and Woolley, G. (2002). Photo-control of peptide helix content by an azobenzene cross-linker: steric interactions with underlying residues are not critical. Protein Eng. Des. Sel. 15: 561–569.

Kuznetsov, G., Goodman, D.B., Filsinger, G.T., Landon, M., Rohland, N., Aach, J., Lajoie, M.J., and Church, G.M. (2017). Optimizing complex phenotypes through model-guided multiplex genome engineering. Genome Biol. 18: 100.

Kwok, Y. and Wong, J.T.-F. (1980). Evolutionary relationship between *Halobacterium cutirubrum* and eukaryotes determined by use of aminoacyl-tRNA synthetases as phylogenetic probes. Can. J. Biochem. 58: 213–218.

Lachmann, D., Lahmy, R., and König, B. (2019). Fulgimides as light-activated tools in biological investigations. Eur. J. Org Chem. 2019: 5018–5024.

Lajoie, M.J., Rovner, A.J., Goodman, D.B., Aerni, H.-R., Haimovich, A.D., Kuznetsov, G., Mercer, J.A., Wang, H.H., Carr, P.A., Mosberg, J.A., et al. (2013). Genomically recoded organisms expand biological functions. Science 342: 357–360.

Larson, A.S. and Hergenrother, P.J. (2014). Light activation of *Staphylococcus aureus t*oxin $YoeB_{Sa1}$ reveals guanosine-specific endoribonuclease activity. Biochemistry 53: 188–201.

Lauxen, A.I., Kobauri, P., Wegener, M., Hansen, M.J., Galenkamp, N.S., Maglia, G., Szymanski, W., Feringa, B.L., and Kuipers, O.P. (2021).

Mechanism of resistance development in *E. coli* against TCAT, a trimethoprim-based photoswitchable antibiotic. Pharmaceuticals 14: 392.

Lee, J., Natarajan, M., Nashine, V.C., Socolich, M., Vo, T., Russ, W.P., Benkovic, S.J., and Ranganathan, R. (2008). Surface sites for engineering allosteric control in proteins. Science 322: 438–442.

Leippe, P., Koehler Leman, J., and Trauner, D. (2017). Specificity and speed: Tethered photopharmacology. Biochemistry 56: 5214–5220.

Leistner, A.-L., Kirchner, S., Karcher, J., Bantle, T., Schulte, M.L., Gödtel, P., Fengler, C., and Pianowski, Z.L. (2021). Fluorinated azobenzenes switchable with red light. Chem. Eur J. 27: 8094–8099.

Lemke, E.A., Summerer, D., Geierstanger, B.H., Brittain, S.M., and Schultz, P.G. (2007). Control of protein phosphorylation with a genetically encoded photocaged amino acid. Nat. Chem. Biol. 3: 769–772.

Lenci, F., Ghetti, F., Colombetti, G., Häder, D.P., and Song, P.S. (2012). *Biophysics of photoreceptors and photomovements in microorganisms*. Springer US, New York City.

Lerch, M.M., Hansen, M.J., van Dam, G.M., Szymanski, W., and Feringa, B.L. (2016). Emerging targets in photopharmacology. Angew. Chem. Int. Ed. 55: 10978–10999.

Liao, Z., Gingras, A.R., Lagarrigue, F., Ginsberg, M.H., and Shattil, S.J. (2021). Optogenetics-based localization of talin to the plasma membrane promotes activation of β3 integrins. J. Biol. Chem. 296: 100675.

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. (2018). Small near-infrared photochromic protein for photoacoustic multi-contrast imaging and detection of protein interactions *in vivo*. Nat. Commun. 9: 2734.

Lim, C.H., Kaur, P., Teo, E., Lam, V.Y.M., Zhu, F., Kibat, C., Gruber, J., Mathuru, A.S., Tolwinski, N.S., Kaeberlein, M., et al. (2020). Application of optogenetic Amyloid-β distinguishes between metabolic and physical damages in neurodegeneration. eLife 9: e52589.

Lin, W.-C., Tsai, M.-C., Rajappa, R., and Kramer, R.H. (2018). Design of a highly bistable photoswitchable tethered ligand for rapid and sustained manipulation of neurotransmission. J. Am. Chem. Soc. 140: 7445–7448.

Liu, C.C. and Schultz, P.G. (2010). Adding new chemistries to the genetic code. Annu. Rev. Biochem. 79: 413–444.

Liu, D.R. and Schultz, P.G. (1999). Progress toward the evolution of an organism with an expanded genetic code. Proc. Natl. Acad. Sci. U.S.A. 96: 4780–4785.

Liu, J., Fu, T.-M., Cheng, Z., Hong, G., Zhou, T., Jin, L., Duvvuri, M., Jiang, Z., Kruskal, P., Xie, C., et al. (2015). Syringe-injectable electronics. Nat. Nanotechnol. 10: 629–636.

Liu, J. and Nussinov, R. (2016). Allostery. An overview of its history, concepts, methods, and applications. PLoS Comput. Biol. 12: e1004966.

Liu, J., Hemphill, J., Samanta, S., Tsang, M., and Deiters, A. (2017a). Genetic code expansion in zebrafish embryos and its application to optical control of cell signaling. J. Am. Chem. Soc. 139: 9100–9103.

Liu, Q. and Tucker, C.L. (2017). Engineering genetically-encoded tools for optogenetic control of protein activity. Curr. Opin. Chem. Biol. 40: 17–23.

Liu, Y., Gao, X., Wei, D., and Ren, Y. (2017b). Reversible photocontrol of lipase activity by incorporating a photoswitch into the lid domain. ChemPhotoChem 1: 393–396.

Lórenz-Fonfría, V.A. and Heberle, J. (2014). Channelrhodopsin unchained: structure and mechanism of a light-gated cation channel. Biochim. Biophys. Acta Bioenerg. 1837: 626–642.

Losi, A., Gardner, K.H., and Möglich, A. (2018). Blue-light receptors for optogenetics. Chem. Rev. 118: 10659–10709.

Luo, J., Uprety, R., Naro, Y., Chou, C., Nguyen, D.P., Chin, J.W., and Deiters, A. (2014). Genetically encoded optochemical probes for simultaneous fluorescence reporting and light activation of protein function with two-photon excitation. J. Am. Chem. Soc. 136: 15551–15558.

Luo, J., Torres-Kolbus, J., Liu, J., and Deiters, A. (2017a). Genetic encoding of photocaged tyrosines with improved light-activation properties for the optical control of protease function. Chembiochem 18: 1442–1447.

Luo, J., Kong, M., Liu, L., Samanta, S., van Houten, B., and Deiters, A. (2017b). Optical control of DNA helicase function through genetic code expansion. Chembiochem 18: 466–469.

Luo, J., Samanta, S., Convertino, M., Dokholyan, N.V., and Deiters, A. (2018). Reversible and tunable photoswitching of protein function through genetic encoding of azobenzene amino acids in mammalian cells. Chembiochem 19: 2178–2185.

Luca, R., Jean-Jacques, F., Catherine, C., Davide, F., Andrew, O., Eirini, K., Ralf, B., Eberhard, S., Nagy, F., Jenkins Gareth, I., et al. (2011). Perception of UV-B by the *Arabidopsis* UVR8 protein. Science 332: 103–106.

Maleeva, G., Nin-Hill, A., Rustler, K., Petukhova, E., Ponomareva, D., Mukhametova, E., Gomila, A.M.J., Wutz, D., Alfonso-Prieto, M., König, B., et al. (2021). Subunit-specific photocontrol of glycine receptors by azobenzene-nitrazepam photoswitcher. eNeuro 8: 0294–20.

Mansouri, M., Hussherr, M.-D., Strittmatter, T., Buchmann, P., Xue, S., Camenisch, G., and Fussenegger, M. (2021). Smart-watch-programmed green-light-operated percutaneous control of therapeutic transgenes. Nat. Commun. 12: 3388.

Marblestone, A., Zamft, B., Maguire, Y., Shapiro, M., Cybulski, T., Glaser, J., Amodei, D., Stranges, P.B., Kalhor, R., Dalrymple, D., et al. (2013). Physical principles for scalable neural recording. Front. Comput. Neurosci. 7: 137.

Mathony, J. and Niopek, D. (2021). Enlightening allostery. Designing switchable proteins by photoreceptor fusion. Adv. Biol. 5: 2000181.

Mayer, G. and Heckel, A. (2006). Biologically active molecules with a "light switch". Angew. Chem. Int. Ed. 45: 4900–4921.

Melançon, C.E. and Schultz, P.G. (2009). One plasmid selection system for the rapid evolution of aminoacyl-tRNA synthetases. Bioorg. Med. Chem. Lett 19: 3845–3847.

Mentel, M., Laketa, V., Subramanian, D., Gillandt, H., and Schultz, C. (2011). Photoactivatable and cell-membrane-permeable phosphatidylinositol 3,4,5-trisphosphate. Angew. Chem. Int. Ed. 50: 3811–3814.

Meyskens, F.L., Jr., Farmer, P., and Fruehauf, J.P. (2001). Redox regulation in human melanocytes and melanoma. Pigm. Cell Res. 14: 148–154.

Miller, J.C., Silverman, S.K., England, P.M., Dougherty, D.A., and Lester, H.A. (1998). Flash decaging of tyrosine sidechains in an ion channel. Neuron 20: 619–624.

Möglich, A. and Moffat, K. (2007). Structural basis for light-dependent signaling in the dimeric LOV domain of the photosensor YtvA. J. Mol. Biol. 373: 112–126.

Möglich, A., Ayers, R.A., and Moffat, K. (2009). Design and signaling mechanism of light-regulated histidine kinases. J. Mol. Biol. 385: 1433–1444.

Möglich, A. and Moffat, K. (2010). Engineered photoreceptors as novel optogenetic tools. Photochem. Photobiol. Sci. 9: 1286–1300.

Möglich, A., Yang, X., Ayers, R.A., and Moffat, K. (2010). Structure and function of plant photoreceptors. Annu. Rev. Plant Biol. 61: 21–47.

Montgomery, K.L., Yeh, A.J., Ho, J.S., Tsao, V., Mohan Iyer, S., Grosenick, L., Ferenczi, E.A., Tanabe, Y., Deisseroth, K., Delp, S.L., et al. (2015). Wirelessly powered, fully internal optogenetics for brain, spinal and peripheral circuits in mice. Nat. Methods 12: 969–974.

Mony, L., Zhu, S., Carvalho, S., and Paoletti, P. (2011). Molecular basis of positive allosteric modulation of GluN2B NMDA receptors by polyamines. EMBO J. 30: 3134–3146.

Morstein, J. and Trauner, D. (2019). New players in phototherapy: photopharmacology and bio-integrated optoelectronics. Curr. Opin. Chem. Biol. 50: 145–151.

Morville, C., Chaud, J., Bolze, F., and Specht, A. (2021). Photolytical reactions for light induced biological effectors release: on the road to the phototherapeutic window. J. Inclusion Phenom. Macrocycl. Chem. 101: 291–304.

Mostad, A., Rømming, C., Hammarström, S., Lousberg, R.J.J.C., and Weiss, U. (1971). A refinement of the crystal structure of *cis*-azobenzene. Acta Chem. Scand. 25: 3561–3568.

Mowery, P.C. and Stoeckenius, W. (1981). Photoisomerization of the chromophore in bacteriorhodopsin during the proton pumping photocycle. Biochemistry 20: 2302–2306.

Mukai, T., Hoshi, H., Ohtake, K., Takahashi, M., Yamaguchi, A., Hayashi, A., Yokoyama, S., and Sakamoto, K. (2015). Highly reproductive *Escherichia coli* cells with no specific assignment to the UAG codon. Sci. Rep. 5: 9699.

Müller, K., Engesser, R., Timmer, J., Nagy, F., Zurbriggen, M.D., and Weber, W. (2013). Synthesis of phycocyanobilin in mammalian cells. Chem. Commun. 49: 8970–8972.

Müller, T.D., Finan, B., Bloom, S.R., D'Alessio, D., Drucker, D.J., Flatt, P.R., Fritsche, A., Gribble, F., Grill, H.J., Habener, J.F., et al. (2019). Glucagon-like peptide 1 (GLP-1). Mol. Metabol. 30: 72–130.

Muranaka, N., Hohsaka, T., and Sisido, M. (2002). Photoswitching of peroxidase activity by position-specific incorporation of a photoisomerizable non-natural amino acid into horseradish peroxidase. FEBS Lett 510: 10–12.

Nadler, D.C., Morgan, S.-A., Flamholz, A., Kortright, K.E., and Savage, D.F. (2016). Rapid construction of metabolite biosensors using domain-insertion profiling. Nat. Commun. 7: 12266.

Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A.M., Bamberg, E., and Hegemann, P. (2002). Channelrhodopsin-1. A light-gated proton channel in green algae. Science 296: 2395–2398.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc. Natl. Acad. Sci. U.S.A. 100: 13940–13945.

Nakayama, K., Endo, M., and Majima, T. (2004). Photochemical regulation of the activity of an endonuclease *Bam*HI using an azobenzene moiety incorporated site-selectively into the dimer interface. Chem. Commun.: 2386–2387, https://doi.org/10.1039/b409844g.

Nakayama, K., Endo, M., and Majima, T. (2005). A hydrophilic azobenzene-bearing amino acid for photochemical control of a restriction enzyme *Bam*HI. Bioconjugate Chem. 16: 1360–1366.

Narayan, O.P., Mu, X., Hasturk, O., and Kaplan, D.L. (2021). Dynamically tunable light responsive silk-elastin-like proteins. Acta Biomater. 121: 214–223.

Naro, Y., Darrah, K., and Deiters, A. (2020). Optical control of small molecule-induced protein degradation. J. Am. Chem. Soc. 142: 2193–2197.

Nguyen, D.P., Mahesh, M., Elsässer, S.J., Hancock, S.M., Uttamapinant, C., and Chin, J.W. (2014). Genetic encoding of photocaged cysteine allows photoactivation of TEV protease in live mammalian cells. J. Am. Chem. Soc. 136: 2240–2243.

Nguyen, T.-A., Cigler, M., and Lang, K. (2018). Expanding the genetic code to study protein–protein interactions. Angew. Chem. Int. Ed. 57: 14350–14361.

Niopek, D., Wehler, P., Roensch, J., Eils, R., and Di Ventura, B. (2016). Optogenetic control of nuclear protein export. Nat. Commun. 7: 10624.

Nödling, A.R., Spear, L.A., Williams, T.L., Luk, L.Y., and Tsai, Y.-H. (2019). Using genetically incorporated unnatural amino acids to control protein functions in mammalian cells. Essays Biochem. 63: 237–266.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. (1989). A general method for site-specific incorporation of unnatural amino acids into proteins. Science 244: 182–188.

Normanno, N., de Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., Carotenuto, A., Feo, G. de, Caponigro, F., and Salomon, D.S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 366: 2–16.

O'Donoghue, P., Ling, J., Wang, Y.-S., and Söll, D. (2013). Upgrading protein synthesis for synthetic biology. Nat. Chem. Biol. 9: 594–598.

Oesterhelt, D. and Stoeckenius, W. (1971). Rhodopsin-like protein from the purple membrane of halobacterium halobium. Nat. New Biol. 233: 149–152.

Oh, T.-J., Fan, H., Skeeters, S.S., and Zhang, K. (2021). Steering molecular activity with optogenetics. Recent advances and perspectives. Adv. Biol. 5: 2000180.

Padmanabhan, S., Pérez-Castaño, R., and Elías-Arnanz, M. (2019). B12-based photoreceptors: from structure and function to applications in optogenetics and synthetic biology. Curr. Opin. Struct. Biol. 57: 47–55.

Park, H., Bradley, P., Greisen, P., Liu, Y., Mulligan, V.K., Kim, D.E., Baker, D., and DiMaio, F. (2016). Simultaneous optimization of biomolecular energy functions on features from small molecules and macromolecules. J. Chem. Theor. Comput. 12: 6201–6212.

Park, S.I., Brenner, D.S., Shin, G., Morgan, C.D., Copits, B.A., Chung, H.U., Pullen, M.Y., Noh, K.N., Davidson, S., Oh, S.J., et al. (2015). Soft, stretchable, fully implantable miniaturized optoelectronic systems for wireless optogenetics. Nat. Biotechnol. 33: 1280–1286.

Patchornik, A., Amit, B., and Woodward, R.B. (1970). Photosensitive protecting groups. J. Am. Chem. Soc. 92: 6333–6335.

Patrick, O., and Zaida, L.-S. (2003). On the evolution of structure in aminoacyl-tRNA synthetases. Microbiol. Mol. Biol. Rev. 67: 550–573.

Petermayer, C. and Dube, H. (2018). Indigoid photoswitches: visible light responsive molecular tools. Acc. Chem. Res. 51: 1153–1163.

Petit, C.M., Zhang, J., Sapienza, P.J., Fuentes, E.J., and Lee, A.L. (2009). Hidden dynamic allostery in a PDZ domain. Proc. Natl. Acad. Sci. U.S.A. 106: 18249–18254.

Polland, H.-J., Franz, M.A., Zinth, W., Kaiser, W., Kölling, E., and Oesterhelt, D. (1986). Early picosecond events in the photocycle of bacteriorhodopsin. Biophys. J. 49: 651–662.

Preston, G.W. and Wilson, A.J. (2013). Photo-induced covalent cross-linking for the analysis of biomolecular interactions. Chem. Soc. Rev. 42: 3289–3301.

Pudasaini, A., El-Arab, K.K., and Zoltowski, B.D. (2015). LOV-based optogenetic devices. Light-driven modules to impart photoregulated control of cellular signaling. Front. Mol. Biosci. 2: 18.

Qazi, R., Yeon Kim, C., Kang, I., Binazarov, D., McCall, J.G., and Jeong, J.-W. (2021). Implantable optofluidic systems for wireless *in vivo p*hotopharmacology. ChemPhotoChem 5: 96–105.

Raab, O. (1900). Über die Wirkung fluorescierender Stoffe auf Infusoria. Z. Biol. 39: 524.

Raja, J.M., Elsakr, C., Roman, S., Cave, B., Pour-Ghaz, I., Nanda, A., Maturana, M., and Khouzam, R.N. (2019). Apple Watch, wearables, and heart rhythm. Where do we stand? Ann. Transl. Med. 7: 417.

Rannversson, H., Andersen, J., Sørensen, L., Bang-Andersen, B., Park, M., Huber, T., Sakmar, T.P., and Strømgaard, K. (2016). Genetically encoded photocrosslinkers locate the high-affinity binding site of antidepressant drugs in the human serotonin transporter. Nat. Commun. 7: 11261.

Raquel, P., Pinheiro, D.P., Evangelista de Oliveira, F.C., Fávero Galvão, G., Alves Marques, L.G., Fonseca Vianna Lopez, R., Pessoa, C., and Eloy, J.O. (2021). Immunoconjugates for cancer targeting. A review of antibody-drug conjugates and antibody-functionalized nanoparticles. Curr. Med. Chem. 28: 2485–2520.

Reis, S.A., Ghosh, B., Hendricks, J.A., Szantai-Kis, D.M., Törk, L., Ross, K.N., Lamb, J., Read-Button, W., Zheng, B., Wang, H., et al. (2016). Light-controlled modulation of gene expression by chemical optoepigenetic probes. Nat. Chem. Biol. 12: 317–323.

Repina, N.A., Rosenbloom, A., Mukherjee, A., Schaffer, D.V., and Kane, R.S. (2017). At light speed: advances in optogenetic systems for regulating cell signaling and behavior. Annu. Rev. Chem. Biomol. Eng. 8: 13–39.

Reynolds, K.A., McLaughlin, R.N., and Ranganathan, R. (2011). Hot spots for allosteric regulation on protein surfaces. Cell 147: 1564–1575.

Ritter, J.M., Rang, H.P., and Dale, M.M. (2020). *Rang and Dale's pharmacology*. Edinburgh: Elsevier.

Robertson, W.E., Funke, L.F.H., La Torre, D. de, Fredens, J., Elliott, T.S., Spinck, M., Christova, Y., Cervettini, D., Böge, F.L., Liu, K.C., et al. (2021). Sense codon reassignment enables viral resistance and encoded polymer synthesis. Science 372: 1057–1062.

Rodríguez-Soacha, D.A., Fender, J., Ramírez, Y.A., Collado, J.A., Muñoz, E., Maitra, R., Sotriffer, C., Lorenz, K., and Decker, M. (2021). "Photo-rimonabant". Synthesis and biological evaluation of novel photoswitchable molecules derived from rimonabant lead to a highly selective and nanomolar "cis-on" CB1R antagonist. ACS Chem. Neurosci. 12: 1632–1647.

Romberg, L. and Levin, P.A. (2003). Assembly dynamics of the bacterial cell division protein FtsZ. Poised at the edge of stability. Annu. Rev. Microbiol. 57: 125–154.

Rosano, G.L., Morales, E.S., and Ceccarelli, E.A. (2019). New tools for recombinant protein production in *Escherichia coli*: a 5-year update. Protein Sci. 28: 1412–1422.

Rustler, K., Maleeva, G., Bregestovski, P., and König, B. (2019). Azologization of serotonin 5-HT3 receptor antagonists. Beilstein J. Org. Chem. 15: 780–788.

Ryu, M.-H., Kang, I.-H., Nelson, M.D., Jensen, T.M., Lyuksyutova, A.I., Siltberg-Liberles, J., Raizen, D.M., and Gomelsky, M. (2014). Engineering adenylate cyclases regulated by near-infrared window light. Proc. Natl. Acad. Sci. U.S.A. 111: 10167–10172.

Salomon, M., Eisenreich, W., Dürr, H., Schleicher, E., Knieb, E., Massey, V., Rüdiger, W., Müller, F., Bacher, A., and Richter, G. (2001). An optomechanical transducer in the blue light receptor phototropin from Avena sativa. Proc. Natl. Acad. Sci. U.S.A. 98: 12357–12361.

Santoro, S.W., Wang, L., Herberich, B., King, D.S., and Schultz, P.G. (2002). An efficient system for the evolution of aminoacyl-tRNA synthetase specificity. Nat. Biotechnol. 20: 1044–1048.

Sarasin, A. (1999). The molecular pathways of ultraviolet-induced carcinogenesis. Mutat. Res. 428: 5–10.

Satzger, H., Spörlein, S., Root, C., Wachtveitl, J., Zinth, W., and Gilch, P. (2003). Fluorescence spectra of *trans*- and *cis*-azobenzene – emission from the Franck–Condon state. Chem. Phys. Lett. 372: 216–223.

Schierling, B., Noël, A.-J., Wende, W., Le Hien, T., Volkov, E., Kubareva, E., Oretskaya, T., Kokkinidis, M., Römpp, A., Spengler, B., et al. (2010). Controlling the enzymatic activity of a restriction enzyme by light. Proc. Natl. Acad. Sci. U.S.A. 107: 1361–1366.

Schlesinger, O., Dandela, R., Bhagat, A., Adepu, R., Meijler, M.M., Xia, L., and Alfonta, L. (2018). Photo-switchable microbial fuel-cells. Biotechnol. Bioeng. 115: 1355–1360.

Schmermund, L., Jurkaš, V., Özgen, F.F., Barone, G.D., Büchsenschütz, H.C., Winkler, C.K., Schmidt, S., Kourist, R., and Kroutil, W. (2019). Photo-biocatalysis: biotransformations in the presence of light. ACS Catal 9: 4115–4144.

Schmidt-Dannert, C. and Lopez-Gallego, F. (2016). A roadmap for biocatalysis – functional and spatial orchestration of enzyme cascades. Microb. Biotechnol. 9: 601–609.

Schrittwieser, J.H., Velikogne, S., Hall, M., and Kroutil, W. (2018). Artificial biocatalytic linear cascades for preparation of organic molecules. Chem. Rev. 118: 270–348.

Schultz, C. (2007). Molecular tools for cell and systems biology. HFSP J. 1: 230–248.

Shao, J., Xue, S., Yu, G., Yu, Y., Yang, X., Bai, Y., Zhu, S., Yang, L., Yin, J., Wang, Y., et al. (2017). Smartphone-controlled optogenetically engineered cells enable semiautomatic glucose homeostasis in diabetic mice. Sci. Transl. Med. 9: eaal2298.

Sharma, M. and Friedman, S.H. (2021). The issue of tissue. Approaches and challenges to the light control of drug activity. ChemPhotoChem 5: 611–618.

Shchelik, I.S., Tomio, A., and Gademann, K. (2021). Design, synthesis, and biological evaluation of light-activated antibiotics. ACS Infect. Dis. 7: 681–692.

Shin, G., Gomez, A.M., Al-Hasani, R., Jeong, Y.R., Kim, J., Xie, Z., Banks, A., Lee, S.M., Han, S.Y., Yoo, C.J., et al. (2017). Flexible near-field wireless optoelectronics as subdermal implants for broad applications in optogenetics. Neuron 93: 509–521.e3.

Simeth, N.A., Kinateder, T., Rajendran, C., Nazet, J., Merkl, R., Sterner, R., König, B., and Kneuttinger, A.C. (2021). Towards

photochromic azobenzene-based inhibitors for tryptophan synthase. Chem. Eur J. 27: 2439–2451.

Smart, A.D., Pache, R.A., Thomsen, N.D., Kortemme, T., Davis, G.W., and Wells, J.A. (2017). Engineering a light-activated caspase-3 for precise ablation of neurons in vivo. Proc. Natl. Acad. Sci. U.S.A. 114: E8174–E8183.

Strickland, D., Yao, X., Gawlak, G., Rosen, M.K., Gardner, K.H., and Sosnick, T.R. (2010). Rationally improving LOV domain–based photoswitches. Nat. Methods 7: 623–626.

Sun, H., Jia, H., Ramirez-Diaz, D.A., Budisa, N., and Schwille, P. (2021). Fine-tuning protein self-organization by orthogonal chemo-optogenetic tools. Angew. Chem. Int. Ed. 60: 4501–4506.

Szymański, W., Beierle, J.M., Kistemaker, H.A.V., Velema, W.A., and Feringa, B.L. (2013). Reversible photocontrol of biological systems by the incorporation of molecular photoswitches. Chem. Rev. 113: 6114–6178.

Tanaka, Y., Bond, M.R., and Kohler, J.J. (2008). Photocrosslinkers illuminate interactions in living cells. Mol. BioSyst. 4: 473–480.

Tang, K., Beyer, H.M., Zurbriggen, M.D., and Gärtner, W. (2021). The red edge. Bilin-binding photoreceptors as optogenetic tools and fluorescence reporters. Chem. Rev. 121: 14906–14956.

Tappeiner, H.v. and Jodlbauer, A. (1904). Über die Wirkung der photodynamischen (fluoreszierenden) Stoffe auf Infusorien. Dtsch. Arch. Klin. Med. 80: 427–487.

Taslimi, A., Zoltowski, B., Miranda, J.G., Pathak, G.P., Hughes, R.M., and Tucker, C.L. (2016). Optimized second-generation CRY2–CIB dimerizers and photoactivatable Cre recombinase. Nat. Chem. Biol. 12: 425–430.

Tian, M. and Ye, S. (2016). Allosteric regulation in NMDA receptors revealed by the genetically encoded photo-cross-linkers. Sci. Rep. 6: 34751.

Tsai, Y.-H., Essig, S., James, J.R., Lang, K., and Chin, J.W. (2015). Selective, rapid and optically switchable regulation of protein function in live mammalian cells. Nat. Chem. 7: 554–561.

Uda, Y., Goto, Y., Oda, S., Kohchi, T., Matsuda, M., and Aoki, K. (2017). Efficient synthesis of phycocyanobilin in mammalian cells for optogenetic control of cell signaling. Proc. Natl. Acad. Sci. U.S.A. 114: 11962–11967.

Umehara, T., Kim, J., Lee, S., Guo, L.-T., Söll, D., and Park, H.-S. (2012). *N*-Acetyl lysyl-tRNA synthetases evolved by a CcdB-based selection possess *N*-acetyl lysine specificity in vitro and in vivo. FEBS Lett. 586: 729–733.

Uprety, R., Luo, J., Liu, J., Naro, Y., Samanta, S., and Deiters, A. (2014). Genetic encoding of caged cysteine and caged homocysteine in bacterial and mammalian cells. Chembiochem 15: 1793–1799.

Veiseh, O., Doloff, J.C., Ma, M., Vegas, A.J., Tam, H.H., Bader, A.R., Li, J., Langan, E., Wyckoff, J., Loo, W.S., et al. (2015). Size- and shape-dependent foreign body immune response to materials implanted in rodents and non-human primates. Nat. Mater. 14: 643–651.

Velema, W.A., Szymanski, W., and Feringa, B.L. (2014). Photopharmacology. Beyond proof of principle. J. Am. Chem. Soc. 136: 2178–2191.

Volkov, O., Kovalev, K., Polovinkin, V., Borshchevskiy, V., Bamann, C., Astashkin, R., Marin, E., Popov, A., Balandin, T., Willbold, D., et al. (2017). Structural insights into ion conduction by channelrhodopsin 2. Science 358, https://doi.org/10.1126/science.aan8862.

Wagner, S., Sudhamalla, B., Mannes, P., Sappa, S., Kavoosi, S., Dey, D., Wang, S., and Islam, K. (2020). Engineering bromodomains with a photoactive amino acid by engaging 'privileged' tRNA synthetases. Chem. Commun. 56: 3641–3644.

Wainberg, M.A. and Erlanger, B.F. (1971). Investigation of the active center of trypsin using photochromic substrates. Biochemistry 10: 3816–3819.

Wang, L. and Schultz, P.G. (2005). Expanding the genetic code. Angew. Chem. Int. Ed. 44: 34–66.

Wang, L., Brock, A., Herberich, B., and Schultz, P.G. (2001). Expanding the genetic code of *Escherichia coli*. Science 292: 498–500.

Wang, X., Chen, X., and Yang, Y. (2012). Spatiotemporal control of gene expression by a light-switchable transgene system. Nat. Methods 9: 266–269.

Wang, H., Vilela, M., Winkler, A., Tarnawski, M., Schlichting, I., Yumerefendi, H., Kuhlman, B., Liu, R., Danuser, G., and Hahn, K.M. (2016). LOVTRAP: an optogenetic system for photoinduced protein dissociation. Nat. Methods 13: 755–758.

Wang, J., Liu, Y., Liu, Y., Zheng, S., Wang, X., Zhao, J., Yang, F., Zhang, G., Wang, C., and Chen, P.R. (2019a). Time-resolved protein activation by proximal decaging in living systems. Nature 569: 509–513.

Wang, W., Huang, D., Ren, J., Li, R., Feng, Z., Guan, C., Bao, B., Cai, B., Ling, J., and Zhou, C. (2019b). Optogenetic control of mesenchymal cell fate towards precise bone regeneration. Theranostics 9: 8196–8205.

Weissleder, R. (2001). A clearer vision for *in vivo* imaging. Nat. Biotechnol. 19: 316–317.

Weissleder, R. and Ntziachristos, V. (2003). Shedding light onto live molecular targets. Nat. Med. 9: 123–128.

Weston, C.E., Richardson, R.D., Haycock, P.R., White, A.J.P., and Fuchter, M.J. (2014). Arylazopyrazoles: azoheteroarene photoswitches offering quantitative isomerization and long thermal half-lives. J. Am. Chem. Soc. 136: 11878–11881.

Wichert, N., Witt, M., Blume, C., and Scheper, T. (2021). Clinical applicability of optogenetic gene regulation. Biotechnol. Bioeng., https://doi.org/10.1002/bit.27895 (Epub ahead of print).

Wu, N., Deiters, A., Cropp, T.A., King, D., and Schultz, P.G. (2004). A genetically encoded photocaged amino acid. J. Am. Chem. Soc. 126: 14306–14307.

Wurm, J.P., Sung, S., Kneuttinger, A.C., Hupfeld, E., Sterner, R., Wilmanns, M., and Sprangers, R. (2021). Molecular basis for the allosteric activation mechanism of the heterodimeric imidazole glycerol phosphate synthase complex. Nat. Commun. 12: 2748.

Wu, Y.I., Frey, D., Lungu, O.I., Jaehrig, A., Schlichting, I., Kuhlman, B., and Hahn, K.M. (2009). A genetically encoded photoactivatable Rac controls the motility of living cells. Nature 461: 104–108.

Yamada, M.D., Nakajima, Y., Maeda, H., and Maruta, S. (2007). Photocontrol of kinesin ATPase activity using an azobenzene derivative. J. Biochem. 142: 691–698.

Yang, L., Dolan, E.M., Tan, S.K., Lin, T., Sontag, E.D., and Khare, S.D. (2017). Computation-guided design of a stimulus-responsive multienzyme supramolecular assembly. Chembiochem 18: 2000–2006.

Yao, X., Rosen, M.K., and Gardner, K.H. (2008). Estimation of the available free energy in a LOV2-Jα photoswitch. Nat. Chem. Biol. 4: 491–497.

Yari, M., Ghoshoon, M.B., Vakili, B., and Ghasemi, Y. (2017). Therapeutic enzymes. Applications and approaches to pharmacological improvement. Curr. Pharmaceut. Biotechnol. 18: 531–540.

Yi, H., Zhang, J., Ke, F., Guo, X., Yang, J., Xie, P., Liu, L., Wang, Q., and Gao, X. (2021). Comparative analyses of the transcriptome and proteome of *Escherichia coli* C321.△A and further improving its noncanonical amino acids containing protein expression ability by integration of T7 RNA polymerase. Front. Microbiol. 12: 2772.

Young, D.D. and Deiters, A. (2007). Photochemical control of biological processes. Org. Biomol. Chem. 5: 999–1005.

Young, T.S., Ahmad, I., Yin, J.A., and Schultz, P.G. (2010). An enhanced system for unnatural amino acid mutagenesis in *E. coli*. J. Mol. Biol. 395: 361–374.

Yu, D., Lee, H., Hong, J., Jung, H., Jo, Y., Oh, B.-H., Park, B.O., and Heo, W.D. (2019). Optogenetic activation of intracellular antibodies for direct modulation of endogenous proteins. Nat. Methods 16: 1095–1100.

Yüz, S.G., Ricken, J., and Wegner, S.V. (2018). Independent control over multiple cell types in space and time using orthogonal blue and red light switchable cell interactions. Adv. Sci. 5: 1800446.

Zemelman, B.V., Lee, G.A., Ng, M., and Miesenböck, G. (2002). Selective photostimulation of genetically ChARGed neurons. Neuron 33: 15–22.

Zemelman, B.V., Nesnas, N., Lee, G.A., and Miesenböck, G. (2003). Photochemical gating of heterologous ion channels. Remote control over genetically designated populations of neurons. Proc. Natl. Acad. Sci. U.S.A. 100: 1352–1357.

Zeng, S., Zhang, H., Shen, Z., and Huang, W. (2021). Photopharmacology of proteolysis-targeting chimeras. A new Frontier for drug discovery. Front. Chem. 9: 70.

Zhang, F., Vierock, J., Yizhar, O., Fenno, L.E., Tsunoda, S., Kianianmomeni, A., Prigge, M., Berndt, A., Cushman, J., Polle, J., et al. (2011). The microbial opsin family of optogenetic tools. Cell 147: 1446–1457.

Zhao, B. and Grosse, R. (2021). Optogenetic control of myocardin-related transcription factor A subcellular localization and transcriptional activity steers membrane blebbing and invasive cancer cell motility. Adv. Biol. 5: 2000208.

Zheng, Y., Lajoie, M.J., Italia, J.S., Chin, M.A., Church, G.M., and Chatterjee, A. (2016). Performance of optimized noncanonical amino acid mutagenesis systems in the absence of release factor 1. Mol. BioSyst. 12: 1746–1749.

Zhou, W., Hankinson, C.P., and Deiters, A. (2020). Optical control of cellular ATP levels with a photocaged adenylate kinase. Chembiochem 21: 1832–1836.

Zhu, S., Stroebel, D., Yao, C.A., Taly, A., and Paoletti, P. (2013). Allosteric signaling and dynamics of the clamshell-like NMDA receptor GluN1 N-terminal domain. Nat. Struct. Mol. Biol. 20: 477–485.

Zhu, S., Riou, M., Yao, C.A., Carvalho, S., Rodriguez, P.C., Bensaude, O., Paoletti, P., and Ye, S. (2014). Genetically encoding a light switch in an ionotropic glutamate receptor reveals subunit-specific interfaces. Proc. Natl. Acad. Sci. U.S.A. 111: 6081–6086.

Ziegler, T. and Möglich, A. (2015). Photoreceptor engineering. Front. Mol. Biosci. 2: 30.

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