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# Recent advances in the photochemical control of protein function

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## Abstract

Biological processes are regulated with a high level of spatial and temporal resolution. In order to understand and manipulate these processes, scientists need to be able to regulate them with Nature's level of precision. In this context, light is a unique regulatory element because it can be precisely controlled in location, timing and amplitude. Moreover, most biological laboratories have a wide range of light sources as standard equipment. This review article summarizes the most recent advances in light-mediated regulation of protein function and the application in a cellular context. Specifically, the photocaging of small molecule modulators of protein function and of select amino acid residues in proteins will be discussed. In addition, examples of the photochemical control of protein function through the application of natural light-receptors are presented.

## Introduction

Proteins are indispensable biological macromolecules within cells. They have a wide range of biological function, from structural support and regulatory mechanisms to the catalysis of synthetic transformations. In nature, protein function is precisely regulated by a variety of complex mechanisms. Myriad effector molecules interact to control the activity of proteins in a precise spatial and temporal fashion. To better understand the function, mechanism of action, and regulation of a specific protein, it is desirable to be able to externally modulate the protein's activity and observe the effects produced by its intentional activation and deactivation. In this context, light represents a unique external control element since its intensity can be easily controlled with high spatiotemporal resolution[1–7]. The examples discussed in this review illustrate some of the most successful and most recent strategies implemented to date for controlling protein activity with light.

Light-removable protecting groups, typically composed of aromatic rings, so-called "caging groups", installed on amino acid residues or small organic effector molecules allow for control over the function of a specific protein in a precise spatiotemporal manner using light irradiation. Caging groups are covalently bound to small organic effector molecules or specific amino acid residues in such a way that their natural activities are inhibited. When irradiated (most commonly with UV light), these molecules undergo photolytic cleavage to expose the previously caged small molecule effector or amino acid in its natural form



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(commonly referred to as “decaging”), thereby either inactivating (Figure 1a) or activating (Figure 1b) protein activity. The most commonly employed caging groups for these applications are based on *ortho*-nitrobenzyl moieties8, but other chromophores with enhanced photochemical properties have been developed as well9–12. Furthermore, researchers have developed systems where naturally occurring photoreactive proteins, such as LOV (light-, oxygen- and voltage-sensitive) domains and plant phytochromes, are conjugated to the protein-of-interest, enabling modulation of protein activity by light irradiation, often in a reversible fashion (Figure 1c and 1d). Advantages of such systems are that they do not require any chemical modifications and can often be genetically encoded. A disadvantage is that the protein activity before and after light-switching is difficult to predict, thus often requiring laborious optimization of the system under study through trial and error. Examples of the four different approaches illustrated in Figure 1 are discussed below.

## Caged small organic effector molecules

Many small effector molecules are known that can bind to proteins, prompting a protein to assume a biologically active or inactive state. Installing caging groups on the small molecule effectors (organic protein ligands with molecular weights typically <1000 Da) of proteins gives researchers the ability to control protein activity without attaching caging groups to the actual proteins. These caged small molecules can be utilized *in vivo* in a variety of prokaryotic and eukaryotic systems, simply by adding the caged effector molecule to the growth medium. Examples of caged small-molecule effectors that specifically affect gene expression include caged doxycycline[13], ecdysone[14], estradiol[15] and tamoxifen[16].

Caged isopropyl-β-D-thiogalactoside (IPTG) **1** (Figure 2a; the caging group is shown in red) was successfully used to control the expression of genes under control of a *lac* operator in bacterial cells[17]. The caged molecule is unable to bind to the lac repressor; however, UV-mediated decaging produced active IPTG, which subsequently removed the lac repressor from the promoter sequence on the DNA and induced gene expression. Using β-galactosidase as a reporter gene, a tenfold activation of gene expression was observed after a brief 5 min UV irradiation of cells exposed to **1**. Irradiation – and thus decaging - of **1** led to a similar level of gene expression as direct addition of non-caged IPTG to the growth medium. Spatial control of gene expression was also demonstrated and thus enabled bacterial lithography (Figure 2b). Cultures grown on solid media were irradiated on half of the area of the plate, with the other half masked from irradiation. The irradiated side showed light-induced gene expression, with the masked side remaining unaffected. Two different constructs were used for the bacterial lithography experiments, one being the same β-galactosidase expression system used before, and the other having a GFP gene under control of the *lac* operator. Expression from both constructs was controlled equally well spatially using the developed caged IPTG molecule. Another excellent example of bacterial lithography took advantage of a photoreactive cyanobacterial protein (an approach similar to the PhyB/PIF system discussed herein) to produce images on a lawn of bacteria with high resolution[18].

More defined spatial control over protein expression was recently demonstrated in mammalian cells using a caged doxycycline molecule **2** (Figure 2c; the caging group is shown in red)[19]. The doxycycline-inducible gene expression system RetroTET-Art (containing a doxycycline-reactive repressor/activator pair)[20] was employed in conjunction with the caged molecule **2** [19]. Patterned GFP expression was achieved in a monolayer of NIH 3T3 mouse fibroblast cells pre-treated with **2** by placing photomasks on the bottoms of culture dishes and irradiating with UVA light of 330–380 nm. Within the irradiated region, 70–85% of cells showed induced expression of GFP, as evidenced by a 5-fold higher level

of GFP expression than non-irradiated areas (Figure 2d). The ability to spatially pattern cells with light was also demonstrated using this system, potentially providing an opportunity for very precise tissue engineering. 3T3 cells expressing a membrane-bound ligand, ephrin A5, under control of RetroTET-Art were grown to a monolayer on tissue culture plates. Spatially restricted irradiation allowed the patterning of HEK293T cells expressing either the membrane receptor EphA7-T1 (which interacts attractively with ephrin A5) or EphA7 (which interacts repulsively with ephrin A5). Upon irradiation, HEK293T cells expressing EphA7-T1 attached to the 3T3 cells, while the HEK293T cells expressing EphA7 preferentially avoided the irradiated 3T3 cells when settling on top of them.

## Reversible photochemical regulation of ion channel activity

An interesting approach to the light-regulation of protein activity is the application of photoswitchable affinity labels (PALs) to introduce photo-activatable ion channels in neurons. Caged molecules have traditionally been used in a wide range of studies of neuronal function[21]. Some of these approaches involved photo-activatable caged glutamate[22, 23], genetically-modified nicotinic acetylcholine receptors[24], potassium ion ($K^+$) channels[25] and glutamate receptors[26], and the incorporation of retinal[27], a naturally-occurring photoactive molecule, into neurons. Light-activated nanopores that penetrate a membrane and form a functional channel after light irradiation have also been engineered[28].

A light-regulated $K^+$ channel was successfully created using the PAL method[29]. The PALs used had three distinct features: a quaternary ammonium group, a photo-isomerizable azobenzene group, and an electrophilic, cysteine-reactive group (e.g. acrylamide, chloroacetamide, epoxide or maleimide). The ammonium group is comparable in size to a potassium cation and thus blocks the pore of the $K^+$ channel; the electrophilic group reacts with the nucleophilic amino acid residues, typically cysteines, creating a covalent attachment to the ion channel. The azobenzene moiety enables reversible photo-isomerization of the molecule. When the PAL is in its *trans* form, the pore is blocked; however, when converted to the *cis* geometry by irradiaton with 360–400 nm UV light, the blockage is removed, enabling the passage of potassium ions (Figure 3a). Conversion from *cis* back to *trans* can be accomplished either by slow thermal reversion in the dark or rapidly by irradiation with 450–560 nm light. Through the reversible blocking of a $K^+$ channel, it was possible to produce changes in membrane potential a cultured cell via irradiation with 380 nm and 500 nm light (Figure 3b). This approach was also applied to the photochemical control over neuronal firing in cultured cells and live tissues (rat cerebral slices and ganglia taken from medicinal leeches) by light-triggering of action potentials.

## Genetically encoded caged amino acids

Instead of photochemically regulating protein function through the caging of small molecule effectors of protein function, it is also possible to directly install caging groups on the protein itself. Traditionally, caged proteins were produced by reacting surface lysine residues of the isolated protein with caging groups in a non-specific fashion (similar to the PAL installation discussed above)[30, 31]. This approach is problematic because it requires purification of the protein-of-interest, attachment of caging groups, and re-introduction of the caged protein into cellular systems (for *in vivo* applications). Perhaps most importantly, the caging of residues crucial to activity cannot be guaranteed using this method, and the location and number of caged residues cannot be controlled. *In vitro* transcription/translation systems can be used instead to successfully engineer caged proteins to increase residue specificity[32]. Rather than targeting the side chains of amino acids, caging groups can be installed on α-amino groups, as demonstrated recently in the creation of a photo-activatable intein splicing system[33]. In contrast, unnatural amino acid incorporation through site-

directed mutagenesis allows incorporation of caged amino acids at specific sites, and caged proteins can be expressed in bacterial, yeast, and mammalian cells using orthogonal synthetase/tRNA pairs[34–36]. Caged tyrosine[37–40], cysteine[41], serine[42] and lysine[43,44] have been incorporated successfully into proteins using site-directed unnatural amino acid mutagenesis.

Site-directed mutagenesis and an evolved orthogonal cellular machinery have recently been used to incorporate the caged lysine **3** (Figure 4a) into proteins in human embryonic kidney (HEK293) cells[44]. The proteins contained the nuclear localization sequences (NLS) of nucleoplasmin and the tumor suppressor p53, which allowed photo-regulation of the cellular localization of those proteins. The orthogonal cellular machinery was first evolved by using the pyrrolysyl-tRNA synthetase of the methanogenic bacterium *Methanosarcina barkeri* and its cognate amber codon tRNA, both of which are functional in bacterial and mammalian cells and are orthogonal to all endogenous tRNAs and tRNA synthetases[45]. The active site of the synthetase was mutagenized and the obtained library of synthetase mutants was subjected to a double-sieve selection yielding a synthetase/tRNA that selectively incorporated **3**, but no endogenous amino acid, in response to the amber stop codon (UAG)[44]. This pair was then used to incorporate **3** into a fusion protein featuring the NLS of tumor suppressor p53 and EGFP (p53-EGFP; gene diagram shown in Figure 4b), where the crucial lysine residue K305 was replaced by the amber stop codon, thus encoding the caged lysine analog (K305→**3**). Without irradiation, fluorescence microscopy showed the EGFP localized to the cytoplasm, thus demonstrating that the caging group on the lysine had blocked nuclear import. After irradiation at 365 nm for 5 s, an increase in nuclear import was observed, indicating that the function of the NLS could be restored by photolysis of the caging group (Figure 4c). The generality of this process was demonstrated by obtaining similar results for the caging of a lysine residue necessary for nuclear import in the NLS of nucleoplasmin.

## Genetically encoded photoresponsive protein domains

The incorporation of caged amino acids into proteins in live cells enables precise, light-mediated regulation of protein function in an *in vivo* environment. However, once decaged, no photochemical deactivation of the protein is possible, thus preventing the reversible regulation of protein activity. The reversible photochemical switching of protein function was recently achieved by fusing LOV domains, which are naturally found in plant phototropin proteins, to the protein-of-interest[46]. The LOV domain is bound by a flavin mononucleotide (FMN) cofactor, which triggers a conformational change in the protein when exposed to blue light (approximately 450–470 nm). The conformation change occurs as an unwinding of a helical domain (Jα) adjacent to the LOV domain, serving as a signaling message to an attached effector protein. Once blue-light irradiation ceases, the protein assumes its original, inactive conformation. Fusing the LOV domain to effector proteins has enabled the light-regulation of a histidine-kinase[47], a GTPase48, 49 and dihydrofolate reductase50.

A system that illustrates a successful light-activated LOV domain fusion is LovTAP (Lov- and tryptophan-activated protein) – a fusion of a LOV domain to an *E. coli trp* repressor protein that requires L-tryptophan and blue light irradiation for activity[51]. In this protein, the conformation of the allosteric Jα region separating the LOV domain from the *trp* repressor region determines whether or not the repressor region can bind DNA. In the dark, the fusion protein is inactive because Jα is associated with the LOV domain, and the repressor region becomes distorted and cannot bind DNA. When exposed to blue light, the FMN chromophore cofactor of the LOV domain induces a conformational change in the protein. As a result, Jα dissociates from the LOV domain, restoring activity to the Trp repressor

protein and enabling DNA binding (Figure 5a). A DNA protection assay has been conducted where an RsaI cleavage site was inserted into the *trp* operator region of a plasmid (Figure 5b). When irradiated with blue light, the fusion protein would bind to the *trp* operator and prevent cleavage by the RsaI restriction enzyme, with the proportion of digested DNA decreasing as the concentration of LovTAP (μM) increased (dashed lines). In the dark, however, the cleavage site was left unprotected and the DNA was cleaved to a greater extent (solid lines). The preference of the fusion protein for the *trp* operator for successful protection indicated that the Trp repressor region of the fusion protein retained its native activity, and the photoactive properties of the LOV domain enabled light-regulation of the system. The next logical step will be the application of LOV-regulated *trp* repressor in the photocontrol of gene function in live cells, as has been demonstrated for other LOV-protein fusions, including the histidine kinases FixL[47] and Rac[48, 49].

## Genetically encoded protein dimerization

Plant phytochromes are photosensory molecules that alternate between two conformations in response to red and infrared light52. They are two-domain proteins bound to a chromophore, phycocyanobilin (PCB), which absorbs light of the appropriate wavelengths. One protein conformation is “active”, meaning that it can interact with other proteins in signaling pathways related to light absorption in plants, whereas the other conformation is “inactive” in this context[53]. The inactive form, referred to as Pr, absorbs red light of approximately 660 nm, which converts it to the active form, Pfr. When Pfr absorbs IR light (~750 nm), it is converted back into Pr, resulting in a loss of biological activity. These conversions are extremely fast, and can be conducted repetitively without degradation of the phytochrome.

PhyA and PhyB are two plant phytochromes which have been used to create photoactivatable fusion proteins. In their active Pfr forms, they undergo a binding interaction with the protein PIF (phytochrome interaction factor)[54]. This interaction has been exploited to modulate the dimerization of other proteins, where one protein of interest is fused to PhyA or PhyB, and the other protein is fused to PIF. A system similar to the one described here, utilized a cyanobacterial phytochrome in the afore-mentioned bacterial lithography experiment to display high-resolution images on a bacterial lawn18. The coupling of a cyanobacterial phytochrome to a bacterial kinase has recently been engineered as a light-sensing component of a genetically-encoded “edge detection program”, where bacteria incubated in the dark secreted the chemical messenger homoserine lactone to nearby cells. If nearby cells detected both the chemical messenger and light, they catalyzed production of a dark pigment, allowing the bacteria to mark the edge of a photomask55. A necessary component of these systems is the addition of the chromophore co-factor, which imparts photoactivity to the phytochrome molecule. While other chromophores can be used effectively, the most common is PCB56.

An elegant application of the light-regulated Phy-PIF interaction is the creation of a light-controlled transcriptional activation system[57]. Here, the transcriptional activation domain of GAL4 was fused to PIF, and the DNA-binding domain of GAL4 was fused to the photoactive N-terminal domain of PhyB (Figure 6a). Yeast expressing these constructs were used to demonstrate transcriptional control over β-galactosidase expression with constant red light. Upon further investigation, it was found that maximal activation of gene expression could be achieved by using shorter pulses of red light rather than constant exposure. By using increasing red light pulses of 5, 10 and 30 min, they observed 2-, 4- and 30-fold increased *LacZ* expression and thus β-galactosidase activity. The reversibility of this light-controlled gene expression system was demonstrated with 5-min pulses of IR light (Figure 6b). β-Galactosidase accumulation slowed within 10 min after IR light exposure, and completely ceased within 15 min. Even though the expression levels of the light-

inducible system were three orders of magnitude above background expression after light activation, they only reached one-sixth of wild-type expression levels observed with the native GAL4 promoter.

Recently, the Phy-PIF dimerization was utilized in the construction of a light-controlled system for the regulation of protein localization to the cell membrane in mammalian cells[54, 58]. A membrane anchored PhyB was used in the red light-activated recruitment of yellow fluorescent protein. Spatial control over this system was demonstrated by irradiation of sub-cellular areas with red or IR light, thus projecting patterns onto the cell surface. In addition, light-induced localization of the guanine nucleotide exchange factor Tiam to the cell membrane using the same system, resulted in photo-controlled lamellipodia formation in mammalian cells. Phy-PIF dimerization has also been used recently in yeast to engineer a light-activated conditional protein splicing system[59].

## Conclusion

Photochemical control over protein function has been achieved through a variety of distinct approaches. Small organic effector molecules that regulate the activity of proteins have been caged, allowing the activities of the proteins to be regulated by light. Proteins themselves have been rendered inactive through the installation of caging groups or photoswitchable groups, and have then been activated through UV irradiation. Such light-responsive groups have been attached through chemical reactions (i.e. photochemical regulation of $K^+$ channels) and through the site-specific incorporation of genetically encoded unnatural amino acids using orthogonal tRNA/tRNA synthetase pairs in bacterial and mammalian cells (i.e. photochemical control of intracellular protein localization). Further optimization of these existing technologies and development of new caging groups for amino acids and the corresponding engineered cellular machinery will enable a higher degree of precision and versatility in controlling protein activity with light.

Naturally occurring photoresponsive proteins have also been adopted for the purpose of controlling the activities of other proteins-of-interest with light. These systems involve fusing the protein that will be the object of light-regulation to a photoactive domain that changes its conformation through irradiation (i.e. LOV domain-fusion proteins), or take advantage of light-induced protein dimerization to reconstitute a dissociated protein-of-interest, thereby restoring its activity (i.e. the PhyB-PIF system). An advantage of those light-regulatory elements is their reversibility while being fully genetically encoded. Each type of system discussed here illustrates how light can be used as a tool to control protein activity both *in vitro* and *in vivo*, often with a high degree of spatiotemporal precision. These technologies will prove useful in further investigations of important biological questions regarding the regulation and function of proteins and genes in cells and multi-cellular model organisms. However, moving forward, open sharing of developed genetic constructs between laboratories and the commercial availability of photocaged monomeric building blocks (e.g. amino acids) and small molecule modifiers will be necessary.

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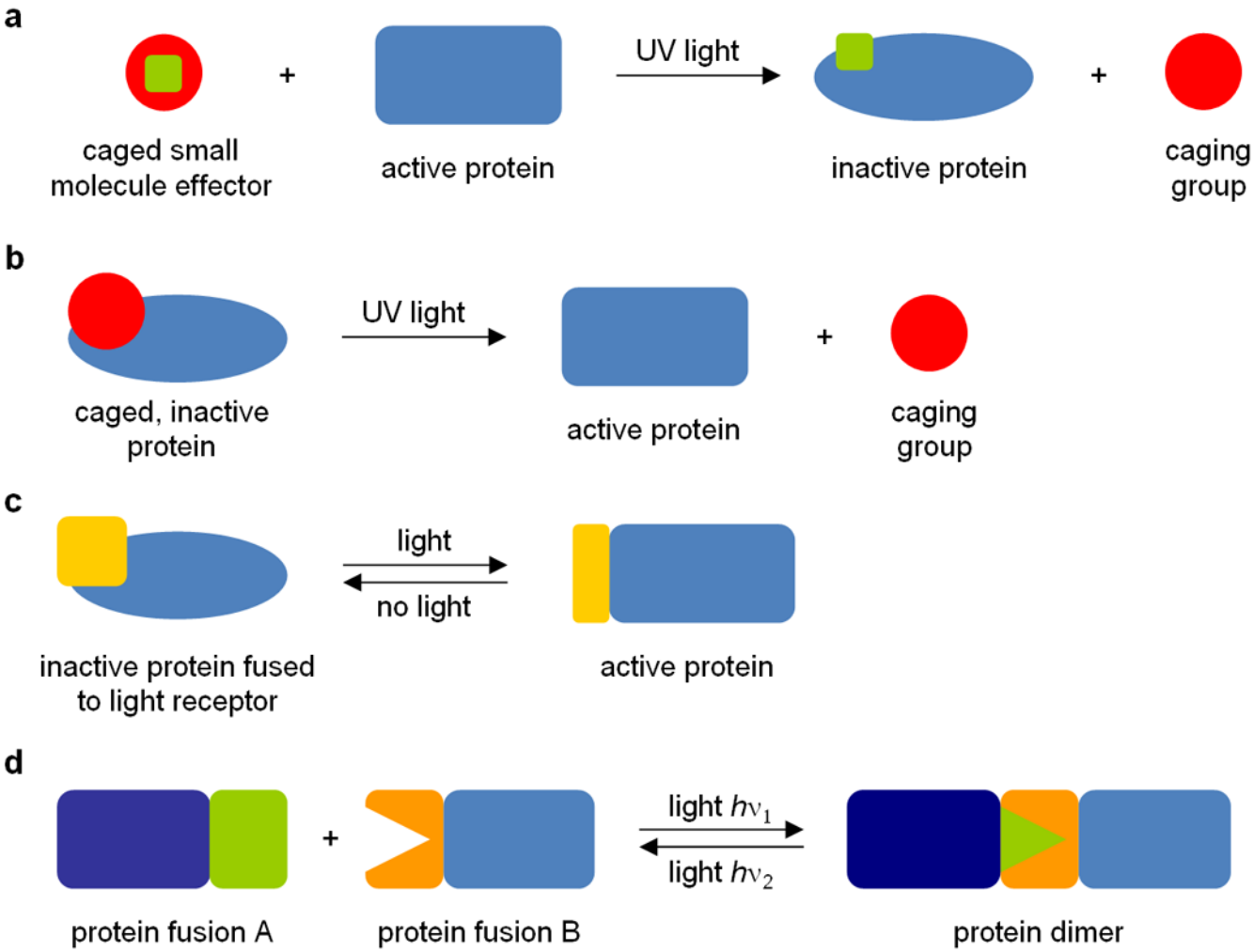


**Figure 1.**

Four different approaches to control protein function with light. (a) Caged small effector molecules can inhibit protein function after caging group removal via UV irradiation. (b) Caged proteins, expressed using genetically encoded caged amino acids, can be activated via UV irradiation through caging group removal. (c) The activity of proteins can be reversibly regulated by light irradiation when fused to a light receptor (e.g. a natural light oxygen voltage (LOV) domain or a synthetic photoswitchable affinity label (PAL)). (d) Two proteins can be dimerized by light irradiation when fused to a natural plant phytochrome (e.g. PhyB) and a phytochrome interaction factor (PIF).

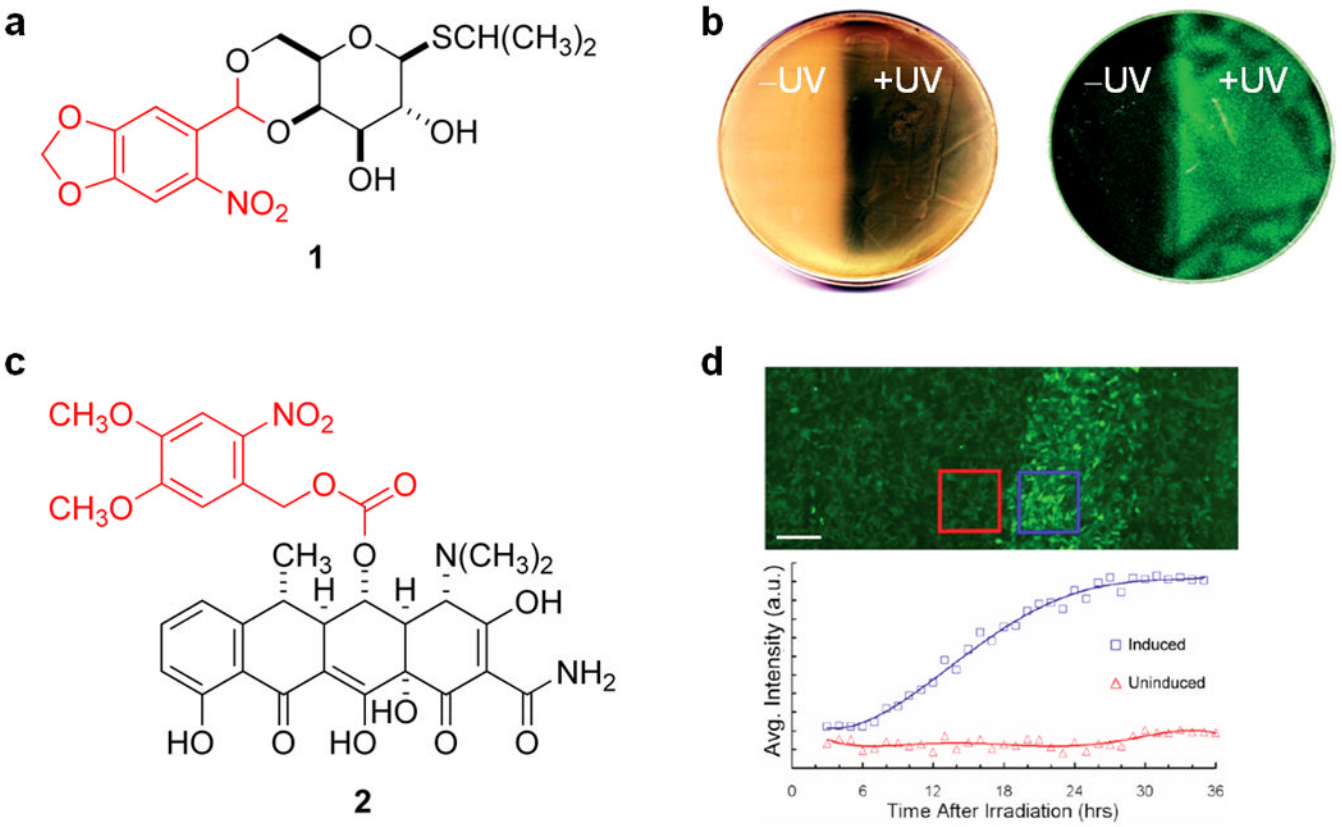


**Figure 2.**

Light-activatable small molecule inhibitors of gene expression repressor proteins. (a) Caged IPTG **1**. (b) Bacterial lithography experiment with **1**, showing light-controlled induction and spatial control of β-galactosidase (left) and GFP (right) in a bacterial lawn (∅ 10 cm). (c) Caged doxycycline **2**. (d) Spatial control of GFP expression using **2**, with irradiation through a 344-μm photomask (scale bar = 250 μm); irradiated (blue) and non-irradiated (red) cells were quantified and fluorescence intensity is shown as a function of time. The light-removable caging groups are shown in red.

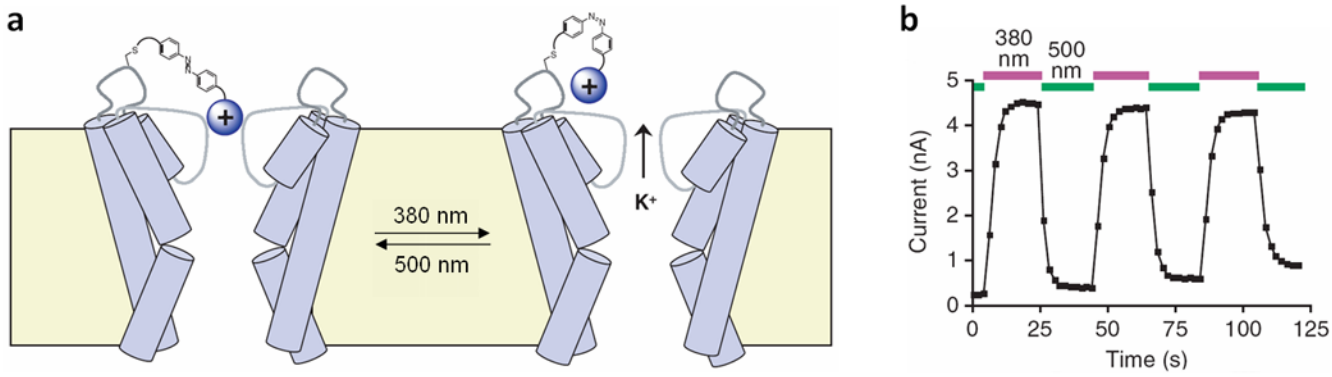


**Figure 3.**

Reversible photochemical activation of a potassium ion ($K^+$) channel. (a) Schematic of PAL-gated $K^+$ channel. The sphere labeled with a plus sign represents a quaternary ammonium group, which blocks the channel when the diazobenzene is in the *trans* conformation. Irradiation at 380 nm switches the diazobenzene from *trans* to *cis*, thus enabling $K^+$ flow. Irradiation with 500 nm light reverses the switching event, thus blocking the ion channel. (b) Light-controlled membrane potential of a PAL-gated $K^+$ channel measured in dependence of light irradiation.

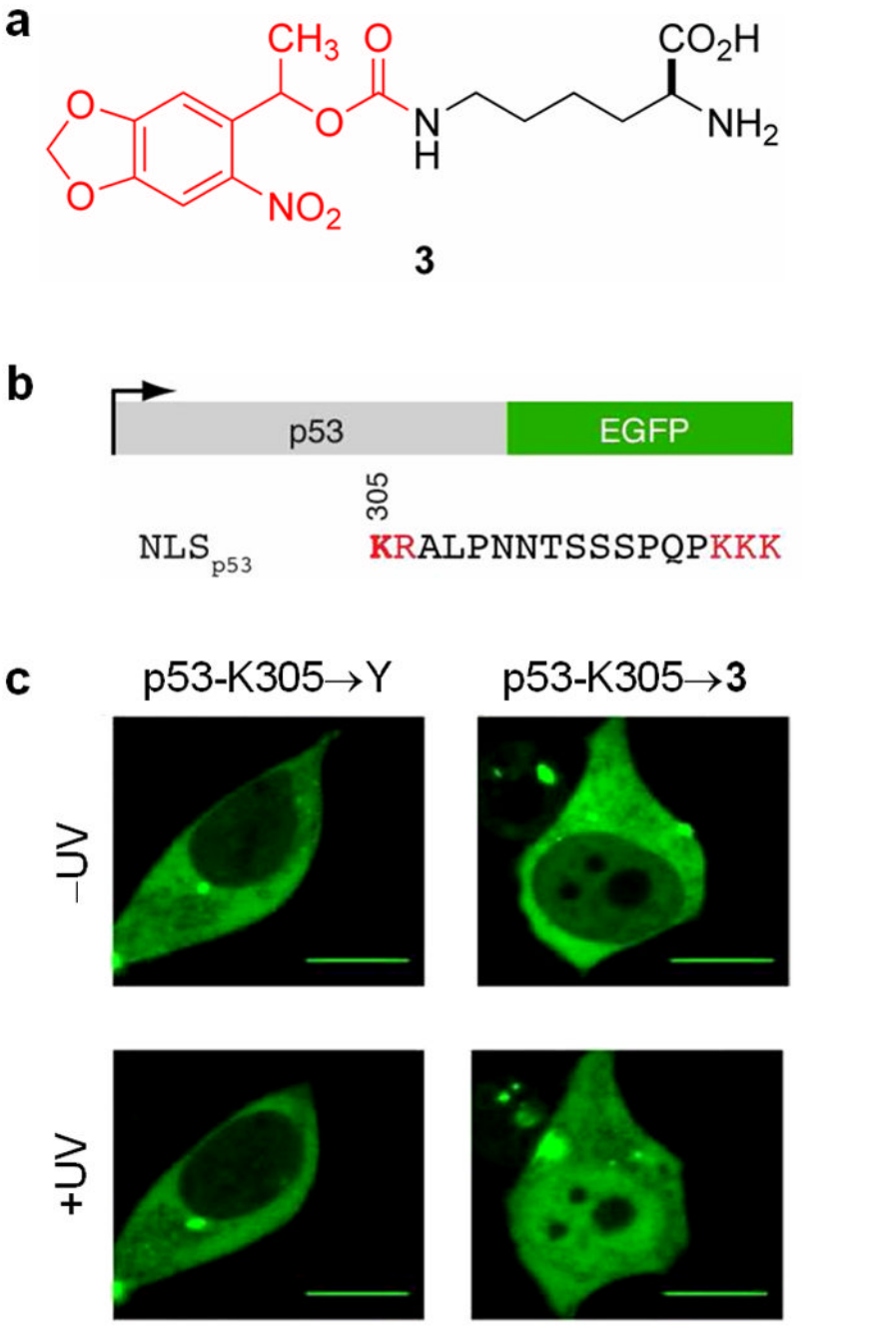


**Figure 4.**

Photochemical control of protein localization in mammalian cells. (a) Genetically encoded caged lysine **3**; light-removable caging group shown in red. (b) Gene diagram of *p53-egfp*, with the crucial lysine K305 bolded. (c) Nuclear import of EGFP in HEK293 cells after introduction and photolysis of **3** introduced into position 305 in $NLS_{p53}$. A mutation of K305 to tyrosine (K305→Y) blocks transport into the nucleus, regardless of light irradiation (left). Introduction of the caged lysine **3** at position 305 (K305→**3**) blocks transport of p53-EGFP-HA, but enables translocation into the nucleus after a brief light irradiation (365 nm, 5 s) (right). Scale bar = 10 μm.

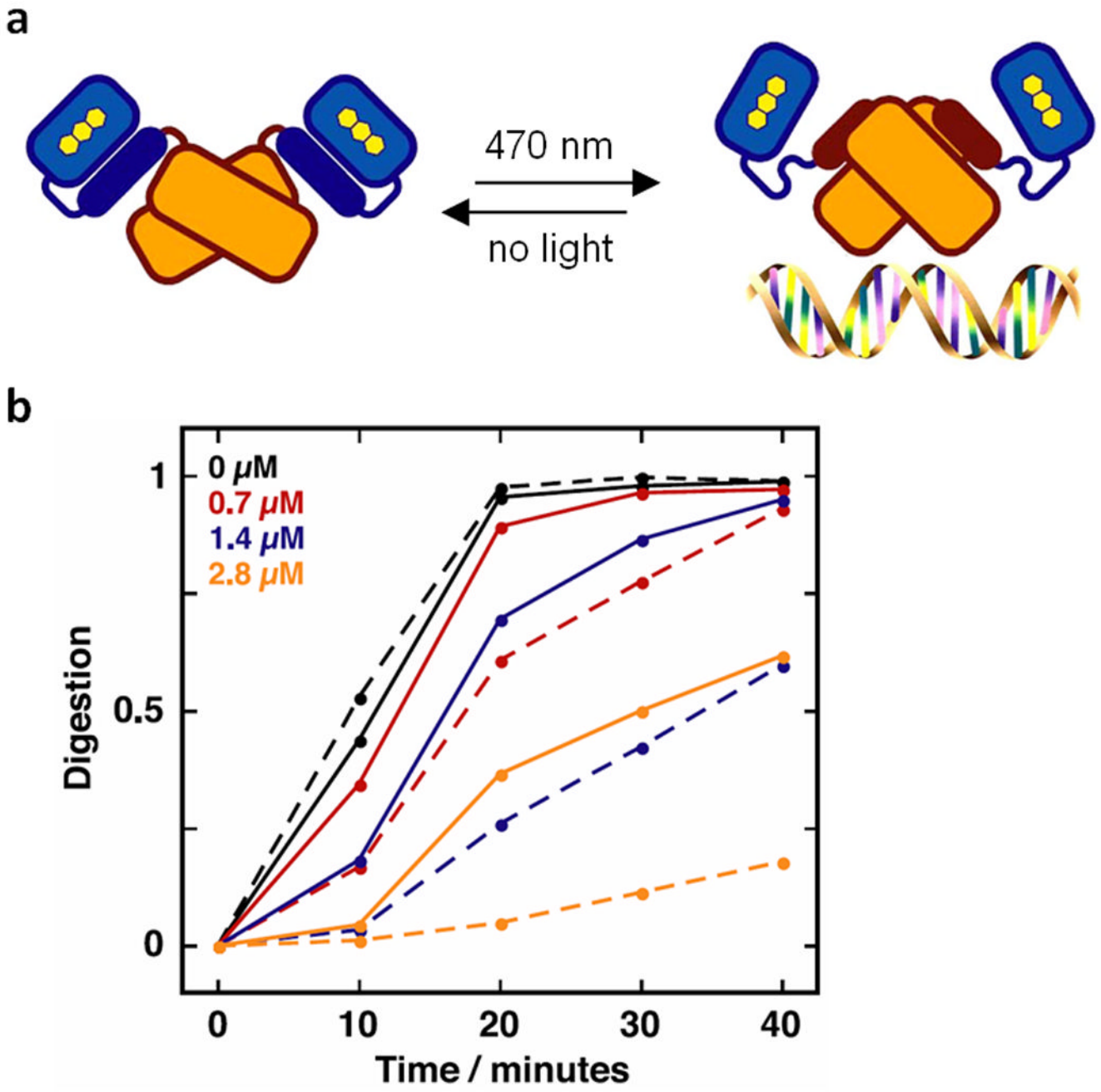


**Figure 5.**

Mechanism of LovTAP, a reversibly switchable DNA binding protein. (a) Before exposure to blue light, Jα (dark blue) is associated with the LOV domain (light blue), which renders the Trp repressor region (light orange) inactive. After irradiation with blue light (470 nm) a conformational change occurs and Jα (now dark orange) dissociates from the LOV domain, in turn activating the Trp repressor. The active protein binds to DNA at a *lac* operator region. The original conformation of the protein is resumed after incubation in the dark, and the Trp repressor region dissociates from the DNA, thus making the activity of LovTAP reversible. (b) LovTAP protects DNA against RsaI digestion at the *lac* operator site.

Increasing the concentration of LovTAP (0–2.8 μM) decreases digestion, as does irradiation with blue light (dashed lines: irradiated; solid lines: non-irradiated).

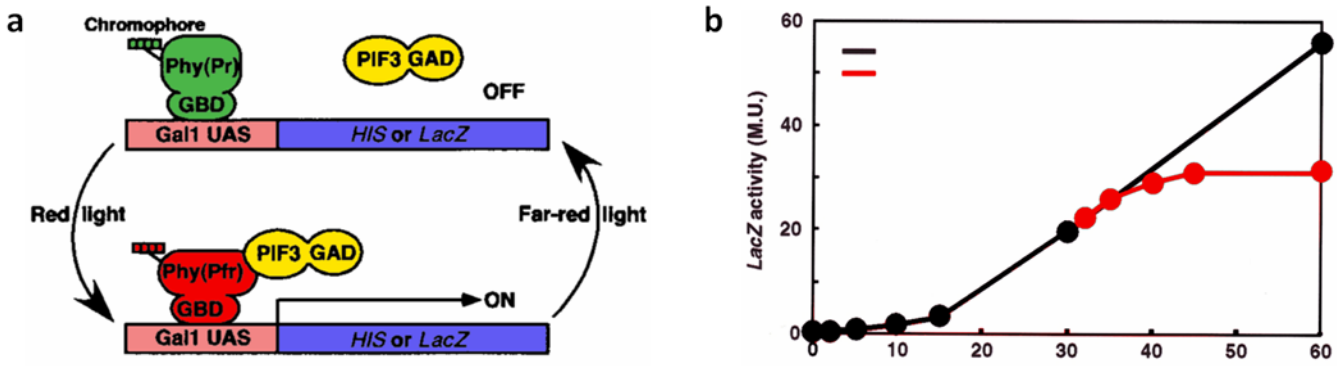


**Figure 6.**

Light-controlled expression of *LacZ* by PhyB-PIF protein dimerization. (a) Schematic of the light-controlled transcriptional activator. Phytochrome (Phy), which is fused to the DNA-binding region of GAL4 (GBD), reversibly binds to PIF. PIF is fused to the transcriptional activating domain of GAL4 (GAD), thus activating gene expression upon exposure to red light and ceasing activity by dissociation upon exposure to IR light. (b) *LacZ* (β-galactosidase) activity induced with pulses of red light (Rp, black line), and arrested with pulses of IR light (FRp, red line).