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Recent Advances in the Optical Control of Protein Function through Genetic Code Expansion

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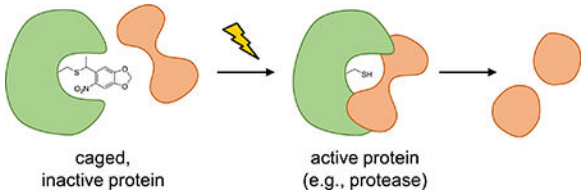
Abstract

In nature, biological processes are regulated with precise spatial and temporal resolution at the molecular, cellular, and organismal levels. In order to perturb and manipulate these processes, optically controlled chemical tools have been developed and applied in living systems. The use of light as an external trigger provides spatial and temporal control with minimal adverse effects. Incorporation of light-responsive amino acids into proteins in cells and organisms with an expanded genetic code has enabled the precise activation/deactivation of numerous, diverse proteins, such as kinases, nucleases, proteases, and polymerases. Using unnatural amino acids to generate light-triggered proteins enables a rational engineering approach that is based on mechanistic and/or structural information. This review focuses on the most recent developments in the field, including technological advances and biological applications.

Table of Content Graphic



Introduction

In order to study and manipulate biological processes with the same precision as nature, chemical biologists have developed a number of optical tools [1]. The use of light to control protein activity provides non-invasive, precise, spatiotemporal control and allows for more acute perturbation than other methods (such as RNA interference or gene editing). Optical control of protein function in live systems has primarily been achieved through two approaches: genetic encoding of light-responsive amino acids or optogenetic methods using natural photoresponsive protein domains. Over the last two decades, more than a hundred non-canonical amino acids have been genetically encoded in a range of organisms to provide

functionalities not found in the common set of 20 amino acids [2]. The incorporation of light-triggered amino acids into proteins has been used to control a wide range of biological processes in cells and animals [3], and this review highlights select examples from the past 5 years in order to demonstrate the versatility of this approach. Due to space limitations, we are not including other important methodologies, such as protein bioconjugation of photoswitchable ligands [4,5]. Purely optogenetic approaches have been extensively reviewed elsewhere [6–9].

Technical Advances in the Field

Caged lysine:

The photocaged lysine **1** (Figure 1) has been applied toward optical triggering of Cas9 nuclease [10], T7 RNA polymerase [11], Cre recombinase [12], MEK [13,14] and LCK [15] kinases, isocitrate dehydrogenase [16], and protein-protein interactions [17,18]. Optical control of lysine, which plays an essential role in enzymatic catalysis of many biological processes, has been instrumental in gaining a deeper understanding of living systems at the molecular level. This photocaged lysine utilizes 365 nm light for activation and may be incompatible with certain experiments performed in *E. coli* due to the abundance of nitroreductases. In order to develop a system that is compatible with a range of organisms and to provide activation with blue (405 nm) and near-IR (two-photon 760 nm) light, the coumarin-caged lysines **2** and **3** were developed [19]. Both were applied in mammalian cells for the optical control of luciferase function and of GFP folding and the different decaging wavelengths for **2** (405 nm) and **3** (760 nm) enable sequential, wavelength-selective activation. Additionally, the coumarin chromophore provides fluorescent tracking of the incorporated amino acid prior to decaging, thus **2** and **3** can act as both fluorescent and photo-activatable probes in live cells. Introduction of the additional methylene group in **4**, blocks photolysis and provides a stable and small fluorophore that can be site-specifically placed into proteins. The caged lysine **2** has subsequently been applied to control MEK kinase in zebrafish embryos [14] (see *Optical Control of Cell Signaling* section) and DNA helicase [20] (see *Optical Control of Nucleic Acid Processing* section).

While lysine often plays an essential role in enzymatic catalysis, replacement of the ε-carbon with oxygen generates an amino-oxy functionality which can undergo bio-orthogonal oxime ligation with a ketone or aldehyde. The Virdee group generated the corresponding lysine analog **5** with a nitrobenzyl caging group to render it unreactive until UV-induced photolysis and encoded it using the same synthetase/tRNA pair engineered for **1** [21]. While incorporation efficiency was low, masking a reactive bio-orthogonal handle with a caging group may minimize off-target reactivity and may enable the encoding of other, more reactive bio-orthogonal handles.

Caged tyrosine:

Photocaged tyrosine **6** was genetically encoded a decade ago and has been applied to the optical control of several enzyme classes [22–27]; however, in order to facilitate decaging through red-shifting of the chromophore's absorption maximum, the Deiters group developed three additional photocaged tyrosine derivatives **7** - **9** [28]. Use of a dual-

luciferase reporter allowed for simultaneous assessment of incorporation and decaging efficiency. While **8** delivered the most efficient optical activation of protein function, the caged tyrosine **7** proved to be the better analog when both incorporation efficiency and decaging efficiency are considered. Thus, caged tyrosine **7** was subsequently applied to the demonstration of spatial control of luciferase activity and the efficient optical triggering of TEV protease (TEVp) activity in mammalian cells. Optical control of TEVp may enable the engineering of precise spatiotemporal activation/deactivation of a protein of interest at a desired subcellular region or protein translocation through light-triggered peptide cleavage. Furthermore, caged amino acids may be applicable to the photocontrol of other proteases [29].

Caged cysteine and caged selenocysteine:

Although it is the least abundant amino acid found in proteins, cysteine plays an essential role in nucleophilic catalysis, redox signaling, metal binding, and structural support through disulfide formation [30]. In order to optically control these different functions, several caged cysteine analogs have emerged in the last few years. Schultz genetically encoded **10** in yeast using an *E. coli* leucyl synthetase/tRNA pair to cage the active site of caspase-3 [29]. More recently, the Chin lab developed the caged cysteine **11**, which was incorporated by an engineered pyrrolysine tRNA synthetase/tRNA pair and applied in mammalian cells for the photo-activation of TEVp (Figure 2a) [31].

Photoactivated proteins are typically generated through replacement of an essential amino acid with a caged analog. In contrast, the Ai lab developed an approach that can be utilized in cases where such a critical amino acid residue is not available, by developing a light-triggered intein [32]. Inteins are protein segments which can self-cleave and excise, thereby rejoining the cleaved N- and C-terminal fragments to form a new, truncated protein. This cleavage event often utilizes a nucleophilic cysteine residue, making it amenable to optical control through incorporation of the caged cysteines **12** and **13**. As proof-of-concept, a caged *Nostoc punctiforme* DnaE intein (splicing occurs with a reaction half-life of one minute) was placed in the middle of the mCherry protein sequence such that an inactive, non-fluorescent protein was expressed. Following UV irradiation and protein splicing, full-length, active mCherry was generated. In a second application, the caged intein was placed into the catalytic domain of Src kinase in order to control its enzymatic activity and downstream phosphorylation (Figure 2b). One limitation of the approach is the necessity of a cysteine residue at the splice site; which, if not naturally present, will leave a scar following intein excision. In addition, optically controlled inteins have been applied to protein splicing in yeast [33] and generation of cyclic peptides in *E. coli* [34].

The light-activated cysteines discussed above required tRNA synthetase engineering for genetic encoding. Alternatively, the Deiters lab engineered the caged cysteine structure and developed the caged cysteine **14** and the homocysteine **15**, which structurally mimic the caged lysine **1** and serve as substrates for the corresponding tRNA synthetase [35]. Not surprisingly, the homocysteine **15** was incorporated as efficiently as caged lysine **1**, while incorporation of **14** was slightly less efficient. Light activation of **14** and **15** was demonstrated through optical control of *Renilla* luciferase. While both **14** and **15** showed an

increase in luminescence following UV irradiation, the activity of the homocysteine containing enzyme was substantially lower. Thus, site-specific incorporation of homocysteine through light-activation of **15** may enable perturbation of an active site with single-atom resolution due to the introduction of an additional methylene unit.

Selenocysteine (Sec) is structurally and functionally similar to its periodic neighbor cysteine. Recently, the role of Sec in functional proteins and enzymes has received much attention [36], and clever approaches to its introduction have been developed [37–39]. The Klimasauskas group masked the nucleophilic selenium with a nitrobenzyl moiety to generate **16** and incorporated it into sfGFP [40]. Following UV irradiation, native Sec was generated and reacted with maleimide-modified biotin.

Photoswitchable amino acids:

While the amino acids described above have enabled optical control of a wide range of protein functions, they are limited to an irreversible activation event. Since many biological processes undergo cycles of activation and deactivation, tools that mimic this reversibility may be better suited for studying these systems. The photoswitchable azobenzene amino acid **17** was first genetically encoded in bacteria in 2006 and applied to controlling catabolite activator protein binding [41] and GFP fluorescence [42]. More recently, additional photoswitchable amino acids have been genetically encoded, in order to provide improved photostationary states, red-shifted wavelengths for photoisomerization, and increased modulation of protein conformation through azo-bridging. The Wang group developed three azobenzene-derived photoswitchable amino acids **18** - **20** with thiol-reactive handles and demonstrated their function in the control of calmodulin conformation [43]. Subsequently, the pentafluoro-azobenzene derivative **21** with red- shifted isomerization wavelength and improved azo-bridging efficiency was reported [44]. The Lin group synthesized and genetically encoded a full set of fluorinated azobenzene analogs with improved photoswitching properties; however, these amino acids have yet to be applied to the control of protein function [45]. Most recently, the Wang group applied **18** to the reversible control of glutamate receptors in mammalian cells without utilizing the inherent cross-linking capabilities [46]. The approaches presented here have established a foundation for obtaining reversible, spatiotemporal control of biological processes in living systems through unnatural amino acid mutagenesis; however, the field is still in its early stages and further work is underway to improve this technology.

Applications

Optical Control of Nucleic Acid Processing:

The CRISPR/Cas9 system is a highly versatile genome-editing tool that enables modification, insertion, or deletion of sequences of genomic DNA [47–49]. While natural Cas9 is constitutively active, conditional control of its function enables applications with spatio-temporal precision and may minimize off-target effects [49]. The Deiters group developed the first optically controlled CRISPR/Cas9 system by replacing a lysine residue in the HNH nuclease domain with the photocaged lysine **1**, in order to prevent the conformational change necessary for nuclease activity (Figure 3a) [10]. Using a dual

fluorescent reporter, which relies on the excision of mCherry and the subsequent expression of GFP, spatial and temporal control of Cas9 activity was achieved in mammalian cells. Additionally, light-triggered gene silencing of an endogenous target, the cell surface receptor CD71, was demonstrated.

In order to expand the toolkit of optically controlled DNA-processing enzymes, the Deiters group developed a caged helicase (UvrD) [20] and a caged DNA recombinase [12] for spatiotemporal control of DNA unwinding and recombination, respectively. UvrD was rendered light responsive through the installation of the photocaged lysine **2** at a conserved lysine residue within the ATPase domain. In conjunction with optical control of kinase function [13,15], this indicates the possibility of universal photochemical triggering of ATP-dependent processes. Cre recombinase was initially photocaged at an active-site tyrosine with **6**; however, in order to improve enzyme expression levels in mammalian cells, a conserved lysine residue was replaced with **1**. Excellent off to on photocontrol of Cre recombinase was achieved and the potential for performing knock- in/knock-out experiments with high spatiotemporal resolution was demonstrated in developing zebrafish embryos [50].

In order to optically control transcription in mammalian cells, the Deiters group developed a photocaged T7 RNA polymerase in which an active site lysine was replaced with the caged lysine **1** (Figure 3b) [11]. This enables light-activation of a transcriptional pathway (gene of interest placed under the T7 promoter) that is orthogonal to the endogenous cellular machinery, and the caged T7 RNA polymerase was applied to the triggering of gene expression (control of EGFP mRNA as a proof-of-concept), as well as, gene silencing (control of shRNA targeting the motor protein Eg5 as a proof-of-concept) with spatial and temporal resolution.

Optical Control of Cell Signaling:

Cell signaling networks exhibit a high degree of spatial and temporal dynamics, suggesting light as a preferred external control element. Unlike genetic tools, which require days or hours to knock down or inhibit signaling proteins, light activation of caged amino acids enables one to study the acute effects of kinase function. Since the caged amino acids are genetically directed to their incorporation site in cell signaling proteins, they provide an unmatched specificity that is difficult to achieve with small molecule inhibitors.

LCK (lymphocyte-specific protein tyrosine kinase) is responsible for initiating the T-cell receptor (TCR) signaling pathway, following MHC protein recognition by the TCR. The Chin and James groups used the photocaged lysine **1** to place LCK function under optical control by following the general strategy of blocking an essential and conserved lysine residue in the ATP binding pocket [15]. Through light-triggering of kinase function, they were able to quantify the phosphorylation kinetics of LCK and identified its ability to stimulate its own activation. This work nicely showcases that acute optical activation of kinase function allows for uncovering of mechanistic details of cell signaling activity.

The photocaged lysine **1** has previously been utilized to render the MEK/ERK pathway light-responsive in mammalian cells [13]. More recently, MEK kinase was placed under

light control using the more sensitive caged lysine **2** in zebrafish embryos (Figure 4a) [14]. Temporal control of MEK function in the developing animal revealed an essential time window in which hyperactive MEK affects dorsal/ventral patterning, a discovery that is relevant to human birth defects caused by Ras/MAPK pathway mutations. The expansion of optical control of cell signaling to multicellular model organisms will enable investigations in complex systems through more precise perturbation of enzymes/pathways required for normal/mutant embryonic development.

Control of neural circuits with light has been extensively explored via optogenetic approaches using light-responsive ion channels [6]. In addition to classical optogenetic methods, the use of unnatural amino acid mutagenesis has been utilized by the Wang group by genetically incorporating the caged cysteine **12** into an inward rectifying potassium channel in mouse neocortex tissue slices in order to obtain precise activation of neuronal suppression as measured by patch-clamp recordings [51,52].

Optical Control of Protein-Protein Interactions:

Most examples of using photocaged amino acids for optical control of protein function target enzymatic processes through caging of an essential residue within the active site. However, even a relatively small nitrobenzyl caging group can be efficiently used in the regulation of protein-protein interactions when strategically placed into the protein binding interface. The Engelke and Deiters groups replaced an important lysine residue in the nuclear localization sequence (NLS) of the transcription factor SatB1 with caged lysine **1** [17]. This led to sequestration of NLS fusion protein in the cytoplasm until UV irradiation generated the native NLS sequence, followed by translocation into the nucleus (Figure 4b). This optical NLS approach was applied to controlling FOXO3 transcription factor-DNA binding and TEVp-nuclear substrate interaction. Due to the small size of this optical NLS (20 amino acids), it can easily be appended to any protein of interest to modulate cytoplasmic to nuclear localization. One limitation of the approach is the relatively slow rate of translocation to the nucleus compared to other NLS.

A similar strategy was applied for optically controlling a virus-host protein-protein interaction using the caged lysine **1** [18]. The Chatterjee group incorporated **1** into VP1, a surface protein, of the adeno-associated virus (AAV) capsid. This blocked interaction with human heparin sulfate proteoglycan (HPSG) and prevented viral infection of HEK293T cells, until photolysis released the caging group, forming the native capsid. This approach provides an innovative tool to probe the cellular entry process of human viruses by disrupting the interactions between the virus and the host cell through introduction of photocaged residues and should be broadly applicable to viruses that utilize Lys/Arg, Cys/Ser/Thr, or Tyr as critical regulatory residues at the binding interface.

Optical Control of other Enzymatic Processes:

Isocitrate dehydrogenase (IDH) is an essential enzyme in the citric acid cycle, and has been found mutated through an active site arginine to lysine mutation in various cancers. In order to better study the effects of mutant IDH2 activation, the Chin group replaced an active site lysine with the photocaged lysine **1** to block substrate binding until UV induced decaging

[16]. Upon photoactivation of IDH2, a decrease in 5-hydroxymethylcytosine was observed, validating a previously proposed sequence of events in cancer cell epigenetic modifications.

The study of pathogenic bacteria has increased over the last several years as the result of a spike in MRSA-related illnesses. Traditionally, it has been challenging to purify S. *aureus* toxinantitoxin proteins due to the toxicity caused in *E. coli* upon overexpression. The Hergenrother group developed a strategy to overcome this limitation by replacing the active site tyrosine with **6**, rendering the toxin inactive until a defined activation time-point post-expression and purification [53]. This approach of expressing toxic proteins as their benign, caged precursors may constitute a general method for obtaining otherwise hard-to-isolate proteins.

Summary

Optical control of proteins in cells and organisms with an expanded genetic code has provided precise, spatiotemporal regulation of a diverse range of protein functions. These include optical control of proteolysis, genome editing, protein splicing, phosphorylation, DNA recombination, RNA polymerization, neuronal activity, and protein translocation. In many of the applications discussed above, replacement of an essential residue with a photocaged analog in either an active site or a binding interface rendered the protein inactive. In instances where an essential residue cannot be identified, incorporation of a photocaged intein into the protein may provide an alternative approach for optical control. However, requirements and kinetics of the splicing event will need to be considered in experimental designs. In recent years, photocaged amino acids with improved optical properties, e.g., red-shifted excitation maxima, have been developed, further broadening the scope of this approach.

Compared to other means to optically control protein function, the site-specific, genetic encoding of photocaged and photoswitchable amino acids in cells and animals has several unique advantages: (i) the small size of the various caging groups (~150–250 Da) results in modification of only the most essential site of the protein of interest; (ii) the location of the caged residue can often be predicted based on structural and mechanistic protein data, thereby minimizing the need for extensive trial-and-error experiments; (iii) light-triggered removal of the caging group yields the native, wild-type protein; and (iv) only the unnatural photocaged/photoswitchable amino acid needs to be synthesized and the unnaturally modified protein is generated by the biosynthetic machinery within the cell or organism. Reversible control of protein function with light-switchable amino acids is promising and improved photostationary states and methods for translating the small configurational change of a single light-switchable amino acids into large changes in protein activity and structure will further improve the applicability of these tools. For structurally complex unnatural amino acids, protein yields can (depending on the protein and the site of incorporation) be significantly reduced, leaving room for improvement of the existing tRNA/tRNA synthetase expression systems. This is particularly important for expansion of photocaged amino acid mutagenesis into multicellular model organisms such as zebrafish, fruit flies, and mice which will enable enhanced developmental studies with spatio-temporal precision in order to better understand the complex underpinnings of metazoan development.

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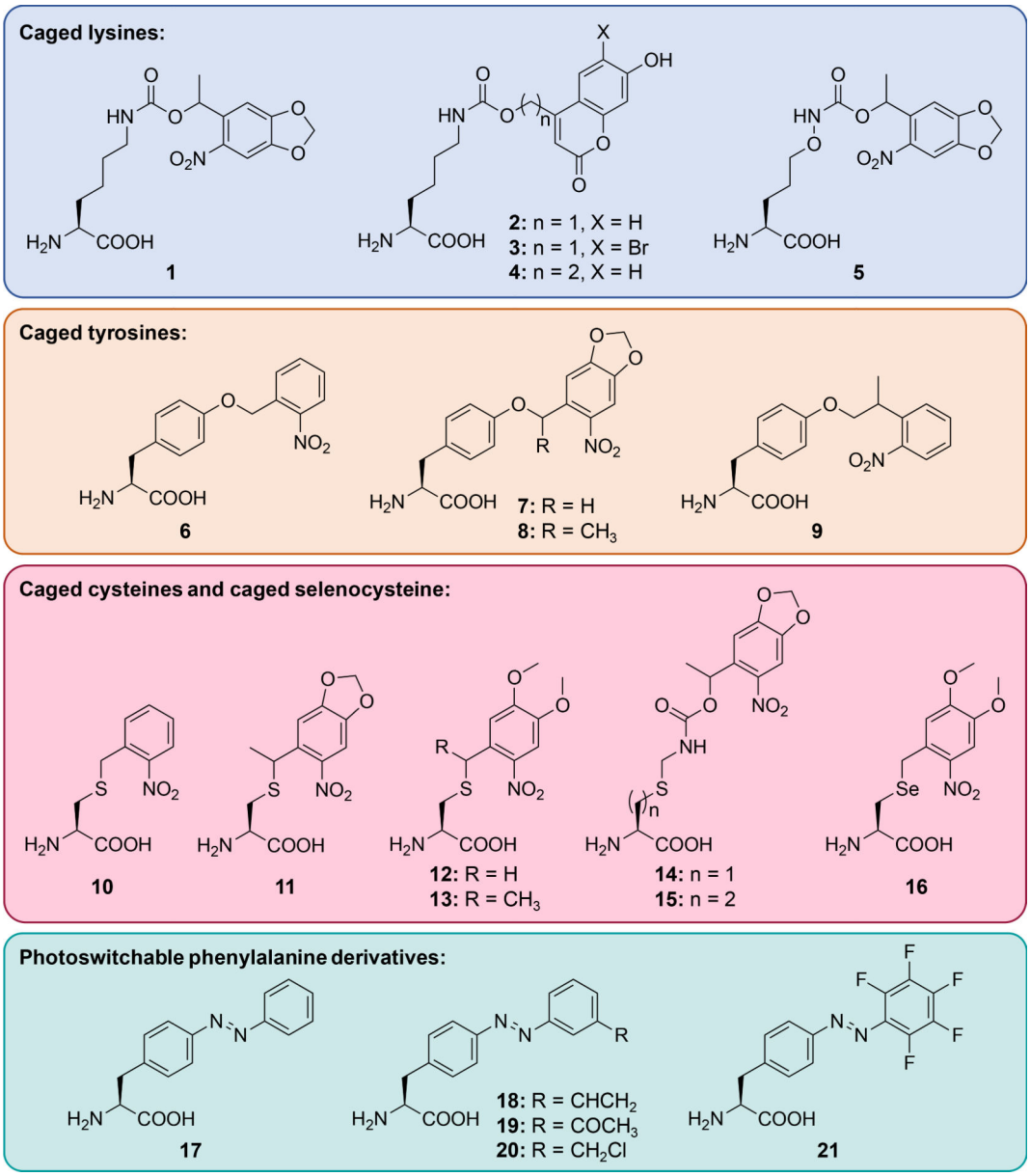

Figure 1. Genetically encoded, light-responsive unnatural amino acids.

These include caged lysines **1** - **5**, caged tyrosines **6** - **9**, caged cysteines **10** - **15**, caged selenocysteine **16**, and photoswitchable phenylalanine derivatives **17** - **21**.

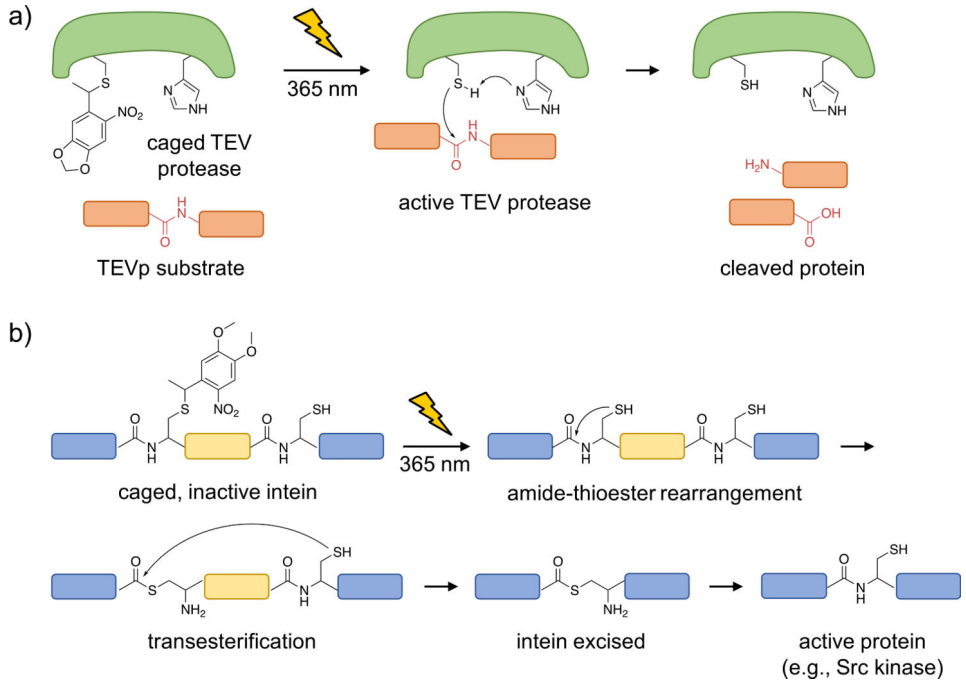

Figure 2. Optical control of TEV protease function and intein splicing using photocaged cysteine.

a) Upon caging of TEVp, the catalytic activity is blocked until photolysis of the caged, catalytically active cysteine, which performs a nucleophilic attack onto the protein substrate to generate cleaved fragments. b) A caged intein, indicated by the region in yellow, can be strategically placed within a protein (shown in blue; e.g., mCherry or Src kinase) such that it is misfolded and inactive prior to irradiation. Upon photo-activation, the native cysteine is generated and the intein is excised, leading to full-length, active protein (with only a cysteine scar).

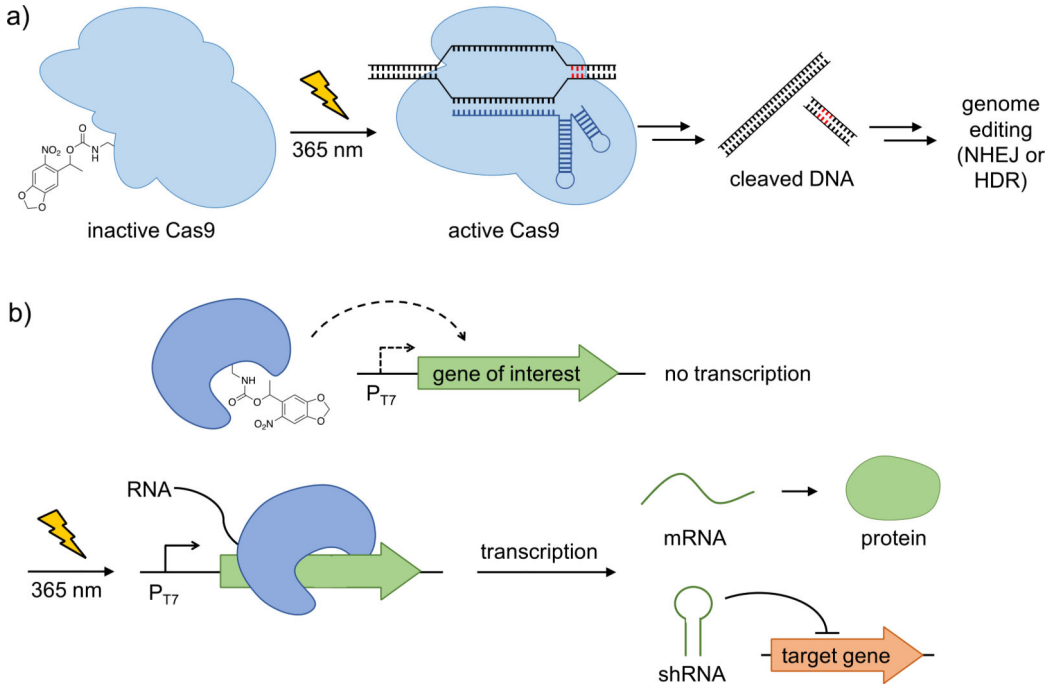

Figure 3. Optical control of nucleic acid processing via caged T7 RNA polymerase and CRISPR/Cas9.

a) CRISPR/Cas9 was rendered inactive by the incorporation of the photocaged lysine **1**, which blocks HNH nuclease activity. UV irradiation generates active Cas9, which results in DNA double strand cleavage and subsequent genomic editing. b) Caged T7 RNA polymerase is catalytically inactive, preventing transcription of genes under control of the T7 promoter. Upon UV-induced decaging, wild-type T7 RNA polymerase is restored and T7- promoter induced genes *(e.g.*, mRNAs to code for protein or shRNA for gene silencing) are transcribed. Adapted with permission from *Angew. Chem. Int. Ed., 55*, 5394. Copyright 2016 Wiley-VCH; and *J. Am. Chem. Soc., 135*, 13433. Copyright 2013 American Chemical Society.

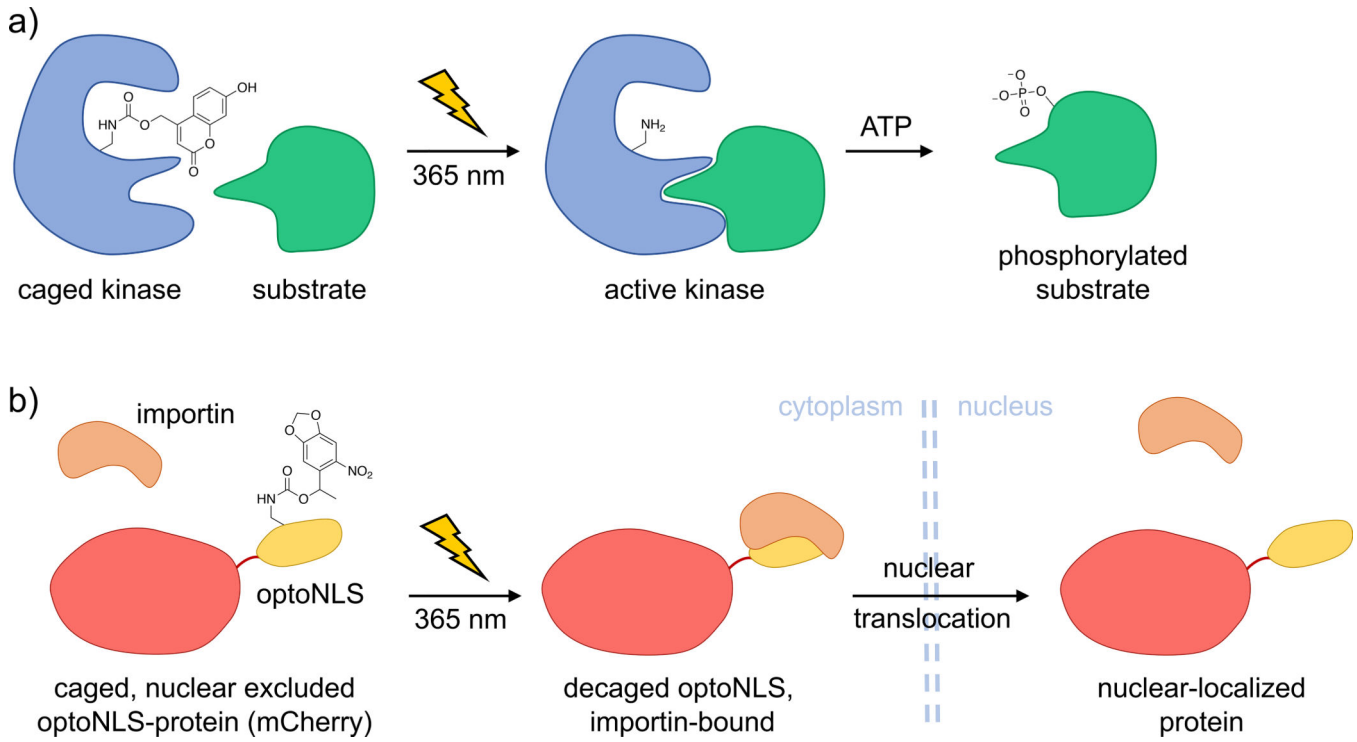

Figure 4. Optical control of kinase activity via genetic incorporation of the caged lysine 2 and optical control of nuclear translocation through caging of a protein-protein interaction.

a) Upon replacement of an essential lysine in the ATP-binding site of a kinase with the caged lysine **2**, the enzyme is rendered catalytically inactive. Following UV irradiation, the native, active kinase is generated and is capable of phosphorylating its downstream substrate. b) Replacement of the endogenous nuclear localization sequence (NLS) in the transcription factor FOXO3 (mCherry labeled) with an optoNLS sequence from SatB1 (indicated in yellow), containing a single caged lysine residue enables optical triggering of nuclear translocation.