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# Photocontrol of Tyrosine Phosphorylation in Mammalian Cells via Genetic Encoding of Photocaged Tyrosine

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Supporting Information

**ABSTRACT:** We report the first site-specific genetic encoding of photocaged tyrosine into proteins in mammalian cells. By photocaging Tyr701 of STAT1 we demonstrate that it is possible to photocontrol tyrosine phosphorylation and signal transduction in mammalian cells.

Signal transduction in mammalian cells transmits information about the environment to the nucleus of the cell, allowing the cell to mount an appropriate response. This process is mediated by cascades of phosphorylation on tyrosine, threonine, and serine residues within proteins and is misregulated in many diseases. To dissect the role of individual steps in signal transduction, it would be advantageous to be able to both rapidly activate protein kinases and rapidly reveal sites of protein phosphorylation. Several approaches have been reported for activating protein kinases in eukaryotic cells,[1−6] and we recently reported a method for activating protein kinases in mammalian cells with a millisecond pulse of light.[1] Genetic code expansion methods for photocaging serine residues in yeast have been reported,[7] allowing a serine residue that is a substrate for a kinase to be revealed with light. These approaches have provided insight into the kinetics and adaptation of signaling pathways that would otherwise be challenging to obtain.[1,7]

In principle it should be possible to genetically encode a photocaged tyrosine[8] in place of a tyrosine residue that is substrate for a protein kinase, making the protein refractory to phosphorylation and signal transmission. Illumination of the cell would remove the caging group from the tyrosine residue in the protein, allowing its phosphorylation. While photocaged tyrosine residues can be genetically encoded in *Escherichia coli*, and the conversion of *o*-nitrobenzyl-*O*-tyrosine (ONBY, **1**, Figure 1a) to tyrosine with light has been explicitly demonstrated,[8−10] the genetic encoding of photocaged tyrosine in eukaryotic cells, where it would be most useful for studying kinase signaling, has not been reported.

Here we demonstrate that the orthogonal pyrrolysyl-tRNA synthetase (PylRS/$tRNA_{CUA}$) pair from *Methanosarcina* species[11−13] can be evolved in *E. coli* to incorporate **1**.[14] We show that this amino acid can be incorporated in mammalian cells using the evolved synthetase and allows the photocontrol of STAT1 phosphorylation in interferon mediated signal transduction.

To evolve the orthogonal PylRS/$tRNA_{CUA}$ pair[15] for the incorporation of **1**, we created a library of *Methanosarcina barkeri* (Mb) PylRS mutants in which five positions within the active site (L270, Y271, L274, N311 and N313) are randomized to all possible amino acids (Supporting Information, SI). We performed three rounds of alternating positive and negative selection in *E. coli* on this library, as previously described.[15] Ten out of the 96 clones we screened from the final round of positive selection conferred survival on 350 $\mu$g/mL of chloramphenicol in the presence of **1**. These clones did not survive at 50 $\mu$g/mL in the absence of **1** (Figure S1, SI). These data suggest the selected synthetases direct the efficient incorporation of **1**. Sequencing reveals that the majority of the selected synthetases contain four mutations: L270F, L274M, N311G, and C313G, with respect to MbPylRS (Table S1, SI). We named the evolved synthetase ONBYRS. In *E. coli*, the ONBYRS/$tRNA_{CUA}$ pair enables the incorporation of **1** in response to the amber codon in sfGFP(150TAG)$His_6$, yielding 3 mg/L of sfGFP-**1**-$His_6$ (Figure 1b). This yield is comparable to the yield obtained for other well-incorporated and useful unnatural amino acids.[15−23]

Since the PylRS/$tRNA_{CUA}$ system is orthogonal in eukaryotic cells,[1,22−25] we transplanted the mutations for the ONBYRS system into a mammalian expression construct, creating pONBYRS-mCherry-TAG-EGFP-HA (encoding ONBYRS and mCherry-amber stop codon (TAG)-enhanced green fluorescent protein (EGFP)-hemagglutinin (HA) tag, both under control of the CMV promoter).[22] To demonstrate the incorporation of **1** in mammalian cells, we introduced pONBYRS-mCherry-TAG-EGFP-HA and p4CMVE-U6-PylT (encoding four copies of PylT, the gene for Mb $tRNA_{CUA}$) into human embryonic kidney (HEK) 293 cells. We detected mCherry expression in the presence or absence of **1**, but EGFP fluorescence only in the presence of **1** (Figure 1c). Anti-HA immunobloting confirms that expression of mCherry-TAG-EGFP-HA is dependent on **1** (Figure 1d). Additional control experiments demonstrate that the incorporation efficiency of **1** in mammalian cells is comparable to that of *Nε-tert*-butyloxycarbonyl-L-lysine (**2**), an established, good substrate for the wild-type PylRS/$tRNA_{CUA}$ pair (Figure 1d). Full-length protein was immunoprecipitated using an antibody against GFP and mass spectrometry conclusively demonstrated that amino acid **1** is incorporated at the genetically encoded site in mammalian cells (Figure 1e).

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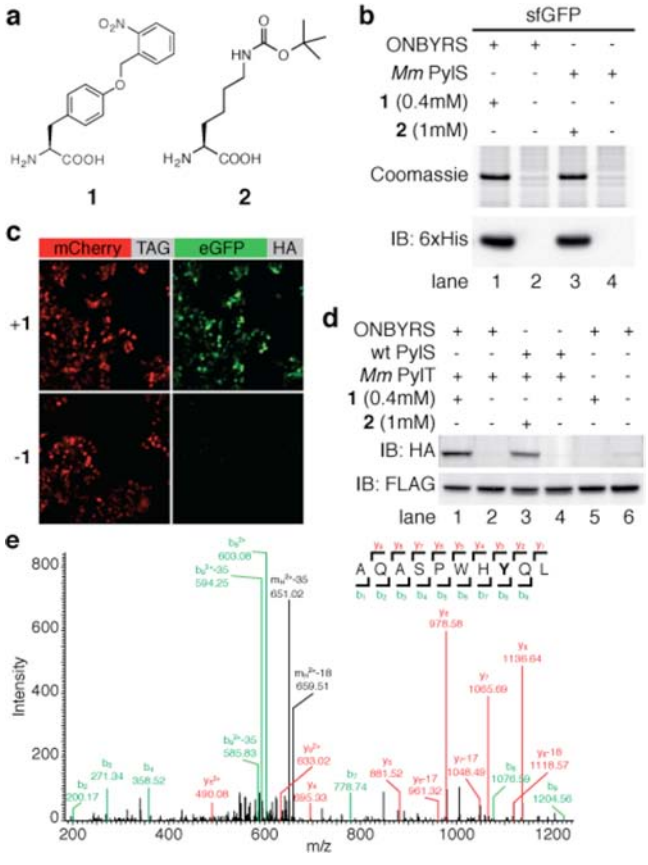


**Figure 1.** Genetic incorporation of photocaged tyrosine using evolved ONBYRS/tRNA$_{CUA}$ pair. (a) Chemical structures of **1** and **2**. (b) Coomassie staining and immunoblot of total-cell extracts from *E. coli* expressing sfGFP(150TAG)His$_6$. Protein coexpressed with the ONBYRS/tRNA$_{CUA}$ pair in the presence or absence of **1** (0.4 mM) (lanes 1, 2) or with the MbPylRS/tRNA$_{CUA}$ pair in the presence or absence of **2** (1 mM) (lanes 3, 4). Data derived from equal number of cells, as judged by $OD_{600}$. (c) Fluorescence confocal microscopy images of HEK293 cells coexpressing the ONBYRS/tRNA$_{CUA}$ pair and mCherry-TAG-EGFP-HA in the presence or absence of **1**. (d) Western blot analysis of HEK293 cells coexpressing mCherry-TAG-EGFP-HA together with the ONBYRS/tRNA$_{CUA}$ pair (lanes 1, 2, 5, 6) or the PylRS/tRNA$_{CUA}$ pair (lanes 3, 4), in the presence or absence of **1** or **2**, respectively. Expression of ONBYRS or PylRS is confirmed by detection with an anti-FLAG antibody. (e) MS/MS fragmentation spectrum of purified and trypsin digested mCherry-TAG-EGFP-HA expressed in HEK293 cells. The position of the photocaged tyrosine in the detected tryptic peptide is marked by a bold **Y**.

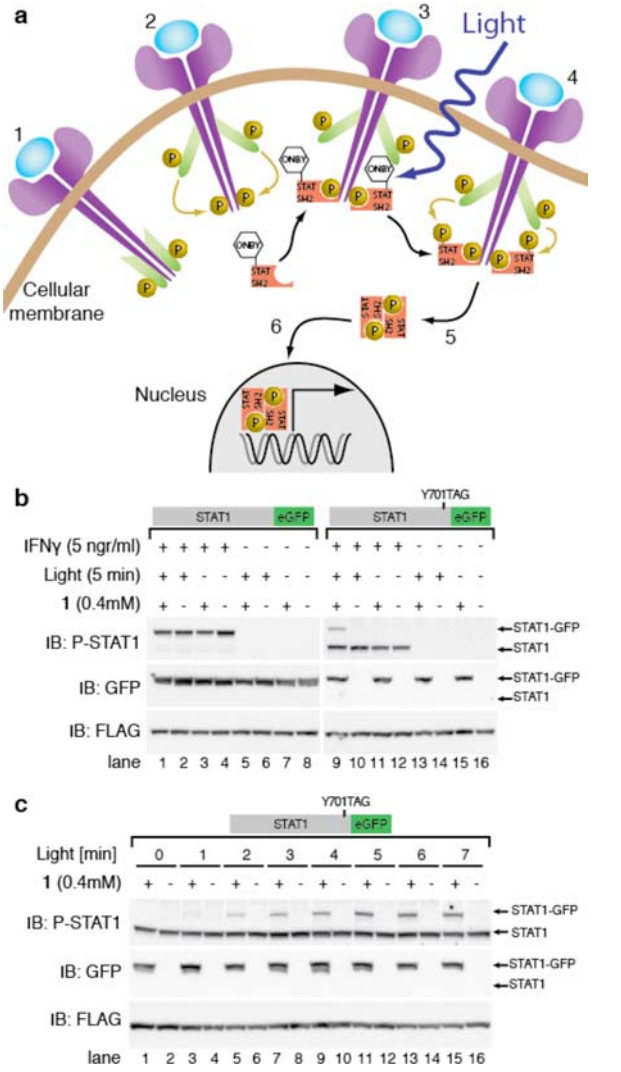


**Figure 2.** Photocontrol of STAT1 Tyr701 phosphorylation. (a) The canonical model for the JAK/STAT pathway. Binding of cytokine drives cytokine-receptor dimerization, mutual phosphorylation of intracellular JAK (1), and receptor phosphorylation (2), followed by binding of (photocaged) STAT (3). Decaging of ONBY protecting group with light allows the phosphorylation (4), dimerization (5), and accumulation of STAT in the nucleuss (6). Figure adapted from Levy and Darnell.[26] (b) HEK cells coexpressing the ONBYRS/tRNA$_{CUA}$ pair and STAT1-EGFP (lanes 1–8) or STAT1-Y701TAG-EGFP (lanes 9–16). In contrast to STAT1-EGFP, the expression of full-length STAT1-Y701TAG-EGFP is dependent on the addition of **1** (IB: GFP). The Y701-phosphorylated version of endogenous STAT1 and STAT1-EGFP can be detected only in cells stimulated with 5 ng/mL of IFN-$\gamma$ 1 h before lysis (lanes 1–4, 9–12, IB: P-STAT1). Phosphorylation of Tyr701-photocaged STAT1-Y701TAG-EGFP is detected only in cells incubated with **1**, stimulated with IFN-$\gamma$, and exposed to 365 nm light (lane 9). ONBYRS expression was verified with anti-FLAG immunobloting and served as loading and transfection control. (c) The phosphorylation level of STAT1-Y701TAG-EGFP increases with time of exposure to 365 nm light and reached saturation after 5 min (Figure S2, SI).

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway responds to stimulation by cytokines, such as interferon, leading to transcriptional regulation of target genes (Figure 2a).[26,27] Signal transduction is initiated by the binding of cytokines to specific cell-surface cytokine receptors. This promotes receptor dimerization and trans-phosphorylation of JAK kinases, which are bound to the intracellular domain of the receptor. Activated JAK kinases phosphorylate tyrosine residues in the cytoplasmic domain of the receptors. The phosphorylated receptor then provides binding sites for the Src-homology-2 (SH2) domain of STAT. Receptor-bound STAT proteins are then phosphorylated by the activated JAK kinases on tyrosine 701 (STAT1 numbering).[28] STAT phosphorylation promotes its release from the receptor and STAT monomers then dimerize via interactions between the SH2 domain of each monomer and the phosphotyrosine of the other monomer. Conformational changes induced by dimerization allow STAT to interact with nuclear import factors, and the resulting nuclear STAT dimers lead to specific transcriptional responses. In recent years it has become clear that signaling through the JAK/STAT pathway is more complicated than suggested by the canonical model.[29−31]


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We demonstrated photocontrol of STAT1 phosphorylation in HEK293 cells cotransfected with p4CMVE-U6-PylT and pMmONBYRS-STAT1-Y701TAG-EGFP (expressing ONBYRS and Y701TAG STAT1 as a C-terminal EGFP fusion).

Interferon stimulation of HEK cells transfected with p4CMVE-U6-PylT and pMmONBYRS-STAT1-EGFP (expressing ONBYRS and nonmutated STAT1 as a C-terminal EGFP fusion, Figure 2b) led to phosphorylation of STAT1-EGFP and endogenous STAT1, and control experiments demonstrate that this phosphorylation is not induced by the addition of the amino acid **1** or by light or by the combination of **1** and light (Figure 2b, lanes 1−8). HEK cells transfected with p4CMVE-U6-PylT and pMmONBYRS-STAT1-Y701TAG-EGFP led to production of full-length STAT1-**1**-EGFP only in the presence of **1**, as judged by anti-GFP Western blot (Figure 2b, lanes 9−16). Interferon treatment led to phosphorylation of endogenous STAT1 but was not sufficient to elicit phosphorylation of STAT1-**1**-EGFP (Figure 2b, lanes 10−12). When cells expressing STAT1-**1**-EGFP are simultaneously treated with interferon and illuminated, we observe accumulation of phosphorylated STAT1-**1**-EGFP (Figure 2b, lane 9). The phosphorylation of STAT1-Y701TAG-EGFP was dependent on illumination time and approached a saturation level after 5 min (Figure 2c and Figure S2, SI). When cells in which STAT1-EGFP is overexpressed are treated with IFN-$\gamma$, STAT1-EGFP is the major phosphorylated species detected, though phosphorylation of endogenous STAT1 is detected. In contrast, in cells expressing STAT1-Y701TAG-EGFP, the levels of STAT1 incorporating **1** that can be phosphorylated upon treatment with IFN-$\gamma$ and light are close to the levels of endogenous phosphorylated STAT1. Taken together these observations demonstrate that we can control the phosphorylation of STAT1-**1**-EGFP with light in live mammalian cells.

Our results demonstrate that it is possible to genetically encode the site-specific incorporation of photocaged tyrosine into proteins in mammalian cells using an evolved pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair. We have shown that this allows tyrosine phosphorylation to be controlled using light and created a system in which the phosphorylation of STAT1-**1**-EGFP displays Boolean AND logic with both light and interferon as inputs. This approach, in combination with approaches for rapid control of serine phosphorylation and protein kinases,[1−7] should provide a powerful set of tools for dissecting the kinetics and contributions to adaptive processes of elementary steps in kinase signaling. While we have focused on photocontrolling tyrosine phosphorylation, tyrosine residues are important in many active sites, and it may be possible to photocontrol enzymatic activity in eukaryotic cells. Notably, it will be possible to photoactivate Cre-mediated recombination or nuclease action.[9,10] Since the pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair can be used to site-specifically incorporate unnatural amino acids in multiceullar model organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster*,[32,33] it may be possible to use extensions of this approach to photocontrol signaling and enzymatic processes in optically targeted single cells in living animals.

## ■ ASSOCIATED CONTENT

### Supporting Information

Table S1, Figures S1−S2, and materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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