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Genetically Encoded Photocontrol of Protein Localization in Mammalian Cells

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Precise photochemical control of protein function can be achieved through the site-specific introduction of caging groups.[1,2] Chemical and enzymatic methods, including in vitro translation[3] and chemical ligation,[4] have been used to photocage proteins in vitro. These methods have been extended to allow the introduction of caged proteins into cells by permeabilization[5] or microinjection,[6] but cellular delivery remains challenging. Recently, *o*-nitrobenzyl (ONB)-caged versions of several amino acids have been genetically encoded in response to the amber stop codon.[7,8] The ONB group is stable under physiological conditions but readily removed with 250−365 nm UV light.

Since lysine residues are key determinants for nuclear localization sequences,[9] the target of key post-translational modifications[10] (including ubiquitination, methylation, and acetylation), and key residues in many important enzyme active sites, we were interested in photocaging lysine to control protein localization, post-translational modification, and enzymatic activity. Photochemical control of the functions mediated by lysine residues in proteins has not previously been demonstrated in intact living cells.

We designed the caged lysine **1** (Figure 1A; see the Supporting Information for a detailed description of the synthesis), since it decages efficiently when irradiated with 365 nm light (as a result of the bathochromic shift in the absorbance spectrum of the caging group induced by the electron-donating substituents).[2] The application of nonphototoxic light to decage **1** prevents potential photoreactions of nucleic acids, destruction of disulfides, and other cellular damage, which may occur when a simpler ONB group is used to cage lysine.[8] Moreover, photolysis of **1** generates a ketone byproduct that, unlike the photolysis byproducts of the simple ONB-caged lysine,[8] does not undergo an undesired condensation with the ε-amino group of lysine. Here we synthesized **1** and evolved a pyrrolysyl-tRNA synthetase/tRNA pair to genetically encode the incorporation of this amino acid in response to an amber codon in mammalian cells. To exemplify the utility of this amino acid, we caged the nuclear localization sequences (NLSs) of nucleoplasmin and the tumor suppressor p53 in human cells, thus mislocalizing the proteins in the cytosol. We triggered protein nuclear import with a pulse of light, allowing us to directly quantify the kinetics of this process.

To evolve the orthogonal *Mb*PylRS/tRNA$_{CUA}$ pair[11] for the incorporation of the photocaged lysine **1** in response to an amber codon, we first created a library of 10^8 mutants of *Mb*PylRS in which 5 positions (M241, A267, Y271, L274, and C313) in the binding pocket of the pyrrolysine ring were randomized to all possible amino acids. We performed three rounds of alternating positive and negative selection on this library in *Escherichia coli*, as previously described.[11,12] Clones that survived the selection were transformed with a plasmid encoding the chloramphenicol resistance gene with an amber codon at a permissive position. The best clones allowed cells to survive on media containing up to 300 μg/mL chloramphenicol in the presence of **1** (1 mM) but did not survive at 50 μg/mL in the absence of **1**. This demonstrated that the selected synthetases have a high specificity for **1** and do not appreciably incorporate any of the common 20 amino acids. The most active synthetase contained the mutations M241F, A267S, Y271C, and L274M with respect to wild-type *Mb*PylRS. This synthetase was named photocaged Lysyl-tRNA synthetase (PCKRS) and was further characterized (see Table S1 in the Supporting Information for all of the variant *Mb*PylRS sequences isolated). Expression of myoglobin and sfGFP genes containing an amber codon at a permissive site[11,12] in the presence of PCKRS/tRNA$_{CUA}$ was efficient and dependent on the addition of **1**. Electrospray ionization mass spectrometry (ESI-MS) and MS/MS sequencing confirmed the incorporation of **1** at a single genetically encoded site (Figure S1 in the Supporting Information). We confirmed that myoglobin containing **1** is efficiently decaged upon irradiation with 365 nm light in vitro (Figure S1E).

To achieve the site-specific incorporation of **1** into proteins in eukaryotes, we required a system to express the orthogonal PCKRS/tRNA$_{CUA}$ pair in mammalian cells.[8,13] Since *PylT*, the gene encoding the pyrrolysine tRNA$_{CUA}$, lacks the consensus internal RNA polymerase III promoter sequences found in eukaryotic tRNAs,[14] it requires an external promoter for transcription. We placed the tRNA expression under the control of a U6 promoter downstream of a CMV enhancer,[15] enabling efficient transcription of *PylT*. To demonstrate that the PCKRS/tRNA$_{CUA}$ pair is functional in human embryonic kidney (HEK293) cells, we examined the red and green fluorescence of cells containing *mCherry-TAG-egfp-ha* (this reporter contains an N-terminal mCherry gene, a linker containing an amber stop codon, a C-terminal enhanced green fluorescent protein (EGFP) gene, and the HA-tag coding sequence) and the PCKRS/tRNA$_{CUA}$ pair in the presence and absence of **1** (Figure 1B). As expected, mCherry fluorescence was detected with or without **1**, but EGFP fluorescence was observed only upon addition of **1** (1 mM). This confirmed that mammalian synthetases do not aminoacylate the pyrrolysine tRNA$_{CUA}$ appreciably in human cells[13] and is consistent with the suppression of the amber codon by the PCKRS/tRNA$_{CUA}$ pair using **1**. Control experiments lacking the pyrrolysine tRNA$_{CUA}$ or PCKRS demonstrated that both are required for amino acid incorporation (Figure S2). Western blot analysis (Figure 1C) showed that the efficiency of incorporation of **1** using the PCKRS/tRNA$_{CUA}$ pair is comparable to the efficiency of incorporation of tyrosine using a human tyrosine amber suppressor tRNA (TyrtRNA$_{CUA}$), which is efficiently aminoacylated by the endogenous human tyrosyl-tRNA synthetase. Similar results were obtained with the *Mb*PylRS/tRNA$_{CUA}$ pair and ε-Boc-protected lysine, a known substrate of PylRS[13,16] (Figure S3). The site-

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specific incorporation of **1** into mCherry-EGFP-HA in mammalian cells was further confirmed by MS/MS sequencing (Figure 1D).

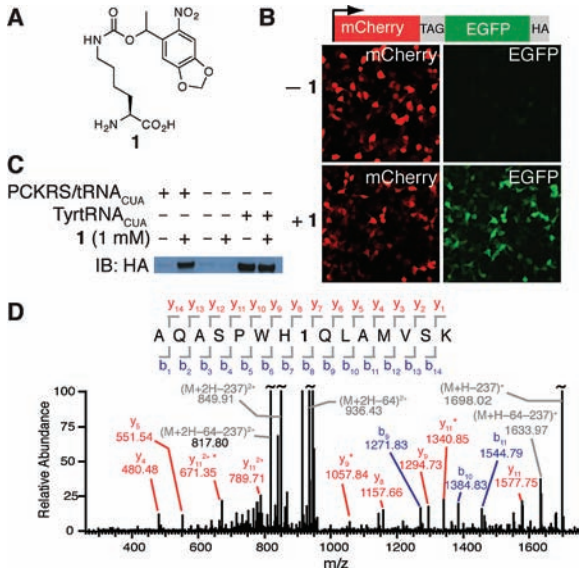

Figure 1. Genetic incorporation of a photocaged lysine in mammalian cells. (A) Photocaged lysine **1**. (B, C) The PCKRS/tRNA$_{CUA}$ pair allows for the specific incorporation of **1** (1 mM) in response to an amber codon in HEK293 cells: (B) fluorescence confocal micrographs of HEK293 cells expressing *mCherry-TAG-egfp-ha* and PCKRS/tRNA$_{CUA}$ without and with **1**; (C) immunoblot (IB) of cells from (B) with anti-HA antibody. TyrtRNA$_{CUA}$ is the human tyrosine amber suppressor tRNA. (D) mCherry-EGFP-HA incorporating **1** expressed in HEK293 cells was purified by anti-HA immunoprecipitation for subsequent MS/MS analysis. The MS/MS fragmentation spectrum of a tryptic peptide derived from the purified protein confirms the incorporation of **1** at the expected site. Fragments labeled with an asterisk (*) result from decaging of peptide fragments during the MS/MS analysis.

To demonstrate the applicability of **1** for functional studies in mammalian cells, we first investigated its utility in the photochemical control of nuclear import processes. Specifically, we investigated the kinetics of nuclear import driven by the classical bipartite nuclear localization signal (NLS) of nucleoplasmin[17] by caging a key lysine residue involved in importin-α binding (Figure 2A). We generated constructs allowing the expression of GFP-HA with an N-terminal wild-type NLS (*nls-gfp-ha*), with an NLS mutant in which a key lysine was replaced by an alanine (*nls-A-gfp-ha*), and with an NLS mutant in which the target lysine was replaced by an amino acid encoded in response to the amber codon (*nls-*-gfp-ha*). Expression of full-length protein incorporating **1** in response to the amber codon (NLS-*-**1**-GFP-HA) from *nls-*-gfp-ha* was dependent on the addition of the PCKRS/tRNA$_{CUA}$ pair and **1** (Figure 2B). We next confirmed by fluorescence imaging that the photocaged lysine **1** blocks the NLS function as efficiently as the alanine mutation, leading to partial relocalization of the GFP fusions to the cytoplasm (Figure 2C). GFP was still present in the nucleus because of passive diffusion. Upon photolysis of NLS-*-**1**-GFP-HA (1 s, 365 nm, 1.2 mW/cm^2), we observed nuclear import of cytoplasmic GFP as a result of the decaging and subsequent nuclear import of GFP (Figure 2C,D and Movie S1 in the Supporting Information). Quantification of 27 representative cells showed a 3.75-fold increase in the ratio of nuclear to cytoplasmic protein following photolysis (Figure 2D). Real-time fluorescence microscopy following photolysis allowed us to measure a half-time of ~20 s for the import of cytosolic GFP (Figure 2E and Movie S1). Irradiation of cells expressing NLS-A-GFP-HA did not lead to any GFP relocalization (Figure 2C). Similar results were obtained when the amber codon in *nls-*-gfp-ha* was suppressed with TyrtRNA$_{CUA}$ (not shown). These results demonstrated that the relocalization is fast and results from specific decaging of **1** upon photolysis.

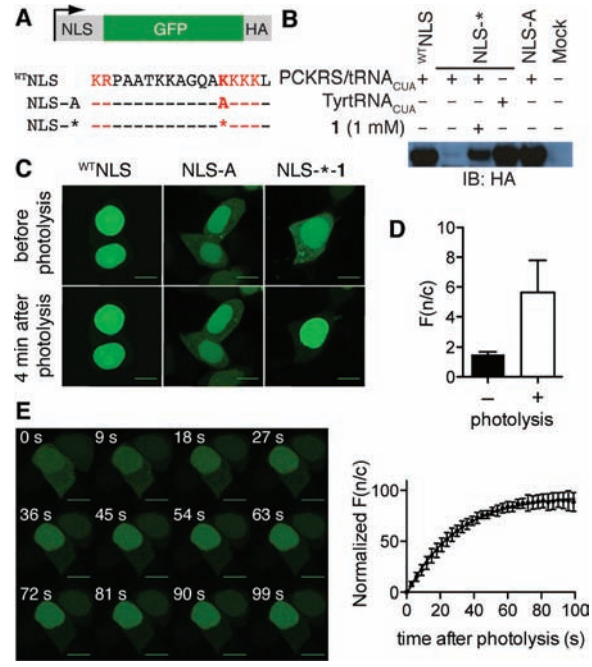

Figure 2. Photocontrol of protein localization. (A) Bipartite nuclear localization signal (NLS) of nucleoplasmin: the codon for lysine in bold was mutated to an alanine codon (NLS-A) or replaced by an amber stop codon (NLS-*). (B) The PCKRS/tRNA$_{CUA}$ pair allows specific incorporation of **1** (1 mM) in response to the amber codon in *nls-*-gfp-ha* (lanes 2 and 3). Controls: expression of ^{WT}NLS-GFP-HA (lane 1), NLS-A-GFP-HA (lane 5), expression of NLS-*-Y-GFP (Y incorporation using Tyr-tRNA$_{CUA}$) (lane 4), and nontransfected cells (lane 6). (C) Fluorescence confocal micrographs showing cellular localization of the GFP fusions (photolysis conditions: 1 s, 365 nm, 1.2 mW/cm^2). (D) Ratio F(n/c) of the mean nuclear and cytoplasmic GFP fluorescence before and 4 min after photolysis in the case of NLS-*-**1**-GFP-HA [data represent mean ± standard deviation (SD) for 27 cells; see Figure S4 for representative examples]. (E) Kinetic analysis of the nuclear import process (see Movie S1): the graph shows the normalized F(n/c) as a function of time (mean ± SD for four cells). A half-time of 20 s was determined. Scale bars represent 10 μm.

To begin investigating the utility of the photocaging approach in more complicated systems and investigate the effect of caging one lysine in a system that is regulated by numerous pathways, we next used the photocaged lysine **1** to control the nuclear import of the tumor suppressor p53. p53 nuclear import is carried out by a bipartite NLS, and K305 is a crucial determinant of nuclear localization[18] (Figure 3A).

We generated constructs allowing the expression of p53 with a C-terminal EGFP-HA tag (*p53-egfp-ha*) and p53 mutants with either the mutation K305A (*p53*-K305A-*egfp-ha*) or an amber codon (*p53*-K305*-*egfp-ha*). The production of full-length p53-EGFP-HA protein from *p53*-K305*-*egfp-ha* was dependent on the addition of the PCKRS/tRNA$_{CUA}$ pair and **1**, confirming the incorporation of **1** in response to the amber codon at position 305 of p53. Western blots (Figure 3B) demonstrated that the levels of p53 containing **1** were comparable to, but slightly lower than, endogenous p53 levels.

We confirmed by fluorescence imaging that p53-EGFP-HA is localized in the nucleus and that p53-K305A-EGFP-HA is mainly localized in the cytosol, as previously reported[18] (Figure 3C). When *p53*-K305*-*egfp-ha* was expressed together with the PCKRS/tRNA$_{CUA}$ pair in presence of **1** (1 mM), we observed that p53 was mainly localized in the cytosol (Figure 3D and Figure S5). This

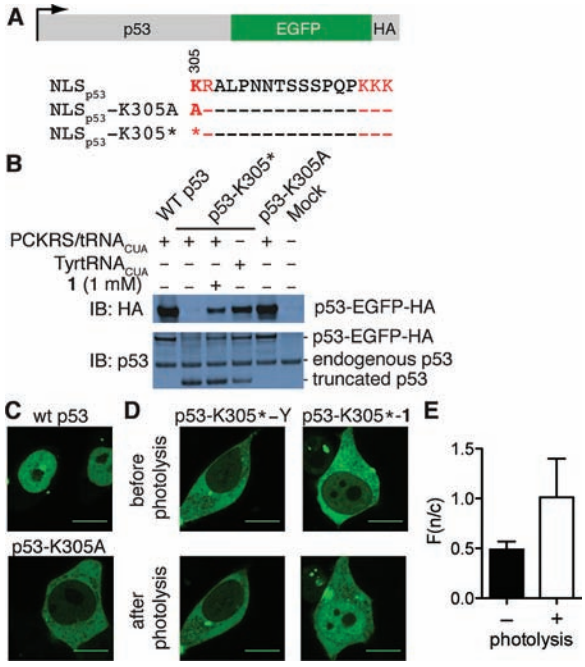

Figure 3. Photocontrol of p53 localization. (A) Bipartite NLS of p53 (NLS_{p53}): the lysine K305 in bold was mutated to alanine (NLS_{p53}-K305A) or replaced by an amber stop codon (NLS_{p53}-K305*). (B) The PCKRS/$tRNA_{CUA}$ pair allows the specific incorporation of **1** (1 mM) in response to the amber codon in p53-K305*-EGFP-HA in HEK293 cells (lanes 2 and 3). Controls: expression of p53-EGFP-HA (lane 1) and p53-K305A-EGFP-HA (lane 5), expression of p53-K305*-Y-EGFP-HA (Y incorporation using Tyr-$tRNA_{CUA}$) (lane 4), and nontransfected cells (lane 6). (C) Fluorescence confocal micrographs showing cellular localization of the EGFP fusions of wild-type p53 and p53-K305A. (D) Confocal micrographs showing cellular localization of the EGFP fusions before and 50 min after photolysis (5 s, 365 nm, 1.2 mW/cm^2). (E) Ratio F(n/c) of the mean nuclear and cytoplasmic EGFP fluorescence before and 30 min after photolysis in the case of p53-K305*-**1**-EGFP-HA (data represent mean ± SD for seven cells). Scale bars represent 10 μm.

demonstrated that the function of the p53 NLS signal was effectively abrogated through introduction of a single caged lysine. Upon photolysis (5 s, 365 nm, 1.2 mW/cm^2), we observed progressive nuclear import of cytoplasmic p53 as a result of the decaging and subsequent nuclear import of p53 (Figure 3D,E, Figure S5, and Movie S2). In line with the greater complexity of this system, we observed greater cell-to-cell variability in nuclear import than in the nucleoplasmin case. In control experiments, we incorporated ε-Boc-protected lysine or tyrosine in response to the amber codon at position 305 of p53. These p53 variants were localized in the cytoplasm and did not localize in the nucleus following photolysis (Figure 3D and Figure S5), confirming that the relocalization results from the specific decaging of **1**.

In conclusion, we have demonstrated the synthesis and site-specific genetic incorporation of the new photocaged lysine **1** into proteins in human cells. We have used this amino acid to cage nuclear localization signals and measure the kinetics of nuclear import via the photochemical control of protein localization in human cells using a rapid pulse of nonphotodamaging UV irradiation.

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Supporting Information Available: Movies S1 and S2, Table S1, Figures S1−S5, and Materials and Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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