LETTER
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Light-Activated Cre Recombinase as a Tool for the Spatial and Temporal Control of Gene Function in Mammalian Cells

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ABSTRACT Cre recombinase catalyzes DNA exchange between two conserved lox recognition sites. The enzyme has extensive biological application, from basic cloning to engineering knock-out and knock-in organisms. Widespread use of Cre is due to its simplicity and effectiveness, but the enzyme and the recombination event remain difficult to control with high precision. To obtain such control we report the installation of a light-responsive *o*-nitrobenzyl caging group directly in the catalytic site of Cre, inhibiting its activity. Prior to irradiation, caged Cre is completely inactive, as demonstrated both *in vitro* and in mammalian cell culture. Exposure to non-damaging UVA light removes the caging group and restores recombinase activity. Tight spatio-temporal control over DNA recombination is thereby achieved.

Site-specific recombinase proteins catalyze strand exchange between defined target sequences on each of two DNA segments (*1*). The well-characterized Cre recombinase enzyme recognizes two palindromic *loxP* sites consisting of two 13-bp repeats flanking an 8-bp asymmetric core; orientation of the core determines deletion, insertion, or inversion of the intervening DNA (Figure 1) (*2*).

Cre has been used in a variety of organisms (*e.g.*, in mice, zebrafish, drosophila, and plants) (*3–7*), toward silencing or inducing gene expression, and has been an invaluable tool for engineering knock-outs and conditional alleles (*3, 8–12*).

While the widespread use of the Cre/*loxP* system is a result of its simplicity and effectiveness, limitations exist in achieving tight control over Cre activity and thus the recombination event. Attempts to regulate Cre activity temporally have involved the use of inducible systems, and spatial control has been achieved with tissue-specific promoters. However, these systems have a variety of limitations (*12*), including the toxicity of inducers, leakiness of the gene expression system, and the unavailability of promoters for every tissue. Importantly, spatial control of the enzyme cannot be achieved on the level of a single cell. Alternatively, Cre has been fused to ligand binding domains that are regulated by small molecules, such as tamoxifen (*13*). However, small molecules are susceptible to problems with diffusion into and out of cells, leading to subsequent off-target recombination. To solve these problems and to provide highly stringent spatial and temporal control with high Cre activity, we herein describe the use of a light-responsive protecting group, termed a caging group, directly installed on an essential amino acid in the catalytic site of the enzyme itself.

Photocaging has become increasingly important in the regulation of gene function (*14–19*), as caging groups can be placed on a biologically active molecule to inhibit its nascent activity. Exposure to non-photodamaging UVA light (*20, 21*) removes the caging group and restores function to the previously inactive molecule. As light can be precisely regulated, photocaging affords a high level of spatial and temporal control over the activity of the biomolecule. Previously, Cre activity was regulated using photocaged tamoxifen (*22*); however, the necessity for multiple irradiations and a low recombination activity, presumably due to diffusion of tamoxifen out of cells, were problematic.

Cre utilizes a nucleophilic tyrosine at position 324 to catalyze sequential strand exchange among its cognate loxP sites *via* formation of a covalent protein-DNA intermediate (Figure 2) (*23, 24*). We hypothesized that the presence of a caging group on the nucleophilic hydroxyl group of Tyr324 would render Cre completely inactive and thus inhibit recombination. Light irradiation would then remove the caging group and restore Cre activity.

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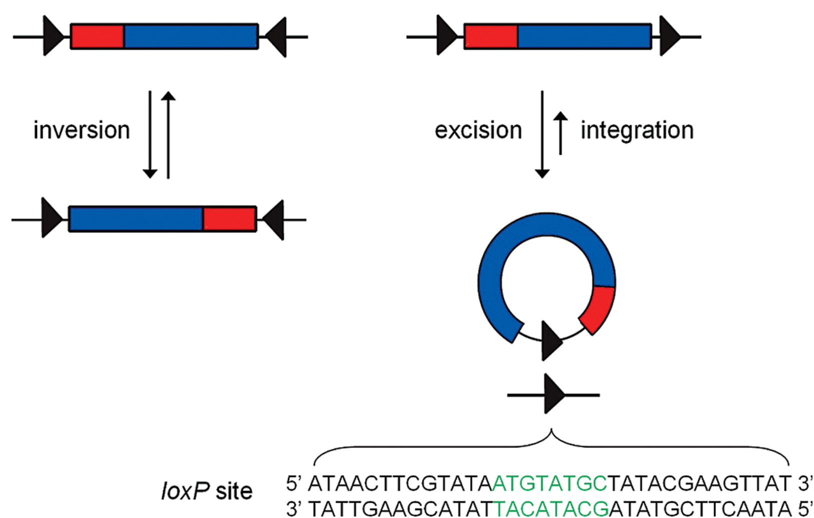

Figure 1. Cre recombinase catalyzes the inversion or excision/integration of DNA between two *lox*P sites. Orientation of the 34-bp *lox*P sites determines whether the intervening DNA will be inverted or removed/integrated. The *loxP* site is composed of two 13-bp inverted repeat elements to which the Cre protein binds and a spacer region (green) separating them, where the DNA cleavage and reunion occurs.

We utilized unnatural amino acid mutagenesis (*25–27*) with the protein biosynthetic machinery of *E. coli* to selectively incorporate a photocaged *o*-nitrobenzyl tyrosine (ONBY) (*28*) at position Tyr324. A wild-type Cre expression vector (pET-21Cre) was constructed, and Tyr324 was mutated to an amber stop codon, TAG324 (pET-21CreY324TAG). Using a previously evolved tRNA/tyrosyl-tRNA synthetase pair from *Methanococcus jannaschii* (*28*) for the recombinant expression in the presence of ONBY yielded photocaged Cre-ONBY, while expression in the absence of ONBY did not result in any Cre protein (Figure 3).

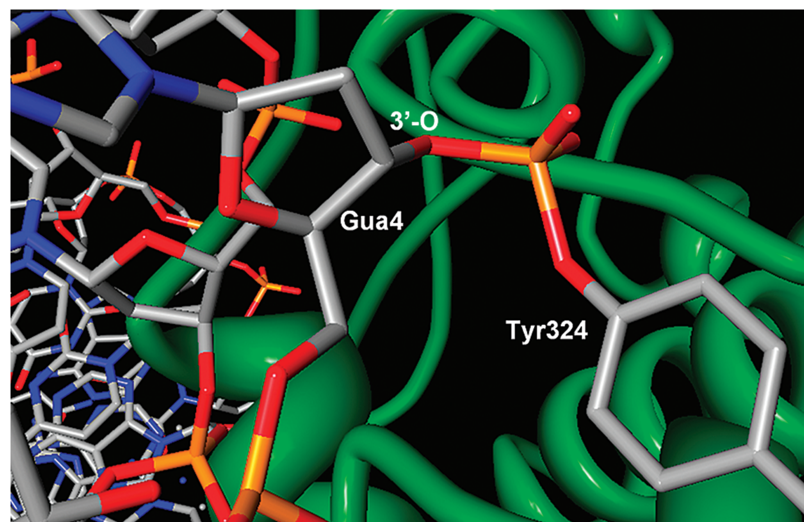

Figure 2. Cre recombinase cleaves DNA strands by the nucleophilic attack of Tyr324 onto the DNA phosphodiester backbone, forming a covalent bond between the 3′-O of guanosine and the Tyr-OH group (PDB 1Q3V).

To verify that our expressed Cre recombinase behaved as expected, assays were performed with the linear substrate pLox2+ (New England Biolabs), composed of two *loxP* sites flanking a β-lactamase gene (*bla*) and an origin of replication (ORI). Cre excises the *bla* and ORI, generating a circular plasmid that upon transformation confers ampicillin resistance to *E. coli*. Cre activity is subsequently determined by counting colonies on ampicillin-containing plates. In separate reactions, wild-type Cre and Cre-ONBY were kept in the dark or irradiated with an LED fiber optic lamp (365 nm, 20 min) and then incubated at 37 °C for 30 min, followed by heat inactivation and transformation. Wild-type Cre and irradiated Cre-ONBY both yielded >1000 colonies upon transformation, while both non-irradiated Cre-ONBY and the pLox2+ control yielded <15 colonies. This confirmed that photocaged Cre-ONBY is completely inactive prior to removal of the caging group and that light irradiation restores recombination activity comparable to that of wild-type Cre.

To demonstrate photochemical regulation of Cre activity in mammalian cell culture, HEK293T cells were transfected with the Cre-Stoplight plasmid (pC-SL) (*29*) and Cre-ONBY. pC-SL encodes DsRed and a transcription termination region, both flanked by lox sites and located upstream of a GFP gene. Prior to Cre-mediated recombination, cells exclusively express DsRed under control of a CMV promoter; after recombination and the ensuing excision of DsRed and its terminator, cells express GFP. Thus, in the absence of Cre recombinase, cells produce DsRed and exhibit red fluoresence (Figure 4, panel A). As expected, transfection of wild-type Cre led to *in vivo* recombination and observation of GFP expression (Figure 4, panel B). Gratifyingly, cells transfected with caged Cre-ONBY exclusively showed DsRed expression, verifying the complete inactivity

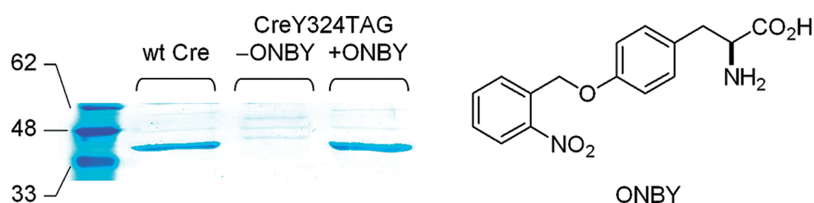

Figure 3. Expression of Cre recombinase in its wild-type form and containing a caged tyrosine at position 324. No protein expression was observed in the absence of ONBY. TAG = amber stop codon. Structure of *o*-nitrobenzyl tyrosine (ONBY).

of the caged Cre protein (Figure 4, panel C). However, a brief UV irradiation with a hand-held UV lamp (365 nm, 23 W, 5 min) almost exclusively yielded GFP expression (Figure 4, panel D). UV light alone had no effect on the conversion of DsRed to GFP (see Supplementary Figures 1 and 2). This confirmed that DNA recombination in HEK293T cells can be effectively triggered with light.

To quantitate the observed recombination activity of Cre-ONBY, flow cytometry data were collected and analyzed with a two-dot plot (see Supplementary Figure 2 and Table 1). As expected, cells transfected with only pC-SL exhibited a high level of red fluorescence (82.0%), with 17.1% of cells exhibiting both red and green fluorescence. Only 1% of cells exhibited solely green fluorescence. It is important to note that DsRed matures through a green intermediate stage (*30*), accounting for green fluorescence in the absence of Cre. The levels of red and green fluorescence after Cre-ONBY transfection but prior to light irradiation remained consistent with those of transfected cells not exposed to Cre (80.4% red fluorescence; 18.3% of cells expressed green and red fluorescence; 1.3% of cells exhibited green fluorescence). However, upon irradiation of Cre-ONBY, a marked decrease in red fluorescence was observed (67.2%), with an increase in the number of cells expressing both green and red fluorescence (27.1%). An increase in cells expressing only green fluorescence was also detected (~6%), indicating activation of the Cre protein and subsequent recombination.

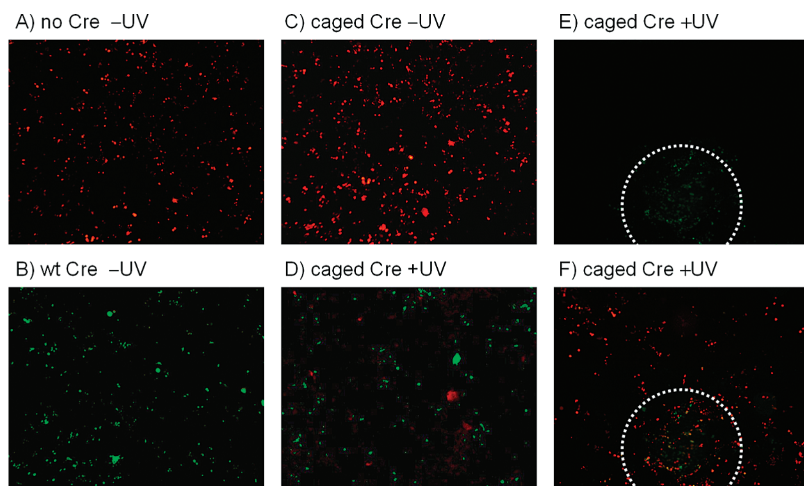

Figure 4. A–D) Photochemical control of Cre-catalyzed DNA recombination in HEK293T cells transfected with the Cre-Stoplight plasmid. E,F) Spatial control of caged Cre. Recombination of the Cre-Stoplight plasmid occurred only in irradiated cells (within the dashed circle), yielding GFP expression. Imaged with Lecia DM5000B microscope 48 h post-transfection, 100X magnification.

To obtain spatial control over DNA recombination in mammalian cell culture, a monolayer of HEK293T cells co-transfected with pC-SL and Cre-ONBY was irradiated in a small area for 20 s with an epi-fluorescence inverted microscope equipped with a 100 W mercury lamp and a DANSA filter cube (330–400 nm). While red fluorescence was detected in all cells, green fluorescence was observed only in the irradiated area of the cell monolayer (dashed circle) demonstrating spatial control over the DNA recombination event (Figure 4, panels E and F).

In summary, we have shown that the activity of Cre recombinase can be stringently regulated both spatially and temporally in mammalian cells through the use of a light-responsive caging group installed on the tyrosine residue essential for catalysis. The described photocaged Cre/lox system has numerous applications toward the creation of knock-in and knock-out organisms in a spatiotemporal fashion.

METHODS

Basic Vector Construction. Top-10 cells were used for cloning and subcloning experiments. The Cre gene was PCR amplified from bacteriophage P1 (a gift from Dr. Eric Miller at NCSU) using primers 5′-AGAGAGGCTAGCTCCAATTTACTGACCGTACACCAA-3′ and 5′-AGAGAGCTCGAGATCGCCATCTTCCAGCAGGCGCACCATTGCCCCTGT-3′, which contain NheI and XhoI restriction sites, respectively. The Cre gene was then inserted into pET-21a(+) as an NheI-XhoI fragment, generating pET-21Cre and yielding hexahistidine-tagged wild-type Cre recombinase upon expression in *E. coli*. The hexahistidine tag not only provides an affinity handle for easy purification but also has been shown to further enhance the cellular uptake of Cre. The expected vector sequence was confirmed by DNA sequencing.

Unnatural Amino Acid Mutagenesis. Following the manufacturer's protocol (Strategene kit), QuikChange mutagenesis was employed to mutate Tyr324 into an amber stop codon (TAT → TAG) with primers 5′-GGACCAATGTAAATATTGTCATGAACTAGATCC-3′ and 5′-ATCCAGGTTACGGATCTAGTTCATGACAATATT-3′, creating pET-21CreY324TAG. Successful amber codon mutation was confirmed by DNA sequencing.

Synthesis of Caged Tyrosine. ONBY was synthesized as previously reported (*28*).

Protein Expression and Purification. To express wild-type Cre, BL21(DE3)Gold cells were transformed with pET-21Cre, inoculated 1:100 mL in LB medium containing 50 μg mL^{-1} ampicillin, and grown at 37 °C, 250 rpm. At an OD_{600} of 0.6, expression was induced with 0.5 mM IPTG for 4 h at 37 °C, followed by 15 h at 25 °C. Cells were then pelleted, and the protein was immediately purified in a 4 °C cold room. Ni-NTA resin (Qiagen) was used to purify both wild-type and caged Cre according to the manufacturer's instructions. To produce caged Cre, BL21(DE3)Gold cells were transformed with both pET-21CreY324TAG and pSupONBY, the vector encoding the *Methanococcus jannaschii* tRNA/tyrosyl-tRNA synthetase pair, and then grown in LB medium containing 50 μg mL^{-1} ampicillin, 30 μg mL^{-1} chloramphenicol, and enriched with 0.1 mM ONBY. A 100× ONBY stock solution (32 mg) was made up in 50 μL of hot DMSO, and then hot water was added to achieve 1 mL total volume. The appropriate amount of ONBY was added to 37 °C prewarmed LB medium dropwise with intense swirling; the pH of the medium was then adjusted, if necessary, to pH 7.0 prior to inoculating the cells. Cells were again induced at an OD_{600} of 0.6, and the protein was expressed and purified as described above. Protein production was verified by 12% SDS−PAGE. Finally, Cre was dialyzed into storage buffer (20 mM Tris-HCl, pH 7.8, 300 mM NaCl, 1 mM EDTA, and 50% glycerol) and stored at −20 °C. No loss of activity was noted after 1 year.

***In Vitro* Recombination.** *In vitro* Cre recombination assays were performed for 30 min at 37 °C in 1× recombination buffer (50 mM Tris-HCl, 33 mM NaCl, 10 mM $MgCl_2$, pH 7.5) with 200 ng of substrate DNA and 100 ng of Cre. Cre was heat-inactivated at 70 °C for 5 min prior to transformation into ultracompetent *recA*- NovaBlue cells (Novagen) following the manufacturer's protocol. The substrate pLox2+ is linearized by restriction digest, resulting in a low number of background colonies. To determine the number of these colonies, control transformations were also performed solely with the substrate. Cre-ONBY was irradiated in the presence of substrate and 1× buffer at 365 nm for 20 min at 37 °C with a fiberoptic LED lamp and then incubated an additional 30 min at 37 °C, followed by heat inactivation and transformation as described above. A timecourse was performed to determine the optimum irradiation time (see Supporting Information Figure 2). Recombination efficiency most comparable to that of wild-type Cre was detected after 20 min of irradiation. Additionally, the enzyme-to-substrate ratio was reduced (50 ng of protein to 200 ng of pLox2+) to ensure that restoration of Cre-ONBY activity was truly achieved without limited substrate. In combination with the timecourse, about 70% of the activity of wild-type Cre was achieved upon irradiation of Cre-ONBY.

***In Vivo* Recombination.** HEK293T cells were either sequentially transfected with pC-SL, followed 6 h later by Cre or co-transfected with both Cre-ONBY and pC-SL using the DOTAP transfection reagent. Select cells were irradiated with a handheld UV lamp (365 nm, 23 W, 5 min), followed by replacement of media with standard growth media and incubation for an additional 48 h. In order to ensure that the irradiation was not harmful, cell viability studies were first performed in the absence of Cre-ONBY and pC-SL; irradiated cells demonstrated no effects of cytotoxicity upon exposure to UV irradiation. Additionally, cells containing pC-SL and not transfected with Cre were irradiated and DsRed was produced, indicating that 5 min of irradiation at 365 nm does not adversely affect protein production or otherwise harm the cell. GFP and/or DsRed expression was then imaged on a Lecia DM5000B microscope 48 h post-transfection. In order to demonstrate spatial control of recombination activity, HEK293T cell transfections were repeated and incubated for 48 h to afford adequate gene expression. Select cells were irradiated for 20 s using a Jenco epifluorescence inverted microscope equipped with a 100 W mercury lamp and a DANSA filter cube (330−400 nm excitation), followed by replacement of media with standard growth media and incubation for an additional 48 h. GFP and/or DsRed expression was then imaged on a Lecia DM5000B microscope 48 h post-transfection.

Flow Cytometry. HEK293T cell transfections were repeated and incubated for 48 h to afford adequate gene expression. The cells were then trypsinized to remove them from the surface of the plate and transferred into eppendorf tubes to be counted. Each reaction condition was individually counted on a Becton-Dickinson FACSCalibur instrument for the number of cells expressing GFP and DsRed (see Supplementary Table 1). Non-transfected 293T cells did not display any fluorescence, as no GFP or DsRed was expressed. Cells transfected with pC-SL and wild-type Cre contained highly fluorescent cells, with a number of them expressing both green and red fluorescence as a result of the DsRed passing through a green intermediate prior to maturation. The 293T cells transfected with only pC-SL predominantly showed red fluorescence (see Supplementary Figure 2, upper left quadrant). A similar result was observed in cells co-transfected with pC-SL and Cre-ONBY that were not irradiated (Figure 4, panel B); however, in the irradiated cells, a statistical increase in green fluorescence was observed, indicating activation of the Cre protein and subsequent recombination (Supplementary Figure 3, right quadrants).

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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