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Photoactivation of the CreER^{T2} Recombinase for Conditional Site-Specific Recombination with High Spatiotemporal Resolution

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We implemented a noninvasive optical method for the fast control of Cre recombinase in single cells of a live zebrafish embryo. Optical uncaging of the caged precursor of a nonendogenous steroid by one- or two-photon illumination was used to restore Cre activity of the CreER^{T2} fusion protein in specific target cells. This method labels single cells irreversibly by inducing recombination in an appropriate reporter transgenic animal and thereby can achieve high spatiotemporal resolution in the control of gene expression. This technique could be used more generally to investigate important physiological processes (e.g., in embryogenesis, organ regeneration, or carcinogenesis) with high spatiotemporal resolution (single cell and 10-min scales).

Introduction

SITE-SPECIFIC RECOMBINATION SYSTEMS have provided powerful genetic tools to manipulate DNA for different applications: lineage tracing (in particular, to know where specific lineages form and what are their precursors) and expression of functional or altered transgenes to define which signals are required for the proper determination and differentiation of these lineages.^{1,2} Among different strategies, the Cre-based genetic switch system is one of the most widely used.³ It mediates recombination by the site-specific DNA recombinase Cre, which excises the sequences between two loxP sites with the same orientation, or inverts sequences flanked by head-to-head loxP sites.⁴ The resulting modification is permanent and transmitted to daughter cells upon division. Recently, the Cre strategy has been successfully transferred from mice to zebrafish^{5,6} and several trans-

genic lines expressing Cre have been described.⁷⁻¹¹ Different strategies were developed to improve the spatiotemporal control of recombination. To regulate Cre expression in a specific lineage, transgenic animals were engineered to express Cre under a specific promoter (Fig. 1A). This standard strategy requires to identify genes specifically expressed in the target lineage and to characterize the promoter–enhancer elements responsible for specific lineage expression. Despite these two restrictions, this approach has been widely used in mice.³ However, it cannot be used to trace unknown cell lineage precursors unless modified to induce sporadic recombination and to go through extensive screening. To activate Cre activity at a specific time, two complementary approaches were previously used (Fig. 1B). The first one makes use of inducible promoters, among which the most popular in zebrafish is certainly the *hsp70* promoter, which allows a transient activation while embryos are taken from

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28°C to 37°C. A heat shock-inducible Cre line Tg (*hsp70:Cre*) was successfully used to induce the human kRASG12D protein expression in a double transgenic fish Tg (*B-actin:LoxP-EGFP-LoxP-kRASG12D; hsp70:Cre*).⁸ The heat shock-inducible system allows to control the time of induction at the minute scale. Although activation in a single cell is possible using a laser beam to generate the temperature increase,¹² there is a practical depth limitation and the 1–2 min needed for the heat shock prevents an induction in multiple cells in a short duration.¹³ The Lewis laboratory set up a two-step recombination assay in which a heat shock induces the expression of Cre. The self-excision of the Cre cassette allows a ubiquitous expression of a Gal4-VP16 activator, which in turn allows the expression of upstream activation sequence (UAS)-driven protein.¹⁴ The second class of strategies for temporal control of recombination makes use of ligand-inducible Cre (Fig. 1B). This system is based on Cre fusion to the hormone-binding domain of the estrogen receptor (ER), making Cre activity dependent on steroid ligand binding.¹⁵ In the absence of ER ligand, the fusion protein is sequestered in the cell cytoplasm by a chaperone complex.^{16,17} Addition of the ligand induces the dissociation of the complex and allows CreER to access the nucleus in an active conformation.¹⁷ The estrogen binding domain was mutated to make it more sensitive to an artificial steroid (4-OH tamoxifen [4-OHT]) and less to natural steroids.¹⁸ Up to now, the ER^{T2} variant (human ER with the triple mutation (G400V/M543A/L544A)) displays the lower background activity and is strongly responsive to 4-OHT.¹⁹ Transgenic animals expressing CreER^{T2} proteins are fed with 4-OHT, which rapidly spreads in the entire body and allows recombinase activity. It is possible to restrict CreER^{T2} expression by using a cell-specific promoter and time scale for 4-OHT uptake can be reduced to less than 2 h.²⁰ A transgenic zebrafish of this kind has been recently reported.²⁰ These approaches have two limitations: (i) the *hsp70* promoter shows some leakiness during development^{7,8} and (ii) cell specificity driven by promoter activity restricts the analysis to cells already fully characterized for regulating sequences, precluding the study of progenitors or other cell types not yet characterized and not allowing for single-cell study in a tissue. Caged precursors²¹ appear to be ideal to circumvent these limitations: indeed they allow to trigger a biological response in a single cell of an organism with excellent temporal resolution.²¹ In that respect the zebrafish is a system of choice to photocontrol protein activity: its transparency, the existence of lines without pigments, its small size, and rapid development allow real-time observations under the microscope. We designed a photocontrol system for CreER^{T2} activity using a caged ligand for the ER^{T2} domain reaching the single cell and the 10-min time-scale resolution (Fig. 1D). The experimental protocol for photocontrol of Cre in zebrafish is straightforward and easy to set up. First, an animal expressing the CreER^{T2} is engineered (preferably permanently by incorporating the appropriate transgene in its genome or alternatively transiently by injecting the corresponding mRNA at the zygote stage). The embryo is incubated in an aqueous solution of a caged inducer (cInd) that penetrates the whole organism. At a chosen stage of development, the embryo is transferred to an illumination chamber where the inducer is uncaged in a single cell by two-photon illumination with a focused IR laser beam. Then the released ligand

activates the fusion protein and allows specific DNA recombination.

Materials and Methods

Plasmid construction and transgenic line

Plasmid coding the CreER^{T2} was a kind gift of D. Metzger (Strasbourg) and the plasmid coding the Cre was a kind gift of W. Wurst (Munich). The *eflα:loxP-GFP-loxP-dsRed2* transgenic line was established with standard conditions using a Tol2 transposon system and the pT2K-XIG plasmid backbone.²² A detailed description of the cloning procedures is available on request.

Microinjection

Tg zebrafish embryos were obtained by natural spawnings according to the zebrafish book,²³ injected at one-cell stage with mRNA synthesized with an *in vitro* transcription kit (mMessage mMachine; Ambion), and stored at –80°C in small aliquots.

Pharmacological treatments and CreER^{T2} activation

4-Hydroxy-cyclofen (4-OH cyclofen or Ind) and caged 4-OH cyclofen (cInd) were prepared as previously described.²⁴ Embryos injected with CreER^{T2} mRNA were subsequently dechorionated by pronase treatment at dome stage before incubation for at least 90 min in an aqueous solution (embryo medium) of the various substrates (e.g., cInd). Ind was photoreleased at different stages of development. Embryos were observed at 2 days postfertilization (dpf) after UV illumination. Embryos positive for either GFP or dsRed fluorescence (Cre-induced recombination) were scored under microscope in a double-blind protocol.

Illumination experiments

One-photon excitation experiments were performed at 20°C, with a bench top UV lamp (365 nm, 8 W; Fisher Bio-block) providing a typical 4×10^{-5} Einstein min⁻¹ photon flux²⁵ (essentially Gaussian spectral dispersion around 350 nm with a 40 nm width at half height). For two-photon excitation, a 40×0.8 NA water-immersion objective (Olympus) was used to image the embryos on a CCD camera (Andor Luca) and locate the focal spot of the two-photon excitation. Illumination (200 fs, 76 MHz, 750 nm) was provided by a mode-locked Ti-Sapphire laser (Mira, Coherent). The incident power at the sample (<20 mW) was measured with a NOVA II power meter (Laser Measurement Instruments). The geometrical characteristics of the focal point were determined by fluorescence correlation spectroscopy measurements using a fluorescein solution of known concentration (100 nM in 0.1 M NaOH).

Image acquisition

The fluorescence images of the embryos were acquired using confocal microscopes Leica TCS SP2 AOBS or Zeiss Axiovert 200M LSM510-Meta. In a given series of experiments, all the conditions (EM gain, exposure duration, lamp power, etc.) were identical to allow for a comparison of the observed fluorescence intensities.

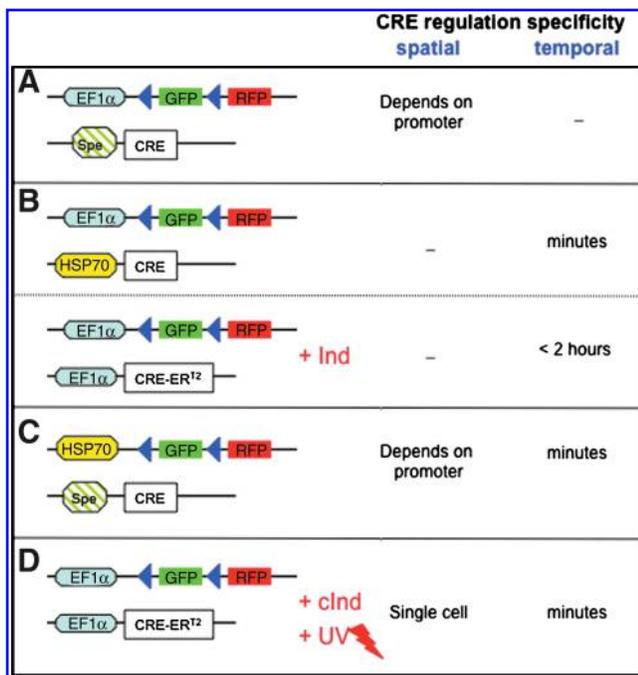


FIG. 1. Strategies used to make accurate spatiotemporal regulation of Cre recombination. (A) Spatial regulation: Cre expression is driven by a cell type-specific promoter. (B) Temporal regulation: heat-inducible Cre (top)/ligand-dependent Cre-mediated recombination (bottom). (C) Spatiotemporal regulation: bitransgenic approach. (D) Spatiotemporal photo-control of CreER^{T2} activity.

Quantitative PCR

Total DNA was extracted from 30 embryos, and 10 ng was used for a quantitative PCR assay. Quantitative PCR was performed using a Roche light cycler 480 detection system and SYBR green labeling system (Qiagen). Details concerning the parameters used are available on request. LoxP recombination was normalized to the number of transgene copies in each sample. Each sample was tested in triplicate. Specific primers were designed by Primer 3 online software (<http://frodo.wi.mit.edu/primer3/input.htm>): Recombine transgene: forward (5'-AGGTCGACCGTTAGGGAAC-3'), reverse (5'-ACCTTGAAGCGCATGAACTC-3'); full transgene: forward (5'-CAACGAGGACTACACCATCG-3'), reverse (5'-TCTAGTTGTGGTTTGTCC-3').

Results

For optical uncaging, one of the most desirable photo-physical properties of the caged inducer is a low photo-damage to the released inducer. Although 4-OHT has been used successfully as an inducer to activate CreER^{T2} recombinase in fish,²⁰ we observed that 4-OHT was susceptible to UV-induced isomerization and degradation on the typical time scales of uncaging.²⁴ Therefore, we looked for another inducer, structurally related to 4-OHT, but not subject to isomerization or degradation upon UV illumination. We adopted the core motif of the estrogen cyclofenil. After grafting on one of its phenol groups the basic pendant chain present in 4-OHT, we obtained the nonendogenous and water-soluble 4-OH cyclofen (Ind), which binds to the ER^{T2} as

efficiently as 4-OHT²⁴ and does not isomerize or degrade upon illumination.

We assayed the toxicity of 4-OH cyclofen in live embryos. The viability test for the *ef1α:loxP-GFP-loxP-dsRed2* transgenic zebrafish embryos was performed at various concentrations of the inducer: it was observed that animals develop normally for concentration of 4-OH cyclofen at least as high as 5 μM. These embryos expressed GFP only and no fluorescence of dsRED was observed irrespective of the presence or absence of the inducer in embryo medium (data not shown). It demonstrates as expected that the transgenic zebrafish line has no endogenous recombination. However, injection of 10 pg of *Cre* mRNA at the one-cell stage causes global recombination in all the cells of the embryo as evidenced by the expression of dsRED in 100% of *Cre*-injected embryos. Figure 2A shows a representative fluorescence image of dsRED in a *Cre*-injected embryo observed at 2 dpf. In Figure 2B–D we show that by controlling the amount of injected *Cre-ER^{T2}* mRNA and the concentration of inducer, we can tune the recombination efficiency in transgenic zebrafish embryos constitutively expressing a *loxP-gfp-loxP-dsRed2* gene construct, and consequently control the activa-

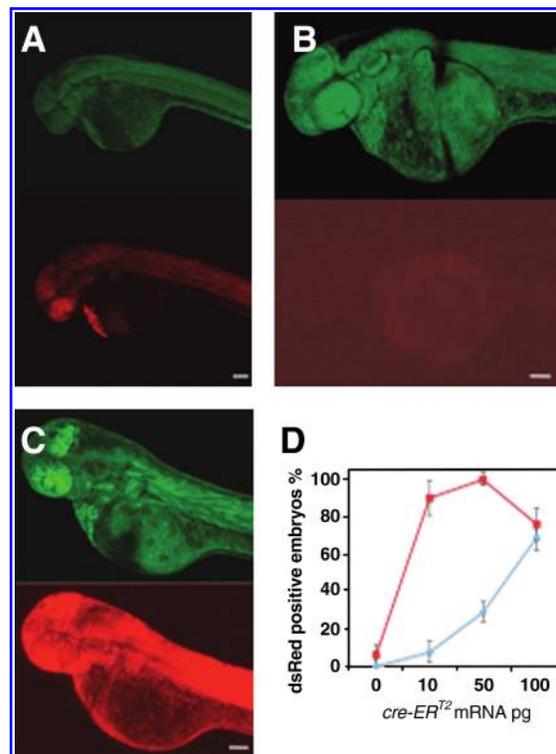


FIG. 2. Cre-mediated recombination in the green-to-red reporter line. In Tg (*ef1α:loxP-GFP-loxP-dsRed2*) embryos injected with 3 pg of *Cre* mRNA at one-cell stage (A), dsRed fluorescence is ubiquitously observed at 36 hpf. Embryos were injected with 3 pg of *Cre-ER^{T2}* mRNA, further incubated with embryo medium (B) or 1 μM cyclofen-OH (Ind) (C), and observed at 2 dpf. Variation of the recombination efficiency with the amount of injected *Cre-ER^{T2}* mRNA (in pg) and inducer concentration: 0 μM (blue triangle) or 3 μM (red square). The percentage of embryos exhibiting dsRed fluorescence was scored by epifluorescence microscopy at 2 dpf (D). The error bars represent statistical errors estimated as $\sqrt{p(1-p)/n}$. Scale bars: 100 μm. dpf, days postfertilization.

tion of dsRED expression. To do this, different amounts of mRNA coding for Cre-ER^{T2} recombinase were injected in transgenic embryos at the one-cell stage. Figure 2B shows that the injection of 3 pg of CreER^{T2} mRNA at the one-cell stage causes no recombination. However, upon addition of (3 μ M) 4-OH cyclofen in the embryo medium at 50% epiboly (Fig. 2C, D), global recombination (dsRED expression) was observed at 2 dpf. Spontaneous (inducer-independent) recombination after CreER^{T2} mRNA injection increased with the amount of injected nucleic acid, presumably because the concentration of the cytoplasmic chaperones was insufficient to complex with every CreER^{T2} protein synthesized. We measured the percentage of embryos that underwent recombination (partial or global) as a function of the amount of injected mRNA, in the presence or absence of Ind (Fig. 2D). Upon addition of 3 μ M Ind in the embryo medium, we found a significant increase in the frequency of recombination for embryos injected with less than 5 pg of mRNA (at concentration 50 ng/ μ L). Thus, by appropriately tuning the amount of injected mRNA, the ratio of induced versus spontaneous recombination can be optimized. Henceforth, we fixed the injection condition at 3 pg of mRNA for all optical uncaging experiments.

To test the ability of our method to locally or globally photocontrol the activity of CreER^{T2}, we investigated the activation of the Cre-ER^{T2} recombinase via uncaging of caged cyclofen (cInd) in the transgenic zebrafish line mentioned earlier. For these experiments, embryos were injected with CreER^{T2} mRNA at the one-cell stage and incubated in embryo medium supplemented with 3 μ M of cInd. In the absence of UV illumination, when observed with epifluorescence microscopy at 2 dpf, embryos showed ubiquitous expression of GFP but no recombination (dsRed expression) as shown in Figure 3A, implying that the caged ligand is indeed inactive. Upon uncaging of cInd with UV illumination at one somite, the embryos ubiquitously expressed dsRed at 2 dpf (Fig. 3B). We also checked that when the embryos were illuminated during early development (50% epiboly to six somites) for up to 4 min with the UV lamp (8 W, 365/40 nm), the recombined embryos developed normally proving that neither UV illumination nor the side product resulting from uncaging (i.e., the caging group) was detrimental to the embryo development (data not shown). One of the limits of the system can be the delay between cInd releasing and Cre recombination. We then analyzed the recombination by the CreER^{T2} upon cInd uncaging. Recombination was measured by quantitative PCR. Figure 3C shows that recombination occurred on the 10-min scale; it can be detected as soon as 3 min after uncaging and 13 min after uncaging 30% of the floxed sequences have been recombined. This time scale is perfectly relevant for zebrafish rapid development.

Finally, to target the activation in a single cell, we used two-photon illumination to release cInd in a specific cell (in a single cell of the forming retina [Fig. 3D] or in a newly formed somite [Fig. 3E]) at three- to four-somite stage of a live zebrafish embryo. This cell's progeny could be observed at 2 dpf to exhibit the characteristic red fluorescence of dsRed in an embryo that is otherwise fluoresced in green (Fig. 3D, E).

Discussion

We used two-photon excitation to quickly and non-invasively achieve spatiotemporal control of CreER^{T2} activity

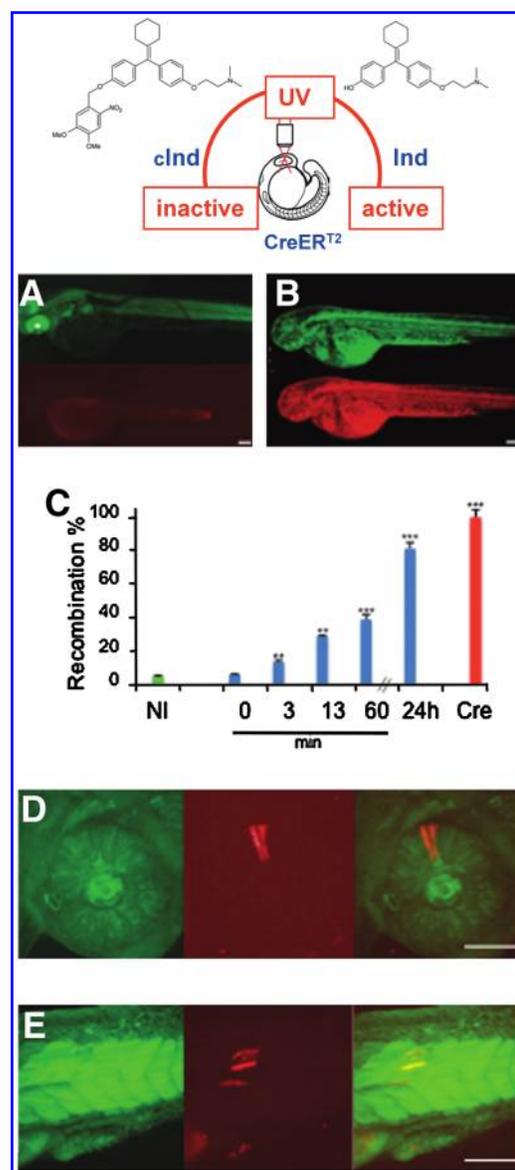


FIG. 3. Photocontrol of CreER^{T2} recombinase activity in Tg reporter line. Transgenic (*ef1 α :loxP-GFP-loxP-dsRed2*) embryos were injected with 3 pg of CreER^{T2} mRNA at the one-cell stage and further incubated with 3 μ M of caged inducer (cInd). Photoactivation of cInd at the 3/6-somite stage resulted in dsRed expression observed here at 2 dpf. In epifluorescence microscopy, the non-UV illuminated embryos (A) do not express dsRed, whereas they do express dsRed in every cell upon global UV illumination (B), in few cells upon two-photon excitation in a single cell in the forming retina (D), or in a just formed somite (E). Scale bars: 100 μ m. Kinetics of recombination was determined by quantitative PCR (C). Genomic DNA was prepared from 30 tg (*ef1 α :loxP-GFP-loxP-dsRed2*) embryos; Green: NI, noninjected embryos; blue: embryos injected at one-cell stage with 30 pg cre-ER^{T2} mRNA, incubated with 3 μ M of caged inducer (cInd), illuminated at 50% epiboly, and then fixed for DNA purification; red: embryos injected at one-cell stage with 30 pg cre mRNA and allowed to develop until 24 hpf. The average values (\pm standard error of the mean) from at least three independent experiments are presented; comparisons with $t = 0$ were performed by a Student's t -test. Values of $p < 0.05$ were considered to indicate statistical significance (** $p < 0.001$; ** $p < 0.01$).

and gene expression at the single-cell level in a live animal. We show that photoreleasing 4-OH-cyclofen from a caged precursor on a second time scale is an efficient strategy to restore the function of Cre fused to the ER^{T2} ligand-binding domain in a live animal down to the single-cell level. This method offers a great versatility: virtually any cell or any cell combinations can be chosen. Complex temporal inductions within the same lineage are also feasible, potentially allowing a dynamic dissection of fate commitment events. Moreover, there is no longer the need for a complicated promoter engineering to achieve the envisioned Cre expression.

The inactivation of a protein fused to the ER domain has been described for various proteins (e.g., Engrailed, Otx2, Gal4, p53, kinases such as Raf-1, Cre, and Flp recombinases).²⁶ The strategy described here is thus compatible with the photoactivation of a wide variety of proteins. This noninvasive optical method opens opportunities for the local spatiotemporal investigation of developmental pathways, the identification of stem cells, and the study of cancer and neuronal (e.g., memory) pathways at the single-cell level in a live organism. In particular, it could be used to label individual cells and track their lineage as well as to interfere with morphogen gradients and signaling pathways with unprecedented spatiotemporal resolution. Ultimately, this strategy may provide crucial information about the regulation networks involved in development and differentiation/dedifferentiation: their timing (i.e., when are the genes/proteins activated in the cell), their dynamics (how fast are they activated), and their spread (how far is their influence felt).

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Disclosure Statement

No competing financial interests exist.

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