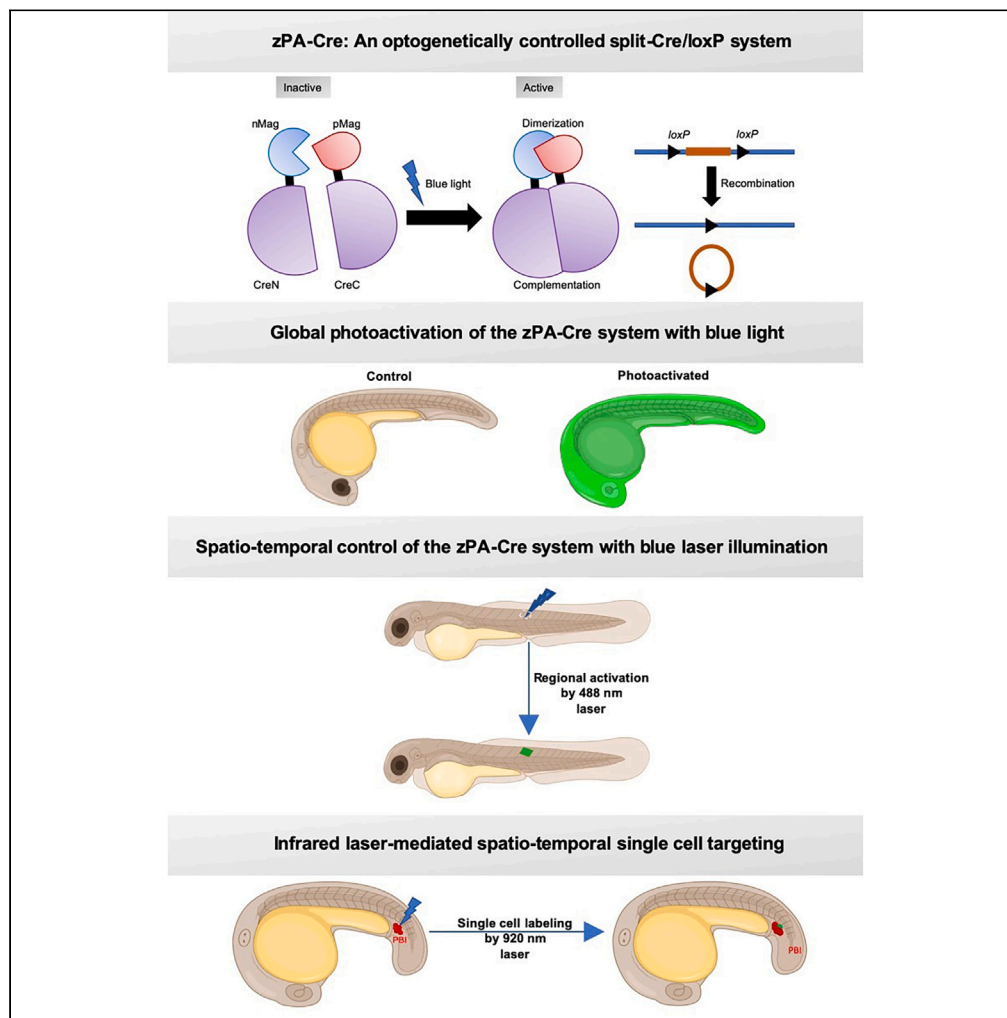


Article

A noninvasive photoactivatable split-Cre recombinase system for genome engineering in zebrafish



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Highlights

zPA-Cre is a novel zebrafish transgenic optogenetic split-Cre tool

zPA-Cre allows loxP-mediated DNA recombination in various cell types

It enables light-induced DNA recombination at different developmental stages

It allows spatiotemporal DNA recombination in a specific tissue region

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Article

A noninvasive photoactivatable split-Cre recombinase system for genome engineering in zebrafish

Ramy Elsaid,^{1,2,3,*} Aya Mikdache,^{1,3} Patricia Diabangouaya,¹ Gwendoline Gros,¹ and Pedro P. Hernández^{1,4,*}

SUMMARY

The cyclic recombinase (Cre)/loxP recombination system is a powerful technique for *in vivo* cell labeling and tracking. However, achieving high spatiotemporal precision in cell tracking using this system is challenging due to the requirement for reliable tissue-specific promoters. In contrast, light-inducible systems offer superior regional confinement, tunability, and non-invasiveness compared to conventional lineage-tracing methods. Here, we took advantage of the unique strengths of the zebrafish to develop an easy-to-use highly efficient, genetically encoded, magnets-based, light-inducible transgenic Cre/loxP system. We demonstrate that our system does not exhibit phototoxicity or leakiness in the dark, and it enables efficient and robust Cre/loxP recombination in various tissues and cell types at different developmental stages through noninvasive illumination with blue light. Our newly developed tool is expected to open novel opportunities for light-controlled tracking of cell fate and migration *in vivo*.

INTRODUCTION

Cell marking by genetic recombination is a powerful technique for noninvasively tracing cell fate and migration in rapidly evolving contexts such as embryonic development.^{1,2} Various recombinases have been used in zebrafish studies^{3,4}; however, the Cre/loxP system remains the primary method for genetic lineage tracing in this species.² The Cre recombinase, derived from the bacteriophage P1, specifically targets two pairs of 34-bp sequences known as loxP sites.⁵ Due to its simplicity and efficiency, the Cre/loxP system has been widely used in applications such as lineage tracing and genome manipulation in transgenic animals.¹ Typically, the Cre recombinase is expressed under a tissue- or cell-specific promoter and can be combined with chemical-inducible systems such as tamoxifen to achieve temporally controlled genetic recombination.^{1,6,7} By crossing transgenic lines containing both Cre and loxP-STOP-loxP constructs, Cre specifically activates the reporter in tissues or cells expressing the promoter by permanently removing the STOP cassette, thereby enabling the tracing of their progeny.^{1,2} Nonetheless, these conventional Cre/loxP recombination approaches have some limitations. For instance, it can be challenging to target and trace cells or tissues that lack a specific promoter during development and/or regeneration. Furthermore, the temporal induction of the Cre/loxP system using chemicals may have undesirable effects, including cytotoxicity and cellular perturbation.^{8,9} Moreover, a critical limitation of conventional Cre/loxP recombination systems is their inability to selectively target specific sub-regions of tissues or cells with similar gene expression patterns.

In contrast to conventional lineage tracing approaches, light-inducible systems offer a significant advantage by enabling noninvasive photoactivation in deep tissues with high spatial and temporal resolution.^{10,11} Consequently, several attempts have been made to develop optically controlled Cre systems in zebrafish, as reported in previous studies.^{12–16} Despite these remarkable advancements, current light-inducible systems tested and used in zebrafish suffer from low efficiency, leakiness in the dark, the need of prolonged exposure to UV light resulting in cytotoxicity, the requirement of advanced microscopy setups, the addition of exogenous chromophores, or the use of harmful chemicals such as caged biomolecules or tamoxifen.^{10,12–17}

On the other hand, a highly efficient genetically encoded photoactivatable Cre recombinase (PA-Cre) has recently been reported in mammals based on magnet dimerization systems.^{18–20} The magnet system was engineered from the blue light photoreceptor Vivid (VVD) which is derived from the filamentous fungus *Neurospora crassa* and requires flavin adenine dinucleotide as its cofactor, which is abundant in eukaryotic cells.^{21,22} This blue light-dependent dimerization system comprises two photoswitches named positive magnet (pMag) and negative magnet (nMag).²¹ Based on the reconstitution of split Cre fragments upon blue light-dependent dimerization of the magnet system, Kawano et al. developed a genetically encoded magnets-based PA-Cre system.¹⁸ Consequently, they showed that their PA-Cre system could be applied to control DNA recombination with high spatiotemporal precision *in vivo*.

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The magnet system has also been shown to be efficient in inducing light-controlled genetic recombination *in vitro* and *in vivo* when used with Dre and FLP recombinases in mammals.^{23,24} Furthermore, it has been recently reported that the magnets-based photoactivatable split Gal4 system enables rapid and robust spatiotemporal gene expression control in *Drosophila in vivo*.²⁵ However, to date, there are no stable transgenic zebrafish models for optogenetic-based Cre/loxP recombination. Therefore, we aimed to develop a PA-Cre system to enable light-inducible lineage tracing *in vivo*, leveraging on the unique strengths of the zebrafish model.

In this study, we evaluated the functionality of the PA-Cre system in zebrafish. We demonstrate that the original PA-Cre system previously used in mammalian cells¹⁸ induces efficient Cre/loxP recombination in zebrafish embryos upon simple light-emitting diode (LED)-mediated blue light illumination. We further developed and assessed the performance of the PA-Cre system under various zebrafish-specific promoters. We illustrate that our new system exhibits no phototoxicity or leakiness in the dark and enables efficient loxP-mediated DNA recombination *in vivo*. Finally, we generated a new transgenic zebrafish line in which the expression of the PA-Cre system is under the control of the zebrafish ubiquitin (*ubb*) promoter.²⁶ By utilizing this *Tg(ubb:PA-Cre)* line, we show that our system allows for efficient and robust Cre/loxP recombination in diverse tissues and cell types through noninvasive blue light illumination. Collectively, our newly developed versatile tool is anticipated to pave the way for light-controlled tracing of cell fate and migration *in vivo*, opening up new opportunities for zebrafish research.

RESULTS

The PA-Cre system enables *in vivo* loxP-mediated DNA recombination in zebrafish embryos

To assess the efficacy of the PA-Cre system (Figure 1A) to induce loxP-mediated DNA recombination in zebrafish, we injected the original PA-Cre construct¹⁸ (Figure 1B) into zebrafish eggs. The PA-Cre construct, in which expression is driven by the cytomegalovirus (CMV) promoter¹⁸ (Figure 1B), was injected into *Tg(-3.5ubb:loxP-lacZ-loxP-eGFP; cry:GFP)* (referred to hereafter as *Tg(ubb:HULK)*²⁷) embryos at one-cell stage and then subjected to global 488-nm blue light illumination from 6 h post fertilization (hpf) to 18 hpf (Figure 1C) utilizing a simple low-cost LED light. It has been shown that the *Tg(ubb:HULK)* zebrafish exhibits ubiquitous green fluorescence protein (GFP) expression only upon Cre-mediated recombination.²⁷ In injected embryos, 488-nm blue light illumination at 6 hpf resulted in Cre/loxP recombination and generated a mosaic GFP expression in various tissues throughout the embryo body, including the head, heart, trunk, and tail (Figures 1D and 1E). All embryos that were injected and exposed to blue light illumination showed mosaic GFP expression. Importantly, we observed no GFP expression in embryos that were injected and kept in the dark (Figure 1D), indicating that the Cre-mediated recombination is solely driven by blue light illumination and does not undergo stochastic magnets dimerization. Fluorescence imaging showed that zebrafish embryos exposed to blue light exhibited a 29-fold induction over embryos that were kept in the dark (Figure 1F). In addition, we observed no photo-induced cytotoxicity upon 488-nm blue light illumination, as we found comparable rates of abnormalities between light-exposed embryos (13.3%) and non-exposed embryos (15.2%). These results demonstrate that transient photoactivation of the PA-Cre system in zebrafish embryos allows robust light-induced Cre/loxP recombination in multiple tissues. Importantly, no background DNA recombination was detected in the absence of illumination and no photo-induced cytotoxicity was observed.

Generation of a stable functional zPA-Cre transgenic line in zebrafish

Having established the functionality of the PA-Cre system in zebrafish embryos, we tested its suitability for generating a stable transgenic line. To this end, we decided to use the zebrafish *ubb* promoter²⁶ in place of the CMV promoter to control the expression of the PA-Cre system. The CMV promoter has been associated with potential silencing of promoter activity *in vivo*, making it less suitable for this type of applications. In contrast, the *ubb* promoter has been shown to have consistent activity throughout zebrafish developmental stages and does not exhibit silencing issues.^{26,28} Therefore, we created the *Tg(-3.5ubb:PA-Cre; cmlc2-EGFP)* line (referred to hereafter as zPA-Cre) in which the expression of the PA-Cre system is driven by the *ubb* promoter. Additionally, the line features *cmlc2-EGFP* as a transgenesis marker in the heart (Figure 2A).

To test whether the newly developed line is capable of inducing DNA recombination upon 488-nm blue light illumination, we crossed it with the *Tg(ubb:HULK)* (Figure 2B). In double-transgenic zebrafish embryos, blue light illumination from 6 hpf to 18 hpf resulted in a robust Cre/loxP recombination, leading to robust GFP expression in various tissues at 24 hpf (Figure 2C), at 48 hpf (Figures 2D and 2E), and at 72 hpf (Figure 2F). All embryos exposed to 488-nm blue light illumination showed GFP expression in various tissues and cells, including the skeletal muscles, the skin epithelia, the motor neurons, the notochord, the neuromasts, and the blood vessels (Figure 2F and Video S1), while no GFP expression was observed in embryos kept in the dark (Figures 2C–2E), confirming the fidelity of the zPA-Cre line and its inducibility exclusively upon blue light illumination. Furthermore, we did not observe any photo-induced cytotoxicity, as there was no difference in numbers of dead cells in the forebrain between light-exposed embryos and the non-exposed embryos, as indicated by acridine orange (AO) labeling (Figures S1A and S1B). In addition, the abnormality rate of blue light-exposed embryos (10.47%; $n = 219$) was not considerably different from that of non-exposed embryos (10.28%; $n = 176$). Moreover, fluorescence imaging demonstrated a 248.8-fold induction of GFP expression in zebrafish embryos exposed to blue light compared to those kept in the dark (Figure 2G). To assess how fast GFP expression appears following exposure to light, we photoactivated *Tg(zPA-Cre; ubb:HULK)* zebrafish embryos at 1-cell stage. We found that light-exposed embryos start to express GFP at the 75% epiboly stage which intensified over developmental time (Figures S2A and S2B). Altogether, these findings demonstrate the robust and efficient induction of DNA recombination *in vivo* upon blue light illumination by the zPA-Cre line.

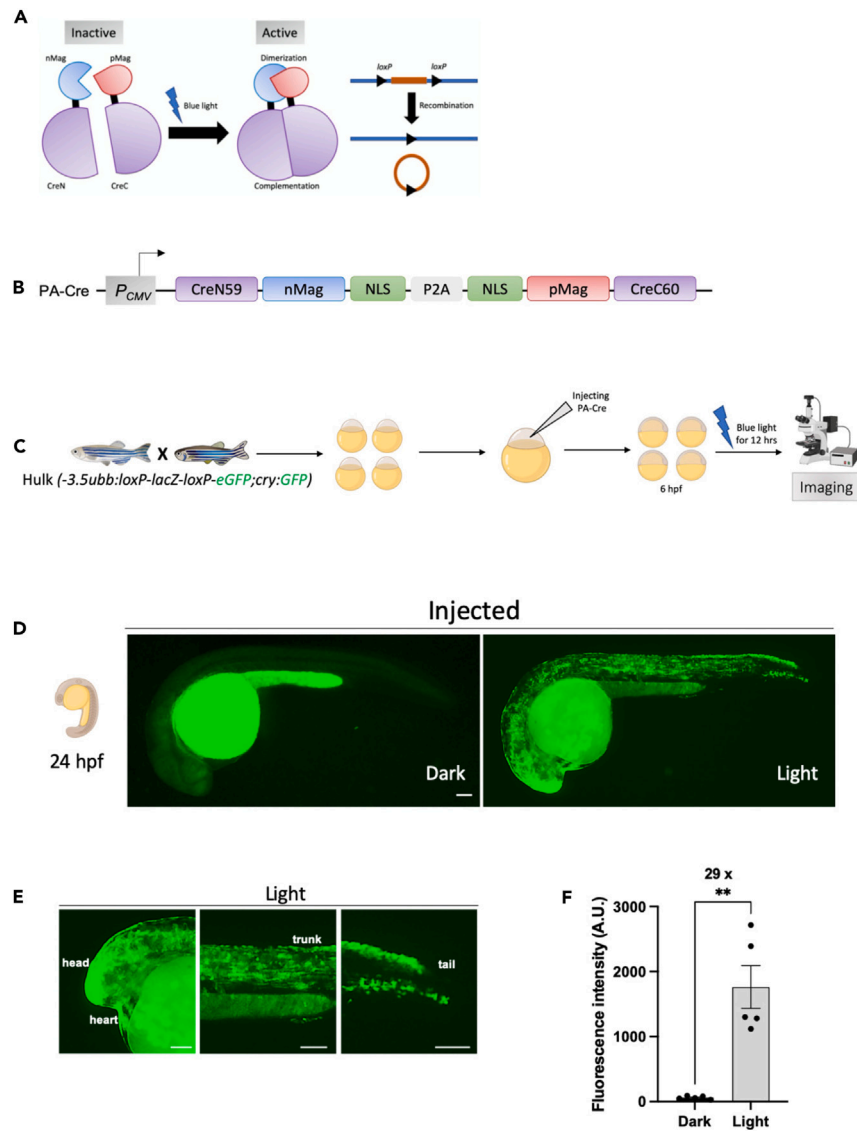


Figure 1. PA-Cre-mediated genetic recombination in zebrafish embryos

(A) Schematic representation of the optogenetic PA-Cre system. CreN and CreC are respectively fused to nMagnet and pMagnet tandems. These fusions are monomers in the dark state (inactive PA-Cre). Upon blue light photoactivation, dimerization of nMag-pMag leads to complementation of CreN-CreC and thus induction of genetic recombination and activation of gene expression (active PA-Cre). nMag, negative magnet; pMag, positive magnet; CreN and CreC, two pairs of split Cre fragments.

(B) Schematic representation of the PA-Cre genetic construct. The PA-Cre system is driven by the CMV promoter. P, promoter; nMag, negative magnet; pMag, positive magnet; NLS, nuclear localization signal; CreN59, split Cre fragment (residues 19–59); CreC60, split Cre fragment (residues 60–343); P2A, 2A self-cleaving peptides.

(C) Schematic representation of the strategy used to induce genetic recombination in zebrafish embryos. *Tg(ubb:Hulk)* embryos were injected at the one-cell stage with the PA-Cre construct, photoactivated with blue light at 6 hpf for 12 h, and then imaged at the desired developmental stage.

(D) Representative fluorescent images at 24 hpf of injected *Tg(ubb:Hulk)* embryos kept in the dark (left panel) or exposed to blue light illumination at 6 hpf for 12 h (right panel). Scale bar: 100 μ m.

(E) Close-ups of image in (D) right panel showing GFP signal of a photoactivated 24 hpf embryo in the head, heart, trunk, and tail. Scale bar: 100 μ m.

(F) Quantification of fluorescence intensity at 48 hpf of embryos injected with the PA-Cre construct and kept in the dark ($n = 5$) or exposed to blue light illumination at 6 hpf for 12 h ($n = 5$). 29x represents the fold induction of the PA-Cre system. Mean \pm SEM of the fluorescence intensity is shown. An unpaired t test with Welch's correction was used for this analysis. $**p \leq 0.01$. A.U., arbitrary unit.

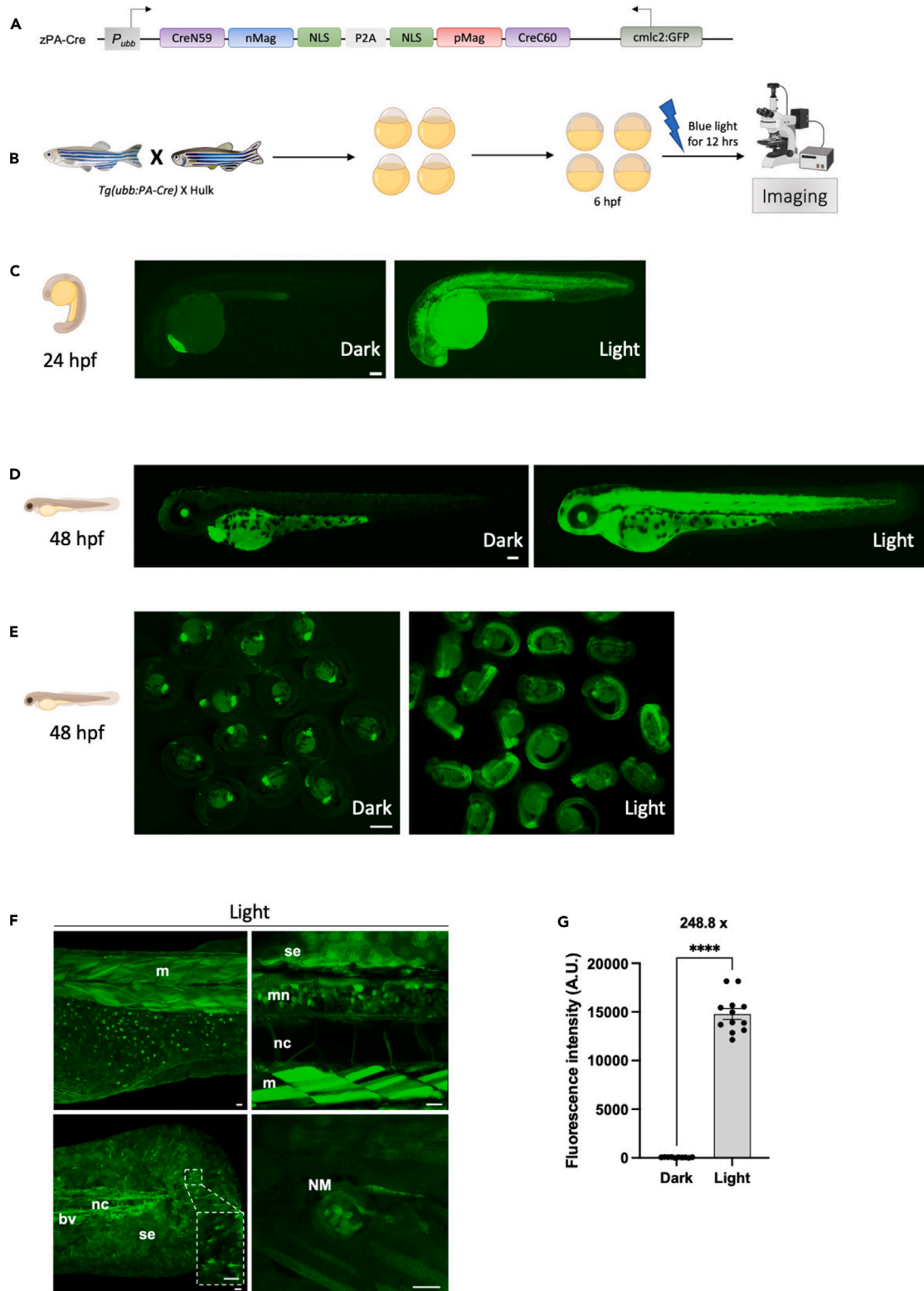


Figure 2. The stable transgenic line *Tg(ubb:PA-Cre)* enables light-induced genetic recombination in zebrafish embryos

(A) Schematic representation of the zebrafish PA-Cre genetic construct (zPA-Cre). The zPA-Cre system is driven by the *ubb* promoter. The green heart is used as a transgenesis marker (*cmlc2:GFP*). P, promoter; nMag, negative magnet; pMag, positive magnet; NLS, nuclear localization signal; CreN59, split Cre fragment (residues 19–59); CreC60, split Cre fragment (residues 60–343); P2A, 2A self-cleaving peptides; *cmlc2*, cardiac myosin light chain 2.

(B) Schematic representation of the strategy used to induce genetic recombination in zebrafish embryos. The stable transgenic line *Tg(ubb:PA-Cre)* was crossed with *Tg(ubb:Hulk)*. The double-transgenic embryos *Tg(ubb:PA-Cre; ubb:Hulk)* were photoactivated with blue light at 6 hpf for 12 h and then imaged at the desired developmental stage.

(C) Representative fluorescent images at 24 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark (left panel) or exposed to blue light illumination at 6 hpf for 12 h (right panel). Scale bar, 100 μ m.

(D) Representative fluorescent images at 48 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark (left panel) or exposed to blue light illumination (right panel) at 6 hpf for 12 h. Scale bar, 100 μ m.

(E) Fluorescent images at 48 hpf of a group of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark (left panel) or exposed to blue light illumination at 6 hpf for 12 h (right panel). Scale bar, 100 μ m.

(F) Representative confocal fluorescent z stack images of photoactivated *Tg(ubb:PA-Cre; ubb:Hulk)* embryo at 72 hpf. The higher magnification highlights GFP+ signal observed in various tissues and cells. m, skeletal muscle; se, skin epithelia; mn, motor neurons; nc, notochord; bv, blood vessels; NM, neuromast. Scale bar, 20 μ m.

(G) Quantification of fluorescence intensity at 48 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark ($n = 10$) or exposed to blue light illumination at 6 hpf for 12 h ($n = 12$). 248.8x represents the fold induction of the zPA-Cre system. Mean \pm SEM of the fluorescence intensity is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$. A.U., arbitrary unit.

zPA-Cre is effective in diverse cell types

To assess the versatility of the zPA-Cre system, we investigated its capacity to induce DNA recombination in various Cre-responsive transgenic zebrafish lines. We crossed the zPA-Cre line with distinct switch lines, in which specific cell types express dsRed under steady-state conditions and switch to GFP expression upon activation of Cre. These included the macrophage-specific *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)*²⁹ (Figure 3A), or the erythrocyte-specific *Tg(α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)*³⁰ line (Figure 3B) or the lymphocyte-specific *Tg(lck:loxP-DsRedx-loxP-GFP)*^{30,31} line (Figure 3C). In double-transgenic zebrafish embryos, exposure to 488-nm blue light illumination from 6 hpf to 18 hpf resulted in Cre/loxP, leading to robust GFP expression at 48 hpf in macrophages (Figure 3A) and in erythrocytes (Figure 3B), and at 120 hpf in thymocytes (Figure 3C). Importantly, we did not observe undesired GFP expression in embryos that were exposed to ambient light (Figure S3A). To evaluate the efficiency of the zPA-Cre system, we quantified the proportion of switched GFP-positive macrophages, erythrocytes, and thymocytes in the *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* line, the *Tg(α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)* line, and the *Tg(lck:loxP-DsRedx-loxP-GFP)* line, respectively. We found that ~95% of macrophages (Figure 3D), ~90% of erythrocytes (Figure 3E), and ~82% of thymocytes in the thymus (Figure 3F) exhibited GFP expression, indicating the high efficiency of our labeling system.

In addition, we tested the functionality of the PA-Cre system under the *kdr1* promoter,^{32,33} which exhibits expression during gastrulation, in the lateral plate mesoderm, and in endothelial cells.^{33–35} To examine the potential of the *kdr1*-PA-Cre system for erythrocyte labeling, we injected a *kdr1*-PA-Cre construct into *Tg(α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)* embryos at one-cell stage, as erythrocytes are known to be derived from the lateral plate mesoderm.^{36–39} Following blue light illumination from 6 hpf to 18 hpf in injected embryos, GFP expression was observed in erythrocytes at 24 hpf, indicating successful Cre/loxP recombination. Conversely, non-exposed embryos did not exhibit GFP expression in erythrocytes (Figures S3B and S3C). These results indicate that the zPA-Cre system enables DNA recombination in different cell types and that the PA-Cre system expression can be driven by distinct promoters. Collectively, our data establish the zPA-Cre system as a versatile tool for DNA recombination in zebrafish.

zPA-Cre enables light-inducible Cre/loxP recombination at different developmental time points

We then evaluated the efficiency of our system in comparison to widely used conventional non-photoactivatable Cre lines. To this end, we utilized the *Tg(ubb:creERT2)* line, in which Cre is ubiquitously expressed but its activity is induced only by incubation with tamoxifen, that allows Cre to enter to the nucleus.²⁶ We found that *Tg(zPA-Cre; ubb:HULK)* zebrafish embryos exposed to blue light overnight at 6 hpf exhibited a 23.2-fold induction compared to *Tg(ubb:creERT2; ubb:HULK)* embryos treated with 4-OH-tamoxifen (4-OHT) (Figures 4A, 4C and S4E), indicating that our system exhibits superior induction efficiency compared to other conventional inducible tools.

The use of light-inducible tools in zebrafish beyond the embryonic stages can be challenging due to limited light penetration in larval tissues. Therefore, we assessed the efficiency of DNA recombination using the zPA-Cre system at different developmental time points. To this end, we exposed double-transgenic *Tg(zPA-Cre; ubb:HULK)* embryos to blue light overnight at 24 hpf, 48 hpf, and 72 hpf and imaged them at 120 hpf (Figures 4B and 4F). We observed GFP expression in all embryos or larvae exposed to blue light, although at lower levels compared to embryos exposed to blue light at 6 hpf. Of note, control siblings that were kept in the dark did not show GFP expression (Figures 4B and 4F). Next, we investigated the relationship between the zPA-Cre activity and the duration of blue light illumination. We exposed double-transgenic *Tg(zPA-Cre; ubb:HULK)* embryos to blue light for different time durations at 6 hpf and 48 hpf. We found that, the longer the exposure time, the more is the GFP expression in exposed embryos, and illuminating 6 hpf embryos with blue light for only 2 h resulted in robust GFP expression at 120 hpf (Figures 4D, 4E and S4A). These results indicate that zPA-Cre enables efficient DNA recombination even through short periods of blue light exposure and that the zPA-Cre activity increases in a light dose-dependent manner.

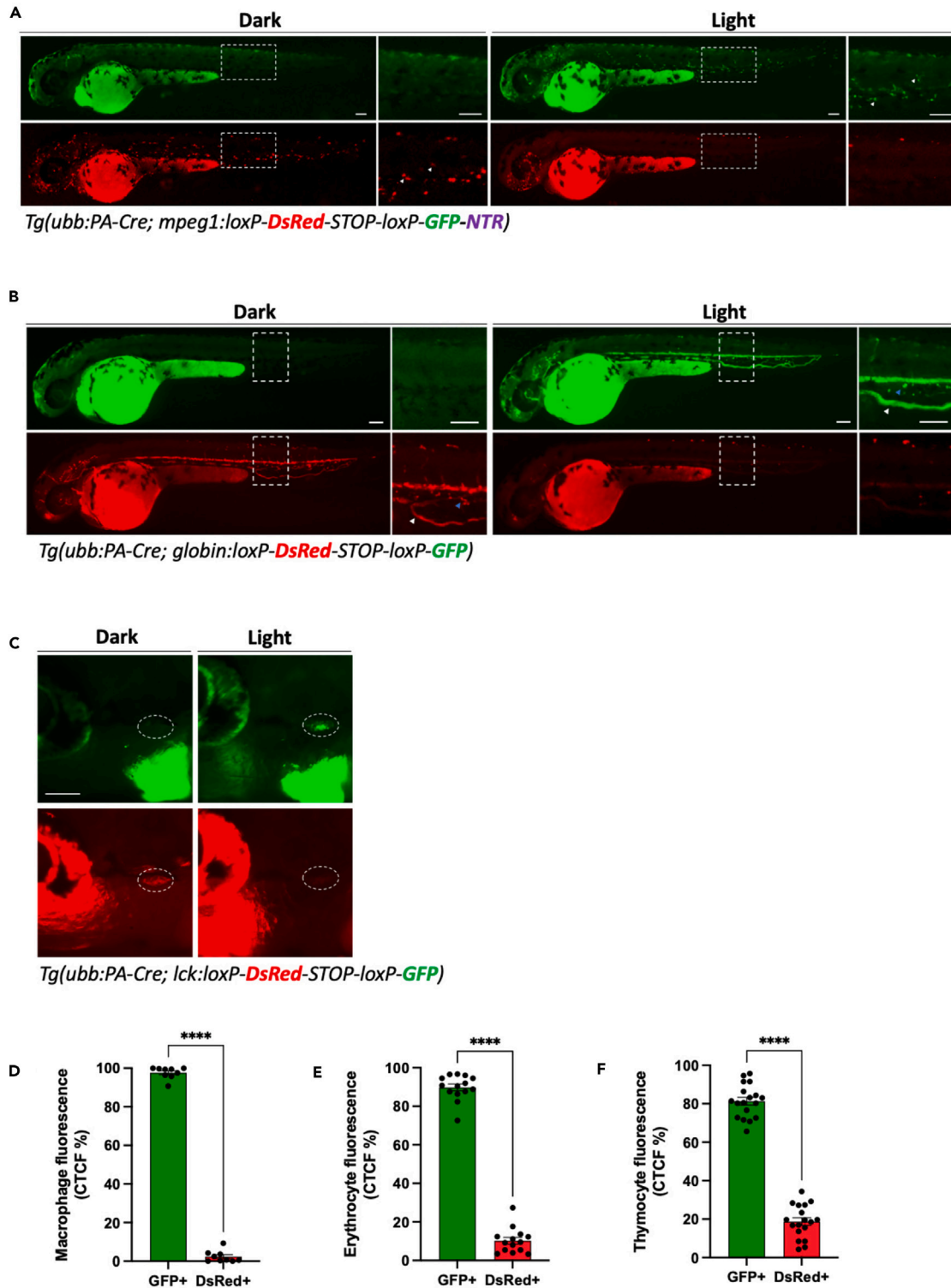


Figure 3. *Tg(ubb:PA-Cre)* enables light-induced genetic recombination with different Cre reporter zebrafish transgenic lines

(A) Representative fluorescent images at 48 hpf of *Tg(ubb:PA-Cre; mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* embryos kept in the dark or exposed to blue light illumination at 6 hpf for 12 h. White dashes indicate the region with higher magnification. White arrowheads highlight macrophages at higher magnification in the trunk. Scale bar, 100 μ m.

Figure 3. Continued

(B) Representative fluorescent images at 48 hpf of *Tg(ubb:PA-Cre; α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)* embryos kept in the dark or exposed to blue light illumination at 6 hpf for 12 h. White dashes indicate the region with close-ups. White arrowheads highlight erythrocytes in circulation, and blue arrowheads highlight erythrocytes in the CHT. Scale bar, 100 μ m.

(C) Representative fluorescent images at 120 hpf of *Tg(ubb:PA-Cre; lck:loxP-DsRedx-loxP-GFP)* embryos kept in the dark or exposed to blue light illumination at 6 hpf for 12 h. White dashes mark the thymus. Scale bar, 100 μ m.

(D) Quantification of macrophage fluorescence intensity was measured at 48 hpf in photoactivated *Tg(ubb:PA-Cre; mpeg1:Switch)* embryos ($n = 9$). Mean \pm SEM of the dsRed+ and GFP+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$.

(E) Quantification of erythrocyte fluorescence intensity was measured at 48 hpf in photoactivated *Tg(ubb:PA-Cre; globin:Switch)* embryos ($n = 14$). Mean \pm SEM of the dsRed+ and GFP+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$.

(F) Quantification of thymocyte fluorescence intensity was measured at 120 hpf in photoactivated *Tg(ubb:PA-Cre; lck:Switch)* embryos ($n = 18$). Mean \pm SEM of the dsRed+ and GFP+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$.

Moreover, to test if our zPA-Cre system is applicable to induce DNA recombination beyond embryonic stages, we photoactivated double-transgenic *Tg(zPA-Cre; ubb:HULK)* 6 days post fertilization (dpf) larvae. We found that the zPA-Cre can be used to induce expression at larval stages, extending the use of this system beyond early embryonic stages (Figures S4B–S4D).

These findings demonstrate that, while the highest efficiency is achieved during early developmental stages, our system remains effective at later developmental stages.

zPA-Cre enables optogenetic-mediated cell ablation in zebrafish embryos

One of the challenges in characterizing the role of specific population subsets is the lack of tools for cell type-specific ablation.⁴⁰ Despite that chemically inducible Cre strains are widely used to achieve this goal, the temporal and spatial resolution is limited. To demonstrate the utility of using our system to perform photoactivation in a specific cell lineage and then perform targeted ablation, zPA-Cre fish were crossed with the macrophage-specific *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* that allows selective ablation of macrophages based on their origin. In this line, the expression of bacterial nitroreductase (NTR) is under the control of the *mpeg1* promoter.²⁹ Thus, the NTR will be expressed exclusively in the photoactivated macrophages; thereby, photoactivated macrophages could be ablated by metronidazole (MTZ) treatment⁴¹ (Figure 5A). First, we photoactivated double-transgenic *Tg(zPA-Cre; mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* between 14 hpf and 24 hpf, a time at which primitive macrophages start to emerge in the developing embryo. To evaluate the efficiency of our labeling strategy, we quantified the proportion of switched GFP-positive macrophages and found that ~65% of macrophages (Figure 5C) exhibited GFP expression, indicating the high efficiency of our labeling strategy.

To assess the efficiency of the zPA-Cre-mediated cell ablation with the NTR-MTZ system, we quantified the total number of GFP+ macrophages of photoactivated embryos after treatment with either DMSO or MTZ for 24 h from 24 to 48 hpf. We found that macrophages were significantly reduced by the MTZ treatment compared to the DMSO-treated controls, suggesting that zPA-Cre also allows for light-induced cell-specific ablation (Figures 5B and 5D). In summary, we concluded that the zPA-Cre transgenic fish could be used for efficient spatiotemporal cell ablation in multiple tissues and would be a valuable approach for dissecting cell functions.

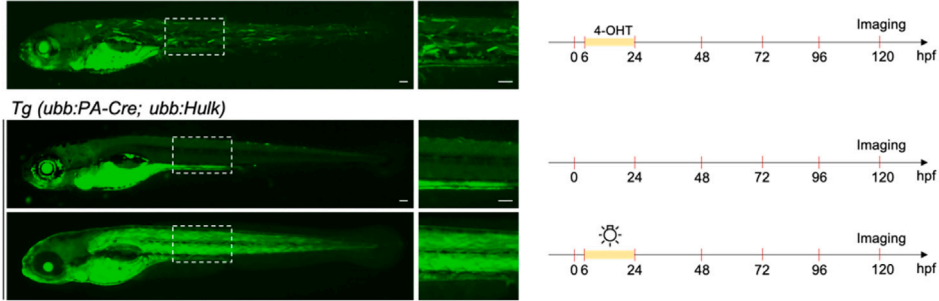
zPA-Cre enables light-inducible Cre/loxP recombination at a specific region of interest and at the single-cell resolution

To test the capability of the zPA-Cre system to drive DNA recombination in specific tissue regions, we used the *Tg(zPA-Cre; ubb:HULK)* embryos and performed photoactivation in targeted regions of interest. Photoactivation with blue light using a 488 nm laser at 100% laser power (2 mW/mm²) in a specific region (a 25 μ m diameter circle) for 5 min within a single skeletal muscle at 48 hpf resulted in GFP expression in the activated region (Figure 6A).

Next, we utilized the *Tg(zPA-Cre)* line in combination with the *Tg(α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)* line to test if *loxP*-mediated DNA recombination could be induced in a small number of few erythrocytes present in the posterior blood island (PBI), a transient hematopoietic tissue in zebrafish.³⁷ Photoactivation with blue light using a 488 nm laser at 100% laser power (2 mW/mm²) in few erythrocytes in the PBI (with a 25 μ m diameter circle) for 5 min at 22 hpf resulted in the presence of GFP+ erythrocytes in the caudal hematopoietic tissue (CHT), the fetal liver counterpart in zebrafish⁹ (Figure 6B), as well as in the circulation (Video S2, and Video S3) at 48 hpf. Finally, we examined if the zPA-Cre allows for optogenetic manipulation with single-cell resolution. To this end, we applied two-photon 920-nm illumination on the double *Tg(zPA-Cre; α/β_{a2} -globin:loxP-DsRedx-loxP-GFP)* to target single erythrocytes in the PBI region at 22 hpf, before the onset of blood circulation in zebrafish³⁷ (Figure 6C). We applied 45 s of 920-nm illumination by scanning the infrared laser beam over an area of 8 \times 8 μ m to cover single erythroid progenitors. After photoactivation, embryos were kept in the dark for 24 h, and live imaging was conducted at this stage to trace the migration of the photoactivated GFP+ cells and their progeny. Results showed that two-photon photoactivation of single erythrocytes resulted in the presence of GFP+ erythrocytes in the circulation, demonstrating that our zPA-Cre system can be used to trace the progenies derived from a small population (Figures 6C and 6D and Video S4, Video S5, Video S6, Video S7).

In conclusion, these experiments demonstrate that the zPA-Cre system enables efficient and precise spatial and temporal control of *loxP*-mediated DNA recombination in zebrafish embryos with high resolution.

A *Tg(ubb:creERT2; ubb:Hulk)*



B *Tg(ubb:PA-Cre; ubb:Hulk)*

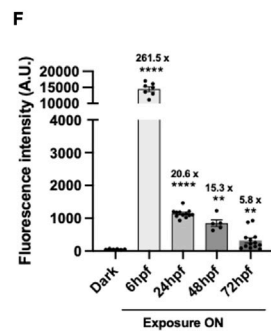
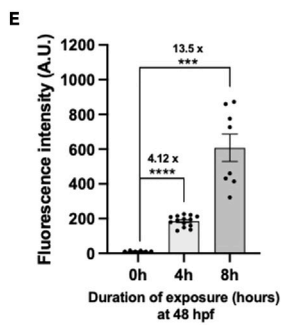
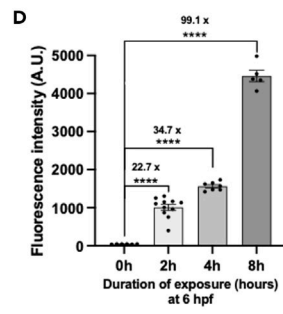
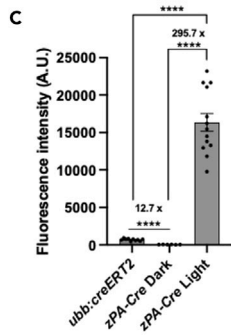
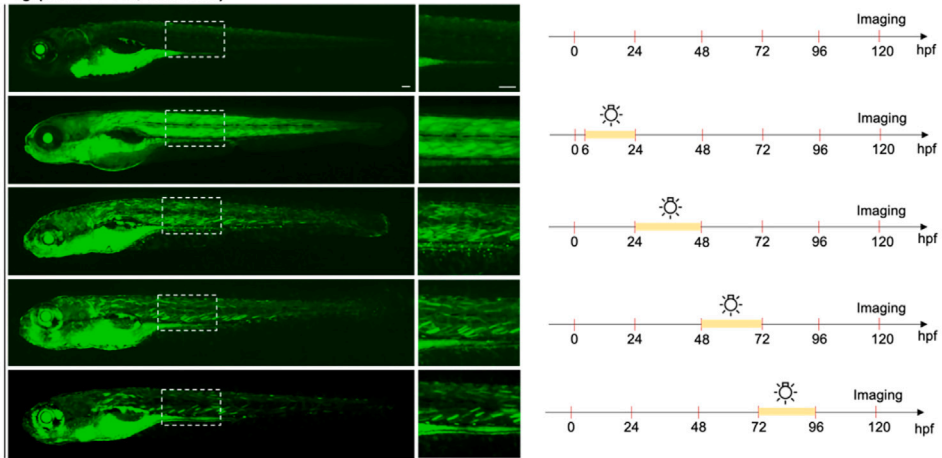


Figure 4. *Tg(ubb:PA-Cre)* enables light-induced Cre-LoxP recombination at different developmental stages

(A) Representative fluorescent images at 120 hpf of 4-OHT-induced *Tg(ubb:creERT2; ubb:Hulk)* embryo or *Tg(ubb:PA-Cre; ubb:Hulk)* embryo kept in the dark or exposed to blue light illumination at 6 hpf overnight (left panel). White dashes indicate the region showing close-ups. Scale bar, 100 μ m. Schematic representation of the strategy used to induce genetic recombination in zebrafish *Tg(ubb:creERT2; ubb:Hulk)* or *Tg(ubb:PA-Cre; ubb:Hulk)* embryos (right panel).

(B) Representative fluorescent images at 120 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark or exposed to blue light illumination overnight at 6 hpf, 24 hpf, 48 hpf, and 72 hpf (left panel). White dashes indicate the region with close-ups. Scale bar, 100 μ m. Schematic representation of the strategy used to induce genetic recombination in zebrafish *Tg(ubb:PA-Cre; ubb:Hulk)* embryos at different developmental stages (right panel).

(C) Quantification of fluorescence intensity at 120 hpf of 4-OHT-induced *Tg(ubb:creERT2; ubb:Hulk)* ($n = 10$), *Tg(ubb:PA-Cre; ubb:Hulk)* embryo kept in the dark ($n = 6$), or exposed to blue light illumination at 6 hpf overnight ($n = 13$). The numbers 23.2x and 295.7x represent the fold induction comparison between the light-exposed group and the 4-OHT-induced group and between the light-exposed group and the non-exposed ones, respectively. Mean \pm SEM of the fluorescence intensity is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$. A.U., arbitrary unit.

(D) Quantification of fluorescence intensity at 120 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos exposed to blue light illumination at 6 hpf for 0 h ($n = 6$), 2 h ($n = 11$), 4 h ($n = 7$), and 8 h ($n = 5$). The numbers 22.7x, 34.7x, and 99.1x represent the fold induction comparison between the 2-h exposed group and the non-exposed group, the 4-h exposed group and the non-exposed group, and the 8-h exposed group and the non-exposed group, respectively. Mean \pm SEM of the fluorescence intensity is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$. A.U., arbitrary unit.

(E) Quantification of fluorescence intensity at 120 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos exposed to blue light illumination at 48 hpf for 0 h ($n = 6$), 4 h ($n = 14$), and 8 h ($n = 8$). The numbers 4.12x and 13.5x represent the fold induction comparison between the 4-h exposed group and the non-exposed group, and the 8-h exposed group and the non-exposed group, respectively. Mean \pm SEM of the fluorescence intensity is shown. Unpaired t test with Welch's correction was used for this analysis. *** $p \leq 0.001$; **** $p \leq 0.0001$. A.U., arbitrary unit.

(F) Quantification of fluorescence intensity at 120 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* embryos kept in the dark ($n = 6$) or exposed to blue light illumination overnight at 6 hpf ($n = 7$), at 24 hpf ($n = 12$), at 48 hpf ($n = 5$), and at 72 hpf ($n = 13$). The numbers 261.5x, 20.6x, 15.3x, and 5.8x represent the fold induction comparison between the different groups and the dark one, respectively. Mean \pm SEM of the fluorescence intensity is shown. Unpaired t test with Welch's correction was used for this analysis. ** $p \leq 0.01$; **** $p \leq 0.0001$. A.U., arbitrary unit.

DISCUSSION

Optogenetic tools play a crucial role in studying development and tissue regeneration in various organisms, offering precise spatial and temporal resolution. Numerous light-inducible tools for Cre/loxP recombination have been developed utilizing UV light, blue light, or red light. Consequently, several attempts have been made to develop optically controlled Cre systems in zebrafish, as reported in previous studies.^{12–16} Despite significant advances, current light-inducible systems tested in zebrafish have been performed in transient and suffer from low efficiency and leakiness, require long light exposure resulting in cytotoxicity, require exogenous chromophores, or need harmful chemicals as caged biomolecules or tamoxifen.^{10,12–17}

Here, we demonstrated the successful development of a novel stable optogenetic zebrafish line in which the PA-Cre system is controlled by the zebrafish ubiquitin promoter.²⁶ By utilizing the *Tg(ubb:PA-Cre)* line, we provide evidence that our system allows for efficient and robust Cre/loxP recombination in various tissues and cell types through noninvasive blue light illumination.

As a general rule, it is crucial for a light-inducible Cre recombinase to maintain low levels of background recombination.¹⁰ In this regard, we show that the *Tg(ubb:PA-Cre)* line exhibits no background recombination in the absence of light stimulation, while displaying a highly inducible efficiency of DNA recombination upon exposure to blue light. Furthermore, we showcase the versatility of our system by demonstrating its ability to induce recombination at different developmental stages and achieve spatial control within specific regions upon blue light illumination. Importantly, after two-photon light illumination, the *Tg(ubb:PA-Cre)* line allows for optogenetic manipulation with single-cell resolution. These distinctive advantages offered by the *Tg(ubb:PA-Cre)* line pave the way for *in vivo* zebrafish studies during development with high spatiotemporal resolution.

As shown in this study, the newly generated *Tg(ubb:PA-Cre)* line enables precise spatial and temporal control of Cre/loxP recombination through blue light illumination. This versatile DNA recombination system holds potential for applications such as fate mapping or the induction of carcinogenesis in specific cell types upon illumination. Moreover, the utilization of the PA-Cre system in zebrafish reveals a striking simplicity and efficiency, thus expanding the repertoire of available genetic manipulation methods.

Our newly developed versatile tool is expected to pave the way for light-controlled DNA recombination *in vivo* in cells or tissues that may not have a specific promoter, thereby facilitating studies on lineage tracing, development, and disease. Furthermore, as we generated a stable transgenic line in which the PA-Cre system is driven by the *ubb* promoter, it holds a particular promise for investigating the utility of our tool in adult zebrafish. Exploring the performance and efficiency of the PA-Cre system under different promoters in zebrafish is also an enticing avenue to achieve more precise cell-specific DNA recombination. Lastly, with the development of the PA-Dre²³ and PA-Flp²⁴ systems, it will become feasible to build additional optogenetic tools in zebrafish and perform intersectional genetics involving more than one recombinase.

Taken together, our findings indicate that the *Tg(ubb:PA-Cre)* line is an efficient optogenetic tool for gene manipulation with greatly expandable applications in developmental biology research.

Limitations of the study

In this study, we have achieved Cre/loxP recombination either by global blue light exposure or through laser-mediated illumination within a defined region or at the single-cell level. However, our zPA-Cre system is driven by the zebrafish *ubb* promoter. Further work will be needed to assess whether the magnet split-Cre system can be expressed from tissue or cell-specific promoters to allow for Cre/loxP recombination upon

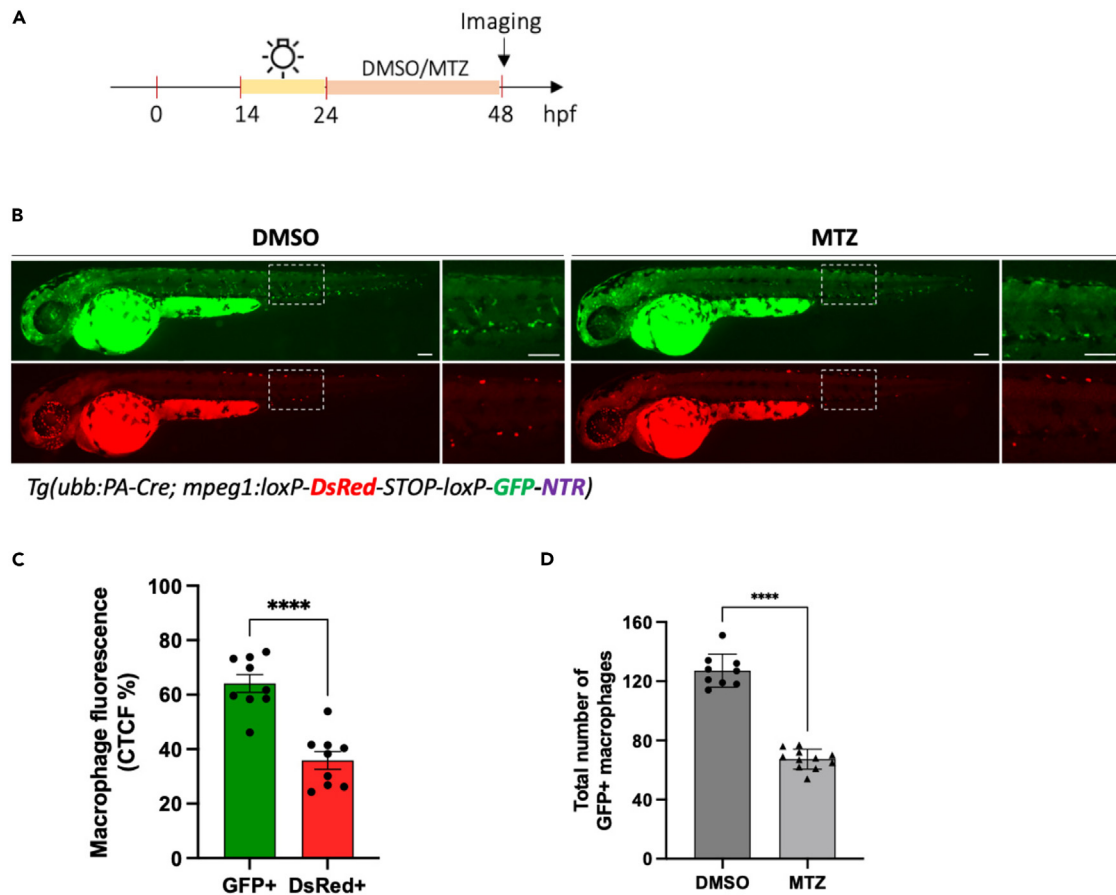


Figure 5. *Tg(ubb:PA-Cre)* enables optogenetic cell ablation in zebrafish embryos

(A) Schematic representation strategy used to selectively ablate macrophages in photoactivated embryos.

(B) Representative fluorescent images at 48 hpf of *Tg(ubb:PA-Cre; mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* embryos treated with either DMSO as control or metronidazole (MTZ) to ablate macrophages. White dashes indicate the region showing close-ups. Scale bar, 100 μ m.

(C) Quantification of macrophage fluorescence intensity was measured at 48 hpf in photoactivated *Tg(ubb:PA-Cre; mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* embryos treated with DMSO ($n = 9$). Mean \pm SEM of the dsRed+ and GFP+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$.

(D) Quantification of the total number of GFP+ macrophages at 48 hpf in photoactivated *Tg(ubb:PA-Cre; mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* embryos treated with either DMSO ($n = 9$) or MTZ ($n = 11$). Unpaired t test with Welch's correction was used for this analysis. **** $p \leq 0.0001$.

global blue light exposure in a tissue-specific manner. In line with that, it is also needed to test whether the two elements of the magnet split-Cre system both can be expressed from two distinct promoters to allow for more lineage-tracing specificity. So far, we have only demonstrated the utility of our system during zebrafish early developmental stages; however, further work is needed to assess whether this zPA-Cre system is also applicable to inducing DNA recombination in juvenile and adult zebrafish. However, global blue light illumination will be likely limited to superficial tissues when applied beyond embryonic developmental stages (beyond 5-dpf). To activate cells/tissues located deep in the body of the animal beyond embryonic or at larval stages, it has to be achieved by applying a two-photon activation strategy to enhance the precision of targeted cells and increase the penetration of light.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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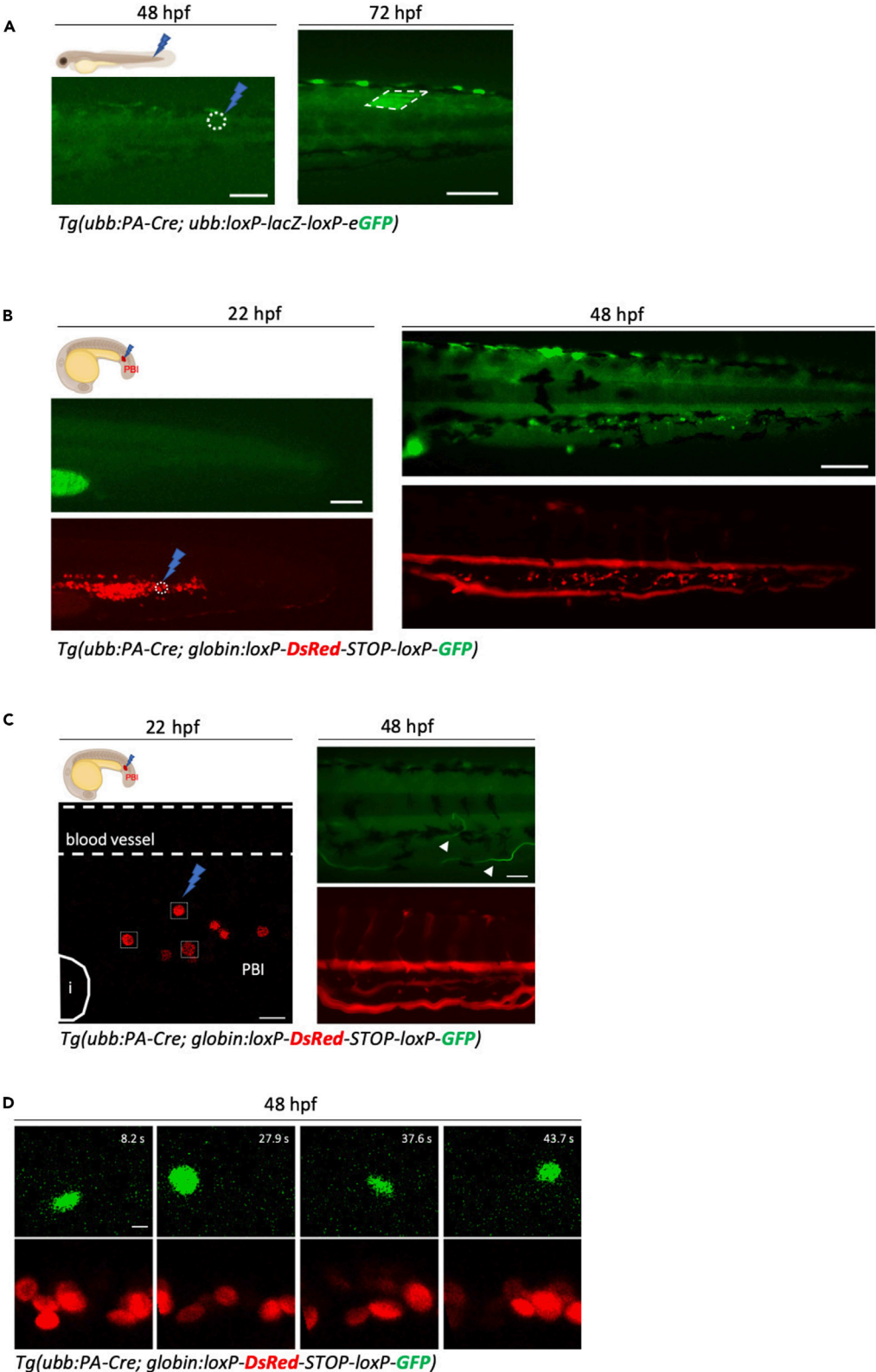


Figure 6. *Tg(ubb:PA-Cre)* enables light-induced spatial and temporal genetic recombination

(A) Schematic representation strategy used to induce local photoactivation within a single skeletal muscle cell using a blue light laser. A 25 μm diameter circle within a single skeletal muscle was targeted by a 488 nm laser for 5 min ($n = 6$). Scale bar, 100 μm . Representative fluorescent images at 72 hpf of *Tg(ubb:PA-Cre; ubb:Hulk)* showing GFP+ skeletal muscle. Scale bar, 100 μm .

(B) Schematic representation strategy used to induce local photoactivation with blue light laser at 22 hpf. Few cells in the PBI (a 25 μm diameter circle) were targeted by a 488 nm laser for 5 min ($n = 6$; left panel). Scale bar, 100 μm . Representative fluorescent image at 48 hpf of *Tg(ubb:PA-Cre; $\alpha/\beta\text{a}2\text{-globin:loxP-DsRedx-loxP-GFP}$)* embryo showing GFP+ erythrocytes in the CHT (right panel). Scale bar, 100 μm .

(C) Schematic representation strategy used to induce two-photon photoactivation at 22 hpf. An area of $8 \times 8 \mu\text{m}$ in the PBI that covers single erythroid progenitors was targeted by a 920 nm two-photon laser for 45 s ($n = 9$; left panel). Scale bar, 20 μm . Representative fluorescent images at 48 hpf of *Tg(ubb:PA-Cre; $\alpha/\beta\text{a}2\text{-globin:loxP-DsRedx-loxP-GFP}$)* showing GFP+ erythrocytes (arrowheads) in the circulation. Scale bar, 50 μm . Higher-magnification images are shown in D.

(D) Higher-magnification images of still fluorescent images of time-lapse imaging in *Tg(ubb:PA-Cre; $\alpha/\beta\text{a}2\text{-globin:loxP-DsRedx-loxP-GFP}$)* at 48 hpf showing GFP+ erythrocytes in the circulation. Scale bar, 5 μm .

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2024.110476>.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.E. and P.P.H.; methodology and data collection, R.E., A.M., P.D., and G.G.; writing – original draft, R.E.; writing – review and editing, R.E. and P.P.H.; funding acquisition, P.P.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
(Z)-4-Hydroxytamoxifen (4-OHT)	Sigma	Cat# H7904
Ethyl 3-aminobenzoate methane sulfonate salt (Tricaine)	Sigma	Cat# A5040
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D5879
Metronidazole (MTZ)	Sigma	Cat# M1547
Acridine orange (AO)	Sigma	Cat#A6014
Experimental models: Organisms/strains		
Zebrafish <i>Tg(-3.5ubb:PA-Cre; cmlc2-EGFP)</i>	This study	NA
Zebrafish <i>Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)</i>	Lin et al., 2019 ²⁹	ZFIN: ZDB-ALT-191122-8
Zebrafish <i>Tg(globin:loxP-DsRedx-loxP-GFP)</i>	Tian et al., 2017 ³⁰	ZFIN: ZDB-TGCONSTRCT-180702-2
Zebrafish <i>Tg(lck:loxP-DsRedx-loxP-GFP)</i>	Tian et al., 2017 ³⁰	ZFIN: ZDB-TGCONSTRCT-180702-3
Zebrafish <i>Tg(ubb:creERT2; cmlc2-EGFP)</i>	Mosimann et al., 2011 ²⁶	ZFIN: ZDB-TGCONSTRCT-110121-1
Zebrafish <i>Tg(ubb:Hulk)</i>	Di Donato et al., 2016 ²⁷	ZFIN: ZDB-TGCONSTRCT-151222-1
Software and algorithms		
ImageJ	Schindelin et al. ⁴²	https://imagej.nih.gov/ij/
Prism 9	GraphPad	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for scripts, resources, and reagents should be directed to and will be fulfilled by lead contact, Pedro P. Hernández (pedro.hernandez-cerda@curie.fr).

Materials availability

The new reagents and datasets generated during the current study are available from the corresponding authors upon request.

Data and code availability

- Datasets reported in this paper will be shared by the [lead contact](#) upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Zebrafish

Embryonic and adult zebrafish (*Danio rerio*) were maintained at 28°C on a 14-h light/10-h dark cycle. The collected embryos were raised in fish water containing 0.01% methylene blue to prevent fungal growth. All fish are housed in the fish facility of our laboratory, which was built according to the local animal welfare standards. Animal care and use for this study were performed in accordance with the recommendations of the European Community (2010/63/UE) for the care and use of laboratory animals. Experimental procedures were specifically approved by the ethics committee of the Institut Curie CEEA-IC #118 (Authorization number - APAFiS#21197-2019062521156746-v2 - given by National Authority) in compliance with the international guidelines."

Transgenic lines

The following lines of the AB stain were used: *Tg(ubb:HULK)*²⁷, *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)*²⁹, *Tg(globin:loxP-DsRedx-loxP-GFP)*,³⁰ *Tg(ubb:CreERT2; cmlc2-GFP)*²⁶ and *Tg(lck:loxP-DsRedx-loxP-GFP)*.³⁰ All Fish used in this study were homozygous and were crossed to produce embryos and larvae by natural mating. Embryo and Larval zebrafish were studied before the onset of sexual differentiation and their sex can therefore not be determined.

METHOD DETAILS

Plasmids

The original PA-Cre construct driven by the CMV promoter was purchased from Addgene (Addgene no. 122960). The PA-Cre coding sequence was synthesized by Genescript and listed in the supplementary note. The (-3.5*ubb*:PA-Cre; *cmlc2*-EGFP) constructs were generated by inserting 3.5 kb ubi promoter fragment, together with the coding sequence of PA-Cre, via Gibson assembly into pTol2 vector that carries *cmlc2*-EGFP as a transgenesis marker. To generate the *kdr1*:PA-Cre constructs, the *kdr1* promoter and the coding sequence of PA-Cre were cloned via Gibson assembly into the pTol2 vector.

Zebrafish embryo microinjection

The CMV:PA-Cre and *kdr1*:PA-Cre DNA constructs (25 ng/μL) were injected into zebrafish embryos at the one cell stage using pressure-controlled microinjector and a micromanipulator. After injection, the embryos were cultured in E3 embryo medium with methylene blue in the dark at 28.5°C.

Acridine orange (AO) staining

Zebrafish embryos were incubated in 5ug/ml AO solution for 1 h and washed for several times until the background is clear.

Generation of transgenic lines

The -3.5*ubb*:PA-Cre;*cmlc2*-EGFP constructs (25 ng/μL) were then co-injected with mRNA transposase (50 ng/μL) into 1-cell stage WT embryos⁴³ and the resulting embryos were grown to adulthood for stable line screening. F0 founders were screened for specific *cmlc2*:GFP expression, raised to adulthood, and screened for germ line transmission. Single-insertion transgenic strains were established and verified through screening for a 50% germ line transmission rate in outcrosses in subsequent generations.⁴⁴

Measurements of laser power

Single photon lasers: Laser powers were measured on the front focal plane of the objective using a compact power and energy meter console (ThorLabs, #PM100D) fitted with a low power photodiode sensor (ThorLabs, #S170C). Infrared multiphoton laser: Laser power on the focal plane was determined by measuring the power at the back focal plane of the objective with a thermopile sensor (PM10V1, Coherent, Santa Clara, USA) and multiplying by a factor of 0.75 (transmission of the 40x oil objective at 920 nm according to Zeiss transmission curves <https://www.micro-shop.zeiss.com/>) for the two-photon Ti:Sapphire laser used in this study.

Photoactivation with global blue light

Global light induction was performed using a MARS AQUA-YWT-165W LED aquarium hood. Actual power of light exposure received by embryos, with lids of plates removed, was measured as ~1.8 mW/cm² at 488 nm. 6 hpf embryos were illuminated with constant blue light for 12 h. To ensure all cells were exposed, embryos were periodically swirled during irradiation. In experiments presented in Figure 4, embryos and larvae at each indicated developmental stage were illuminated with constant blue light overnight and kept again in the dark till the desired developmental stage. Dark controls were placed in a lightproof box within the same 28.5°C incubator as the light-treated samples.

Macrophage ablation and drug treatments

The Tg(*ubb*:PA-Cre;*mpeg1*:loxP-DsRedx-loxP-GFP-NTR) embryos were photoactivated by global blue light illumination and were immersed in system water containing 10 mM metronidazole (M1547, Sigma) for 24 h, which caused an acute depletion of GFP-NTR⁺ cells. Photoactivated controls were incubated in the equivalent amount of DMSO solution during the same period. Light exposure was avoided by using foil to cover the plates as MTZ is light sensitive. Zebrafish were further analyzed by fluorescence imaging at 48 hpf.

Regional activation

The blue-light activation was performed on a Zeiss LSM780 inverted confocal microscope. The desired region of the embryo was focused under a 25X lens (Zeiss Plan-Apochromat 25X, NA = 0.8 WD = 0.55), with the background fluorescence signal excited by a 405 nm laser. The desired region was selected under the bleach mode in ZEN software. A 2 mW 488 nm laser was used for activation the region for 5 min at 100% laser power. After activation, the embryos were incubated in dark at 28.5°C and then examined at indicated developmental stages.

Two photon photoactivation for single cell targeting

Two photon based photoactivation was performed on a Zeiss LSM880 NLO inverted confocal microscope equipped with Ti:Sapphire laser (Mai Tai DeepSee, Spectra Physics) at 920 nm. The desired region of the embryo was focused under a 40X lens (Zeiss Plan-Apochromat 25X, NA = 1.3 WD = 0.21), with the red fluorescence signal excited by a 561 nm laser. The desired region was selected under the bleach mode in ZEN software. A 120 mW 920 nm laser was used for activation of single cells for 45 s. After activation, the embryos were incubated in dark at 28.5°C and then examined at indicated developmental stages.

Fluorescence microscopy

Zebrafish embryos, larvae and adults were anesthetized with 0.01% tricaine (A5040, Sigma) and mounted in 2.5% methylcellulose in 35-mm imaging dishes (MatTek).⁴⁵ Fluorescent imaging was performed with either Zeiss LSM780 inverted confocal microscope or Zeiss Axio Zoom V.16 upright microscope with an AxioCam HRm Zeiss camera and Zeiss Zen 3.3 software or with Leica thunder imaging system with Leica LAS-X software. Fluorescence was detected with mCherry, Texas Red (for dsRed labeled cells), and green fluorescent protein (GFP) filters.

Image analysis

All images were analyzed using FIJI software.⁴²

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed by the GraphPad Prism software (Prism 9). All experiments with only two groups and one dependent variable were compared using an unpaired t-test with Welch's correction. Statistical data show mean \pm S.E.M. Each dot plot value represents an independent embryo, and every experiment was conducted three times independently. The corrected total cell fluorescence (CTCF) was calculated using the formula 'Integrated density whole - (area whole embryo x mean fluorescence background)'. This formula is loosely based on a method described for calculating cell-fluorescence.⁴⁶ To calculate the CTCF percentage for dsRed+ cells, the CTCF value of dsRed was divided by the total summation of CTCF values for both dsRed and GFP in each larvae. The same was done for calculating CTCF percentage for GFP+ cells. For fluorescence intensity quantification, the same parameters (excitation/emission, gain for detectors, lasers intensity) were applied for images and fluorescence intensity was measured using FIJI. The fold induction or the GFP-fold induction was calculated by dividing the average of the fluorescence intensity values in the light-exposed group by the average values of the non-exposed one. Finally, Unpaired t-test with Welch's correction was used for quantifications. ** $p \leq 0.01$; **** $p \leq 0.0001$. A.U., Arbitrary Unit.