Optogenetic Control of Apoptosis in Targeted Tissues of *Xenopus laevis* Embryos

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ABSTRACT: KillerRed (KR) is a recently discovered fluorescent protein that, when activated with green light, releases reactive oxygen species (ROS) into the cytoplasm, triggering apoptosis in a KR-expressing cell. This property allows for the use of KR as a means of killing cells in an organism with great temporal and spatial specificity, while minimizing the nonspecific effects that can result from mechanical or chemical exposure damage techniques. Such optogenetic control of cell death, and the resulting ability to induce the targeted death of specific tissues, is invaluable for regeneration/repair studies—particularly in *Xenopus laevis*, where apoptosis plays a key role in regeneration and repair. We here describe a method by which membrane-bound KR, introduced to *Xenopus* embryos by mRNA microinjection, can be activated with green light to induce apoptosis in specific organs and tissues, with a focus on the developing eye and pronephric kidney.

KEYWORDS: optogenetics, KillerRed, apoptosis, *Xenopus*, regeneration

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Introduction

The field of optogenetics, the use of light to control cellular processes, has made tremendous strides in the decade since its inception. Optogenetic control of almost every level of cellular function has become feasible, from the beginnings of the field in light-mediated regulation of membrane potential, to recent, more sophisticated methods of controlling enzymatic function and gene transcription.[1] Within the past eight years, the discovery of genetically encoded photosensitizing agents—in particular the fluorescent proteins KillerRed (KR) and miniSOG—has expanded the optogenetic toolkit further. These proteins have allowed for the development of photoinducible methods of cell death, giving rise to new methods of killing cells and damaging tissues in whole organisms with great spatial and temporal specificity.[2,3] Applications of these new tools so far have ranged from tumor phototherapy to cell cycle control.[4–7] However, these proteins also have a great deal of potential in less immediately obvious fields of research. The ability to selectively induce cell death and ablate tissues in a living organism is of key importance in developmental biology and the growing field of regeneration and repair.

For over a century, cell death has been known to be an essential part of organismal development. Programmed cell death plays a role across a wide range of life stages. It is necessary for the sculpting of tissues, removal of premetamorphic or sex-specific organs, and providing a counteracting force to proliferation to control cell numbers, among other developmental functions.[8,9] In *Xenopus laevis*, one of the first (and still widely used) models of embryonic development, a complex dynamic of cell death takes place throughout the embryo as it develops into a tadpole, and beyond into metamorphosis.[10,11] While methods of inducing cell death to study these processes are prevalent,

in order to truly replicate the dynamics of cell death in an embryo, cell death needs to be controlled with specific timing and targeted only at specific populations of cells. Few tools for inducing cell death exist that meet these criteria, and as yet, none have been exhibited in *Xenopus* that also have the advantages offered by an optogenetic system. The practical applications of such a tool would go beyond development, as well.

The means by which organisms can repair damaged tissues and regenerate missing tissues, or sometimes even entire organs and appendages, have been an outstanding question in biology since at least the 18th century, when Trembley, Bonnet, Spallanzani, and others discovered that numerous species of animals, extending across a large range of phyla, were capable of regeneration.[12] In more recent years, *Xenopus* has also proven an effective and popular model system for experiments in regeneration and repair.[13] Regeneration in *Xenopus* has been studied in a variety of tissues and organs, including the lens of the eye,[14] retina,[15] pronephric kidney,[16] tail,[17] and limb.[18] In order to study regeneration or repair of a particular organ or tissue, it is necessary to damage or remove cells at the target, sometimes with a high degree of specificity that limits the effects of damage—and the ensuing repair response—to the target organ or tissue. Surgery and chemical exposures have, until recently, been the major methods of inducing damage. Light-induced methods of cell death offer a number of advantages over traditional techniques, including greater temporal and spatial specificity, and a corresponding reduction in off-target effects. By inducing death on an individual cell-level basis, photosensitizers like KR also aid in studying the repair of damage on a smaller scale than removal of an entire organ or tissue – for example, the apoptosis of cardiomyocytes observed in a number of forms of cardiomyopathy.[19–21]

KR is a dimeric red fluorescent protein with excitation/emission maxima at 585/610 nm that, upon excitation with green light, releases reactive oxygen species (ROS), in the form of superoxides and singlet oxygen.[2,22] Following exposure to green light, KR-expressing cells undergo cell death via apoptosis, either through direct activation of apoptotic pathways by mitochondrial KR or oxidation of phospholipids in the plasma membrane by membrane-bound KR.[2,23] Additionally, KR targeted to the nuclear lamina or histones can induce DNA breaks and halt the cell cycle.[5,24] KR has been demonstrated in a variety of applications and organisms, including chromophore-assisted light inactivation (CALI) of proteins,[23] induction of mitochondrial death,[25] ablation of brain tissue in zebrafish,[26] blockage of cell division in *Xenopus*,[5] and therapeutic photosensitization of tumor cells.[4,7]

We have developed a method for targeted ablation of tissues by photoinduced cell death in KR-expressing *X. laevis* tadpoles. Following microinjection of membrane-bound KR mRNA into *Xenopus* embryos, tadpoles are selected for KR expression in a region of interest, and exposed to green light under a fluorescent microscope to induce apoptosis in the target tissue. We demonstrate that this process induces morphological and histological changes in the target tissues, including ablation of developing organs.

Methods

mRNA synthesis and preparation. The pCS2-NXE + mem-KR plasmid (Addgene plasmid 45761) was linearized with Acc65I restriction enzyme. Mem-KR mRNA was synthesized using the Ambion mMESSAGE mMACHINE SP6 kit, following the manufacturer's instructions. mRNA injection solution was prepared by diluting mem-KR mRNA to a concentration of less than 30 ng/μL in nuclease-free water.

Tadpoles. All experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee (IACUC) at Tufts University. No Internal Review Board (IRB) approval was required for this study. Adult female *X. laevis* were induced to ovulate by injection of chorionic gonadotropin hormone (Chorulon). Adult male *X. laevis* were euthanized by intraperitoneal injection of 5% tricaine methanesulfonate (MS222; adjusted to pH 7.2), and the testes were removed and stored in 1× modified Barth's saline (pH 7.5). Eggs were fertilized in vitro, and embryos were dejellied in 2% cysteine (pH 8) and raised in 0.1× Marc's Modified Ringer's solution (MMR; 10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.2 mM CaCl, 0.5 mM HEPES, 1 μM EDTA, pH 7.4). Embryos and tadpoles were staged according to Nieuwkoop and Faber.[27]

Expressing KillerRed in *Xenopus* embryos/larvae. Embryos were injected at cleavage stages using a microinjector and pulled capillary glass needles, calibrated to administer 5–10 nL of mem-KR mRNA solution per injection. A *X. laevis* fate map (eg, Ref.[28]) was used to target the injection location for later KR expression. Embryos were kept in 3% Ficoll (in 1× MMR) during injection, then gradually transferred to 0.1× MMR, and raised at 14–20°C in a dark environment to the desired stage.

Activation of KillerRed. Using a fluorescent microscope with a 200 W metal halide lamp and TRITC filter set (EX 545/20; BS 565; EM 595/50), tadpoles were selected for expression of KR in the region of interest. Tadpoles were immobilized in a 0.04% MS222 solution (in 0.1× MMR) and held stationary during light treatment. Light treatment consisted of a focused exposure through a 40× objective lens and TRITC filter of the region of interest, until noticeable photobleaching of KR had occurred (approximately five minutes). After exposure, tadpoles were transferred to fresh 0.1× MMR and raised at 14–20°C until morphological and histological changes were visible. They were then euthanized in tricaine, fixed for one hour in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), rinsed in 1× phosphate buffered saline (PBS), and dehydrated in 100% methanol to be stored at −20°C. Remaining KR protein was photobleached by exposure under white light, in methanol, overnight.

Detection of reactive oxygen species. Tadpoles were selected for KR expression as described above. Tadpoles

were then immersed in a 30 µM solution of dihydroethidium (30 mM stock in DMSO, diluted into 0.1× MMR), as per Owusu-Ansah et al,[29] with 0.04% MS222 to immobilize them. Light treatment was as described above. Before and after light treatment, dihydroethidium fluorescence was photographed using a Mermaid FRET filter set (EX 450/20; BS 460; EM 590).

Immunohistochemistry. Immunohistochemistry was performed on whole-mount tadpoles as described by Caine and McLaughlin.[16] In brief, fixed tadpoles were rehydrated and permeabilized in PBTr [1× PBS with 0.1% Triton and 2 mg/mL bovine serum albumin (BSA)], blocked in 20% heat-inactivated goat serum in PBTr, and incubated overnight at 4°C in anti-active-Caspase 3 primary antibody (1:300; BD Biosciences Pharmingen). They were then rinsed in PBTr for four hours, blocked, and incubated under the same conditions in goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 555 (1:300; Invitrogen). Tadpoles were finally washed in PBTr for five hours, before being imaged under a fluorescent microscope with a TRITC filter. For regions in which tadpoles exhibited punctate expression of apoptosis (eg, the pronephros), the number of individual apoptotic loci within the region was counted for each tadpole. Treatments were compared using an ANOVA test, with a Tukey–Kramer post-test to determine whether individual treatments significantly differed. For regions where individually counting apoptotic cells was not possible (eg, the eye), tadpoles were evaluated based on whether they showed a qualitative increase in active caspase-3 expression over a typical control tadpole. Treatments were compared using Fisher's exact test.

In situ hybridization and measurement of expression. In situ hybridization was performed on whole-mount tadpoles as described by Caine and McLaughlin.[16] Briefly, fixed tadpoles were hybridized with digoxigenin-labeled antisense RNA probes for senescence marker protein 30 (SMP-30). Probes were detected with anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1500; Roche Diagnostics) and 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT), which formed a blue precipitate when developed. The area of SMP-30 expression on both sides of each tadpole was measured with SPOT Advanced Imaging Software, and the ipsilateral and contralateral areas were compared to determine which was larger.

Results and Discussion

This protocol allows for induction of apoptosis in *X. laevis* tissues in a highly spatial and temporally regulated manner, by exposing KR expressing cells via fluorescent microscope and TRITC filter set to a standard metal halide or mercury light source. The eye and pronephric kidney of the developing *X. laevis* tadpole, both known regenerating systems, are presented here as example targets for demonstrating KR's effects on *Xenopus* tissue, and potential as a tool for inducing apoptotic damage in regeneration studies.

KR activation under the fluorescent microscope was confirmed through observation of photobleaching of fluorescence in the illuminated target regions (Figs. 1A and 3A), which has been shown to correlate with KR cytotoxicity.[26] Photobleaching was instantaneously apparent upon photoexcitation, and we found that five minutes of light exposure under a 40× objective at full lamp power (~25 mW/mm^2) was sufficient to ensure approximately 75% reduction in brightness of KR (Supplementary Fig. 1). To confirm that the response to KR is spatially restricted to the illuminated regions of the tadpole, we measured ROS production with the fluorescent superoxide detector dihydroethidium. We found that ROS-induced fluorescence was indeed limited to the illuminated region (Fig. 2).

We next looked at the effects of KR photoactivation in the target tissues. As KR is known to induce apoptosis in other organisms, we looked for an increase in active (cleaved) Caspase-3 expression, as a marker of apoptotic cells, following light exposure. Within 1–5 hours after light exposure, KR-expressing tadpoles showed a significant increase in apoptosis in targeted tissues (Figs. 1B, C and 3B, C). As with ROS production, apoptosis too was limited to the illuminated region. To confirm that the targeted tissues were, in fact, being disrupted by the increase in apoptosis, we examined morphological and molecular phenotypes in the eye and pronephros, respectively. Twenty-four hours post-exposure, tadpoles showed significant increase of phenotypes consistent with cell death in the exposed area, as verified by the ablation of the eye pigment (Fig. 1D, E) and the expression of molecular markers located in proximal tubules found on the exposed side, compared to the unexposed side of tadpoles (Fig. 3D, E). In comparison, we found no increase in apoptosis or tissue ablation from light illumination alone, in tissues not expressing KR (data not shown).

Even without light activation, KR-expressing cells have the potential to undergo increased apoptosis (Fig. 1C). Because of this "leakiness," the concentration of mRNA injected must be titered for each target tissue, to minimize nonspecific cell death and phenotypes. Conversely, too low a concentration of mRNA resulted in a greatly reduced incidence of phenotypes after light activation and may require an increase in time of light exposure to induce a phenotype (Supplementary Fig. 2). The data presented here used a 26 ng/µL dilution in injections targeted for the eye and 18 ng/µL dilution in injections targeted for the pronephros. Concentrations of mRNA at 40 ng/µL or higher drastically reduced the viability of injected embryos.

Compared with traditional methods of tissue ablation, including mechanical damage and whole-organism chemical exposures, KR-induced damage allows for greater specificity in targeting which cells will undergo apoptosis. For example, organisms exhibiting an ablated phenotype in the left pronephros after KR activation showed no corresponding phenotype in the adjacent somites (data not shown). There are two major ways in which spatial specificity can be controlled: limiting the location of KR expression, such as through targeted
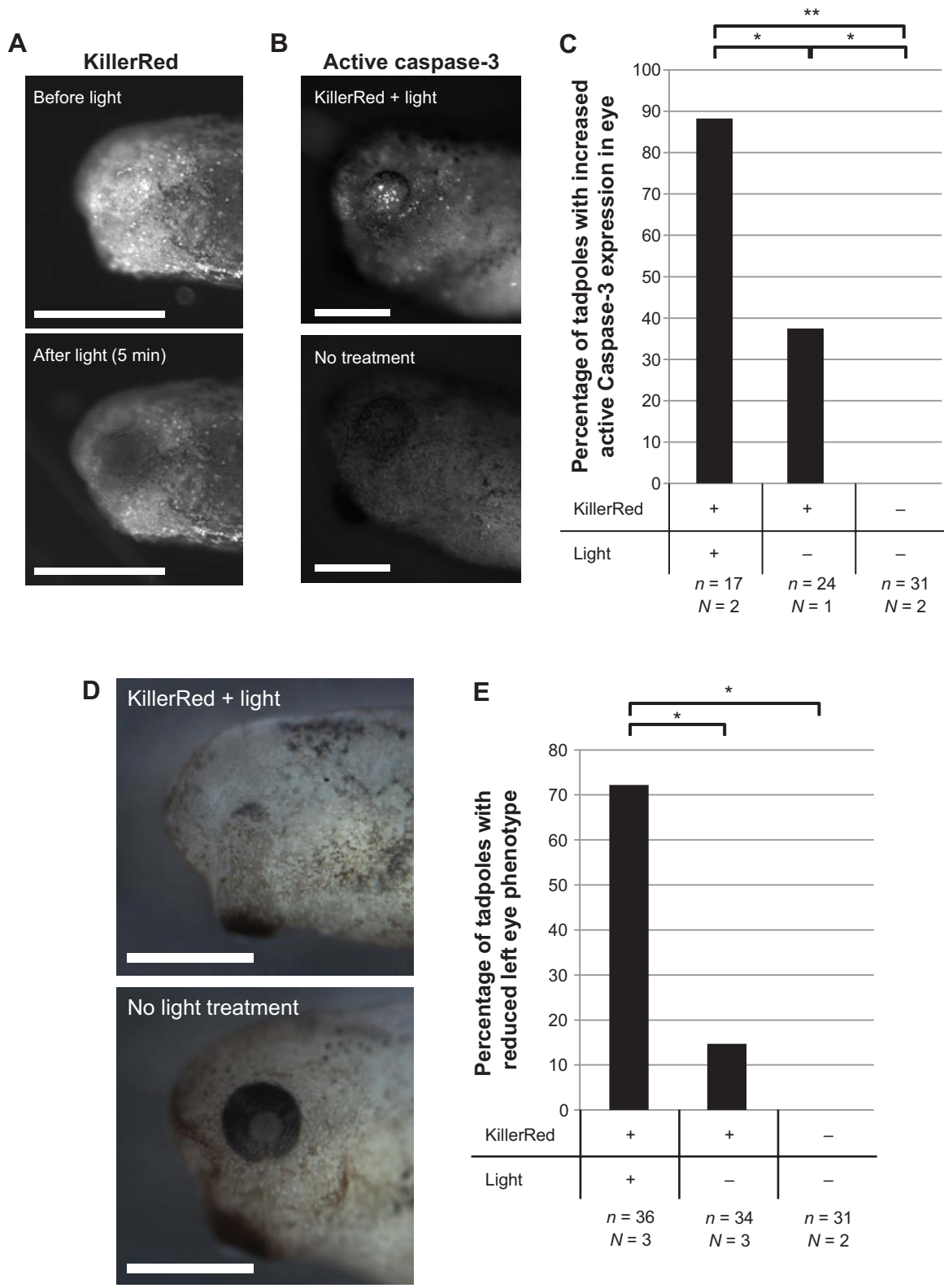
Figure 1. KillerRed (KR)-mediated light-induced apoptosis results in ablated eye phenotype. *Xenopus laevis* embryos were injected with KR mRNA at Nieuwkoop–Faber (NF) stage 2–3 and selected for KR expression in the left eye at NF stage 26–28.

Notes: (**A**) Five minute activation of KR by exposure to green light induced photobleaching. (**B, C**) 1.5–3.5 hours after light treatment in eye (or at matching stages for unlit tadpoles), immunohistochemistry for active Caspase-3 showed that light activation of KR resulted in significantly higher levels of apoptosis over tadpoles not exposed to light, or expressing KR. (Fisher's exact; *$p < 0.01$; **$p < 0.001$). Apoptosis was spatially restricted to the illuminated region. (**D**) After 24 hours, tadpoles were visually inspected for the presence of an ablated phenotype in the left eye. (**E**) Light activation of KR resulted in significantly higher incidence of ablated eye phenotype. (Fisher's exact; *$p < 0.001$). All scale bars are 500 μm. *n* indicates the number of tadpoles across all replicates, and *N* indicates the number of replicates shown. (A, B: TRITC; D: Brightfield).
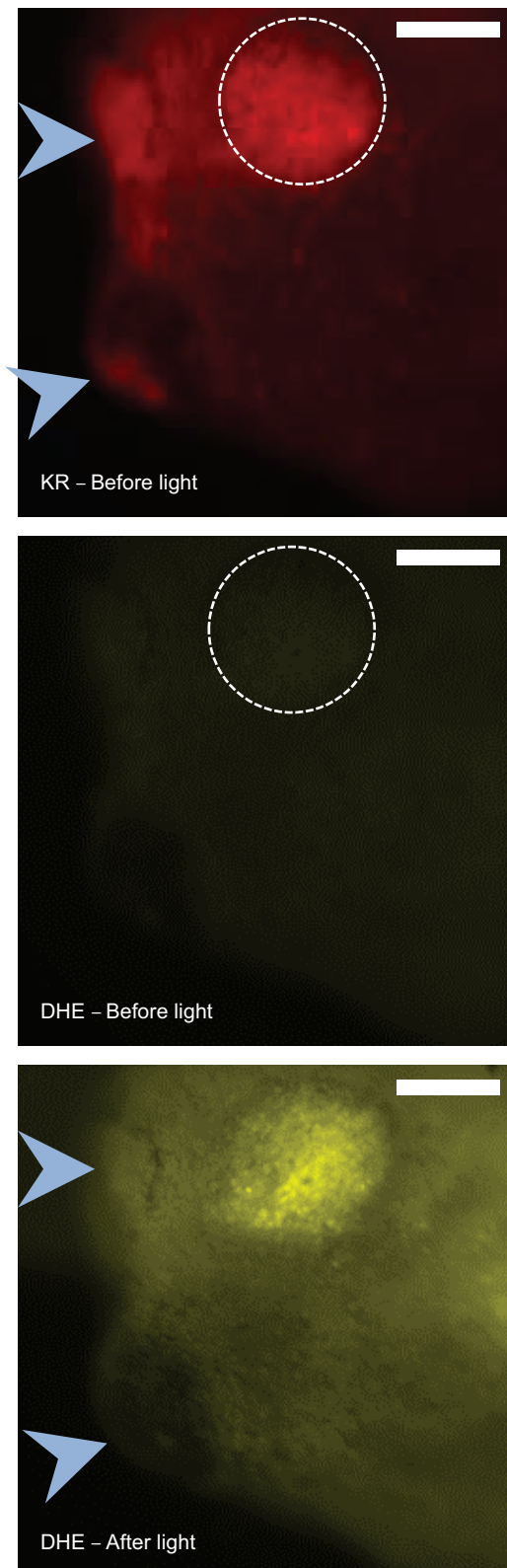
Figure 2. Photoactivation of KillerRed (KR) increases reactive oxygen species only within the illuminated region. Dihydroethidium (DHE) is a fluorescent indicator of superoxides. Following five minute activation of KR by exposure to green light, DHE fluorescence greatly increased within the illuminated region (dashed circle). However, it did not similarly increase in nearby, non-illuminated KR-expressing regions, like the forebrain and cement gland (arrowheads). All scale bars are 200 μm. (Top: TRITC; middle, bottom: Mermaid.)

injections or expression under a promoter, and limiting the light exposure to a particular target region using a fluorescent microscope. One or both methods of spatial control may be used simultaneously. The spatial resolution of KR activation is dependent on the maximum magnification of the fluorescent scope used; a higher level of magnification will produce a tighter, more intense light exposure. Intensity of exposure, and therefore activation of KR, is also strongest in the focal plane of the microscope. Cells that are deep within the organism or obscured by opaque tissue may be exposed to light of reduced intensity, resulting in inefficient photoactivation. For organs or tissues thicker than the microscope depth of field or wider than the field of view, multiple light exposures may be necessary to activate KR across the entire target region.

KR's ability to induce apoptosis in a variety of organisms has been well demonstrated, but its application as a method for tissue ablation in *Xenopus* is novel. While KR-mediated tissue ablation has been demonstrated in zebrafish,[26] these published techniques rely on the use of transgenic organisms, a method that is not always practical, particularly if a promoter for a desired tissue type has not been cloned. The method demonstrated here, by contrast, requires only the presence of KR mRNA and is adapted for use in *Xenopus*, for which protocols for KR as a means of tissue ablation have not yet been described. KR-mediated tissue ablation can be used in *Xenopus* as an alternative to mechanical or chemical damage in regeneration and repair studies. Unlike those generalized models of tissue damage, however, KR can also be used to study the specific effects of oxidative damage and apoptosis. As apoptosis has been shown to play a key role in regeneration of some *Xenopus* tissues, photosensitizers may be particularly useful tools in this system.[16,17] Similar tissue ablation has been shown in invertebrates; in particular, a comparable protocol has been demonstrated in *Caenorhabditis elegans*, where photoactivation of the protein miniSOG, a generator of singlet oxygen, was shown to ablate cells in a cell-autonomous manner.[30] KR's cytotoxicity, in comparison, occurs primarily via superoxides.[4] In conclusion, we believe that this method of inducing targeted apoptosis in *Xenopus* tissues by photoactivation of KR will prove to be a highly useful and adaptable protocol, with novel applications yet to be discovered, in the fields of developmental biology, regeneration, and repair.

Abbreviations
KR, KillerRed; MMR, Marc's Modified Ringer; ROS, reactive oxygen species.

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Author Contributions
Carried out experiments, helped participate in the design of study, and drafted manuscript: KJ. Conceived study, helped
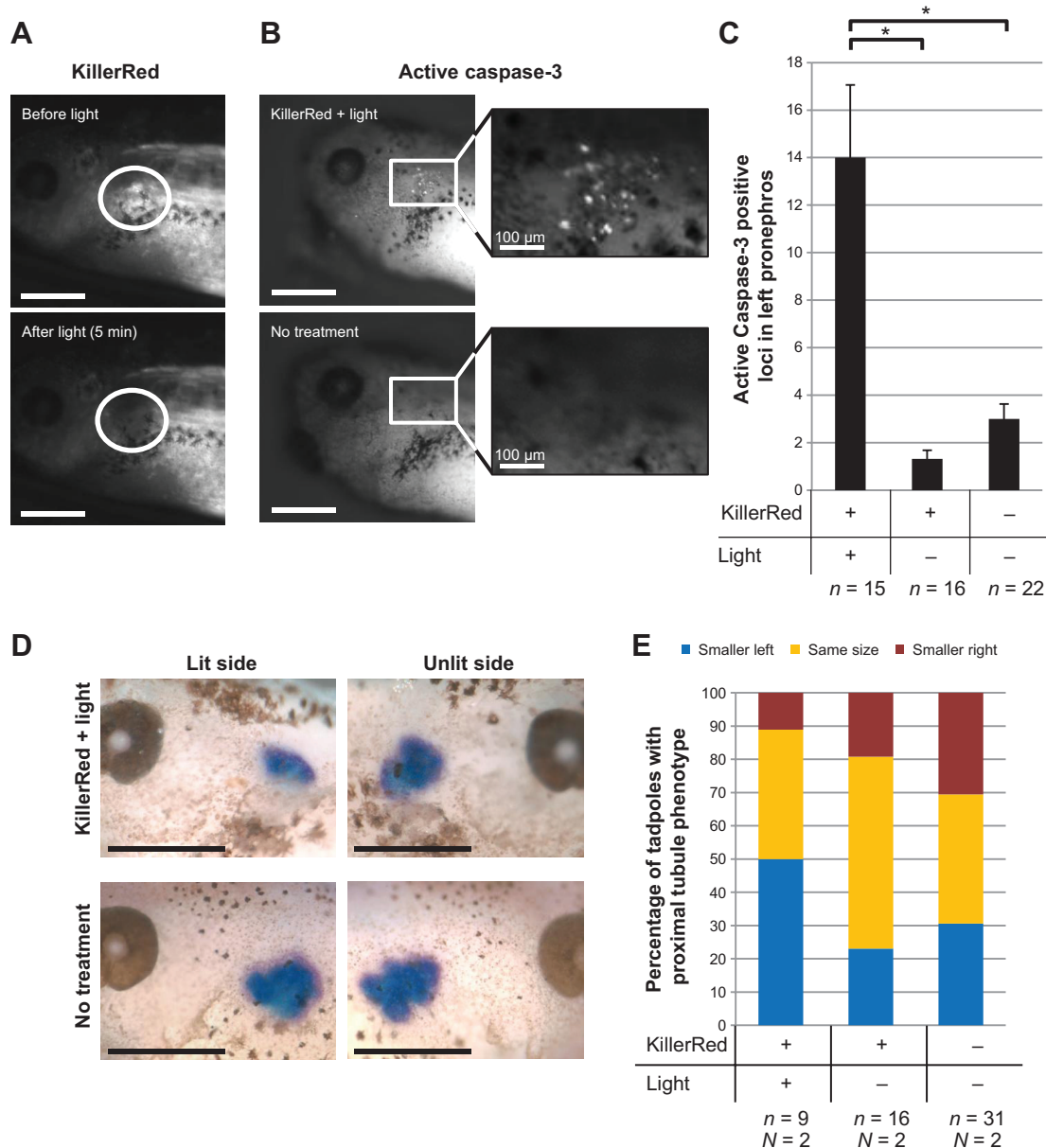
Figure 3. KillerRed (KR)-mediated light-induced apoptosis results in ablated pronephros. *Xenopus laevis* embryos were injected with KR mRNA at Nieuwkoop–Faber (NF) stage 2–3 and selected for KR expression in the left pronephros at NF stage 37.

Notes: (**A**) Five minute activation of KR by exposure to green light induced photobleaching in pronephros (circled). (**B, C**) 1.5–3.5 hours after light treatment in pronephros (or at matching stages for unlit tadpoles), immunohistochemistry for active Caspase-3 showed that light activation of KR resulted in a significant increase in the number of Caspase-positive loci over tadpoles not exposed to light, or expressing KR (Tukey–Kramer; *$p < 0.001$). Mean number of Caspase-positive loci per tadpole pronephros is shown, with error bars indicating the standard error of the mean. Apoptosis was spatially restricted to the illuminated region. (**D**) After 24 hours, in situ hybridization for senescence marker protein 30 (SMP30) was performed to visualize proximal tubules, and areas of SMP30 expression were measured on each side of the tadpole using SPOT Advanced imaging software. (**E**) Light activation of KR resulted in ablation of proximal tubules on the left side, compared to tadpoles with KR expression alone and tadpoles with no treatment at all. For (**B**) (inset) scale bars are 100 μm; all other scale bars are 500 μm. *n* indicates the number of tadpoles across all replicates, and *N* indicates the number of replicates shown. (A, B: TRITC; D: Brightfield.)

participate in its design, and reviewed manuscript: KM and ML. All authors read and approved the final manuscript.

Supplementary Data

Supplementary figure 1. Extensive photobleaching of KillerRed occurs within the first five minutes of light exposure. A tadpole expressing KillerRed in the eye was exposed to green light under the 40× objective for nine minutes, with images taken each minute. Images have been false colored to improve contrast. While the brightness of KillerRed fluorescence continues to diminish with increasing light exposure time, the greatest extent of photobleaching occurs within the first five minutes. Scale bar is 50 μm. (All images: TRITC.)

Supplementary figure 2. Titration of mRNA concentration is necessary for proper penetrance of phenotype. Effects of specific (with light) and nonspecific (no light) activation of KillerRed are shown for varying concentrations of mRNA injection solution, using a typical injection dosage as described in the methods. With a standard injection solution of 26 ng/μL, the injection dosage of mRNA is ~200 pg/embryo. Lower concentrations of mRNA result in markedly reduced incidence of the ablated eye phenotype upon photoactivation of KillerRed, while higher concentrations show a drastic increase in nonspecific KillerRed damage, even without photoactivation. Numbers above bars indicate the number of tadpoles shown for each treatment. Bars for 13 ng/μL show 1–2 replicates, 26 ng/μL show 3 replicates, and 52 ng/μL show 4 replicates.

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