Light-induced blockage of cell division with a chromatin-targeted phototoxic fluorescent protein

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INTRODUCTION

Proteins of the GFP (green fluorescent protein) family are widely used as passive reporters for live cell imaging. In the present study we used H2B (histone H2B)-tKR (tandem KillerRed) as an active tool to affect cell division with light. We demonstrated that H2B–tKR-expressing cells behave normally in the dark, but transiently cease proliferation following green-light illumination. Complete light-induced blockage of cell division for approx. 24 h was observed in cultured mammalian cells that were either transiently or stably transfected with H2B–tKR. Illuminated cells then returned to normal division rate. XRCC1 (X-ray cross complementing factor 1) showed immediate redistribution in the illuminated nuclei of H2B–tKR-expressing cells, indicating massive light-induced damage of genomic DNA. Notably, nondisjunction of chromosomes was observed for cells that were illuminated during metaphase. In transgenic *Xenopus* embryos expressing H2B–tKR under the control of tissue-specific promoters, we observed clear retardation of the development of these tissues in green-light-illuminated tadpoles. We believe that H2B–tKR represents a novel optogenetic tool, which can be used to study mitosis and meiosis progression per se, as well as to investigate the roles of specific cell populations in development, regeneration and carcinogenesis in vivo.

Key words: cell division arrest, chromosome mis-segregation, DNA damage, KillerRed, photodynamics, optogenetics.

Proteins of the GFP (green fluorescent protein) family are widely used as genetically encoded fluorescent labels for imaging structures and processes in living cells and organisms [1]. Previously, we have developed the first phototoxic fluorescent protein KillerRed, capable of light-induced generation of ROS (reactive oxygen species) [2,3]. KillerRed is a GFP-related dimeric red fluorescent protein with fluorescence excitation and emission maxima at 585 and 610 nm respectively. It was developed based on the nonfluorescent red chromoprotein anm2CP of *Hydra zoa* jellyfish. The phototoxic properties of KillerRed are superior to those of all other members of the protein family analysed, exceeding the phototoxicity of EGFP (enhanced green fluorescent protein) by at least 1000-fold. KillerRed combines phototoxicity with the benefits of a fully genetically encoded label, enabling highly specific protein tagging, localization to various cell compartments and expression in specific cell types [4]. Recent studies showed the usefulness of KillerRed for the light-induced inactivation of target proteins and the killing of specific cell populations in vivo [5–8].

Optogenetics is a recently emerged and rapidly developing field and is based on genetically encoded light-sensitive proteins that enable direct light-dependent control of various cellular processes such as neuron activity and signalling cascades [9,10]. KillerRed is a novel optogenetic tool that makes it possible to direct light-induced oxidative stress to various proteins and cell compartments.

It is well recognized that the intracellular localization of a photosensitizer is very important. When ROS generation overwhelms a cell’s antioxidant defences, the radicals can interact with endogenous macromolecules and alter cellular function. Multiple signalling cascades are activated in cells that are exposed to the photodynamic stress. Depending on the subcellular localization of the ROS, these signals can result in different responses. For example, mitochondrially localized photosensitizers efficiently induce apoptosis, whereas necrosis is the major cell death pathway induced by compounds localized to the plasma membrane [11]. The same was observed with KillerRed localized to the mitochondria and plasma membrane [2,3]. However, the dimeric state of KillerRed and its lower phototoxicity, as compared with chemical photosensitizers, impede the broad application of this protein.

In the present paper, we constructed tKR (tandem KillerRed), which can be used as a pseudomonomeric tag, and fused it to H2B (histone H2B). This fusion product (H2B–tKR) allowed correct chromatin labelling and uninterrupted functioning in the dark. At the same time, green-light illumination of cells expressing H2B–tKR resulted in an efficient transient blockage of cell proliferation.

Abbreviations used: BP, band pass; EYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; FLD, fluoresceine lysine dextrane; GFP, green fluorescent protein; EGFP, enhanced GFP; H2B, histone H2B; ROS, reactive oxygen species; tKR, tandem KillerRed; XRCC1, X-ray cross complementing factor 1.

1 Ekaterina O. Serebrovskaya, Sergey Lukyanov and Konstantin A. Lukyanov are authors of a patent application on H2B–tKR.
2 Tatiana V. Gorodnicheva is employed by Evrogen JSC, which commercializes KillerRed-related products.
3 Dmitriy M. Chudakov, Sergey Lukyanov and Konstantin A. Lukyanov have a financial interest in Evrogen JSC.
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This was a novel effect never observed for other KillerRed locations.

EXPERIMENTAL

Genetic manipulations

The vector pKillerRed-N (Evrogen) was used as a template to construct the vector ptKR-N, encoding two KillerRed proteins connected by the HGTGSTGSGGSTASSEDNNMA linker [12]. cDNA encoding human H2B was amplified using the pmKate2-H2B (Evrogen) vector as a template and cloned into ptKR-N in frame with tKR to generate H2B fused to the N-terminus of tKR via the DPPVATLEAT linker. For lentiviral transduction, the NheI-blunt PCR fragment containing the H2B–tKR open reading frame was cloned into NheI- and EcoRV-digested pRRLSIN.EF1.WPRE vector kindly provided by Professor Didier Trono (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) with a modified multiple cloning site.

Cell culture

The strongly adherent HeLa cell line isolate 'Kyoto' was used for both transient and stable expression experiments. Cells were plated at 5×10⁴ cells per 35 mm glass-bottomed culture dish and grown in DMEM (Dulbecco's modified Eagle's medium; PanEco) with 10% (v/v) FBS (fetal bovine serum; Sigma) for 24 h before transfection. Transient transfections were performed with the FuGene® 6 reagent (Roche), according to the manufacturer's protocol using 1 μg of plasmid DNA per transfection. Stable expression of H2B–tKR was obtained by lentiviral transduction, and the cell population with brightest fluorescence was sorted using a MoFlo fluorescence-activated cell sorter (DakoCytomation).

Flow cytometry

For cell-cycle analysis, 5×10⁵ cells per sample were harvested with 0.25% trypsin/EDTA, washed twice with PBS and fixed with 70% ethanol for 20 min at −20°C. After fixation, the cells were washed with PBS and stained with 0.002% propidium iodide solution in PBS, containing 0.2 mg/ml RNase, for 1 h at room temperature (22°C). Samples were analysed by a Cytomine FC500 flow cytometer, equipped with an air-cooled argon-ion laser operating at 488 nm (Beckman Coulter). The following detection parameters were used: 6 mW laser power and 620 nm band pass filter (FL3 channel). A minimum of 10000 events were collected for each sample.

For sterile cell sorting, 2×10⁶ cells were resuspended in 100% FBS at a density of 5×10⁵ cells/ml. The cell suspension was then filtered through a 70 μm nylon mesh cell strainer. Using a MoFlo cell sorter (DakoCytomation), a minimum of 1.5×10⁵ events were collected into a sterile 2 ml tube containing DMEM, 10% (v/v) FBS, 10 units/ml penicillin and 10 μg/ml streptomycin.

Microscopy

Live cell imaging was performed 24–48 h after transfection in Heps-buffered DMEM (PanEco) supplemented with 10% (v/v) FBS at 37°C in a 5% CO₂ atmosphere. For fluorescence microscopy, a Leica AF6000 LX imaging system, based on a Photometrics CoolSNAP HQ CCD (charge-coupled device) camera, was used. A 120 W HXP short arc lamp (Osram) was used as a light source. A standard TX2 filter set [excitation BP (band pass) 560/40 nm, emission BP 645/75 nm] was used to acquire a red fluorescence signal and to illuminate the cells. A Laser Power Meter LP1 (Sanwa) was used to measure the total power of the excitation light. Light power density (W/cm²) was estimated by dividing the total power by the area of the illuminated region.

Embryo manipulation

Synthetic mRNA encoding the H2B–tKR chimaeric protein was generated with an SP6 Message Machine Kit (Ambion) from a pSP64–H2B–tKR plasmid linearized with VspI. The mRNA was then mixed with FLD (fluoresceine lysine dextrane) and injected into embryos at a concentration of 10 pg/blastomere.

To generate the constructs expressing H2B–tKR under the control of the Xenopus laevis Xanf1 and Xag2 promoters, the fragments of these promoters that were sufficient for tissue-specific targeting of the expression ([13] and A. Zaraisky...
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Figure 2 Light-induced transient blockage of proliferation of HeLa Kyoto H2B–tKR cells

Representative cells (n = 75) are shown at designated time points (in hours) after green-light illumination (overlay of red fluorescence and transmitted light). Note that, in contrast with non-transfected cells, H2B–tKR-expressing cells do not divide for 27 h (top row) and then undergo mitosis normally (bottom row; arrows point to mitotic or newly appeared daughter cells). Scale bar, 20 μm.

unpublished results) were subcloned into the pH2B–tKR vector. Transgenic embryos were generated as described previously [14].

mRNA-injected embryos were illuminated with a 120 mW/cm² green light from a short arc lamp (Osram) with a BP 560/40 nm filter (Leica) for 20 min. The transgenic embryos were illuminated with 45 mW/cm² green light (BP 525/30 nm) from an ultrabright diode array (BioSpec) for 1 h. The corresponding control embryos were kept in the dark at room temperature for the time of illumination.

RESULTS AND DISCUSSION

Transient light-induced blockage of cell division with H2B–tKR

We aimed to direct KillerRed to chromatin, which is known to be very sensitive to oxidative stress [15,16]. However, because of the dimeric state of KillerRed, its fusion to core histone proteins renders histones non-functional, as cells expressing such fusions are unable to divide [17]. To overcome this problem, we constructed tKR, which includes two copies of KillerRed connected by a flexible linker. We expected tKR to form intramolecular dimers and thus behave as a monomeric tag, as has been described for other dimeric fluorescent proteins [12,18–20]. Indeed, mammalian cells transiently transfected with H2B–tKR demonstrated correct chromatin labelling and underwent mitosis normally. Moreover, we successfully established a HeLa Kyoto cell line stably expressing H2B–tKR, further demonstrating that H2B–tKR expression does not interfere with cellular division (Figure 1).

We then studied the phototoxic effects of H2B–tKR. Using a fluorescence microscope we illuminated HeLa Kyoto cells with green light (540–580 nm, 0.5 W/cm², 2 min), which resulted in bleaching of about 70% of the red fluorescent signal. We then tracked cell behaviour by capturing time-lapse images over 48 h. Complete light-induced blockage of cell division for approx. 24 h was observed in cells that were either transiently or stably transfected with H2B–tKR (Figure 2). During this time, the cell nuclei had interphase morphology and no cells underwent division. However, most cells remained viable with no membrane blebbing, loss of attachment, cell shrinkage or other signs of cell death. In contrast, non-transfected cells were insensitive to illumination and had a normal rate of proliferation, as almost 100% of the non-transfected cells underwent mitosis during the 24 h interval. Over a second 24 h period (24–48 h after light illumination), approx. 90% of the H2B–tKR-transfected cells underwent mitosis (Figure 2). Notably, two repeated light illuminations (at 0 and 24 h time points) resulted in the blockage of cell division for 48 h (results not shown). Thus cells can be maintained in a non-dividing state by regular iterative green-light illuminations.

To access a possible DNA damage via H2B–tKR, we used visualization of EYFP (enhanced yellow fluorescent protein)-tagged XRCC1 (X-ray cross complementing factor 1) distribution within the target nuclei. XRCC1 acts as a central loading platform in DNA repair [21]. It was demonstrated that XRCC1
shows immediate recruitment to the sites of DNA damage [22]. We co-transfected HeLa Kyoto cells with plasmids encoding EYFP–XRCC1 and H2B–tKR and then analysed them by fluorescence microscopy. As we expected, the fluorescence signal of EYFP–XRCC1 was uniformly distributed (often with a few bright spots) in the nuclei of interphase cells. Immediately after illumination with KillerRed-activating green light (540–580 nm, 0.5 W/cm², 30 s), EYFP–XRCC1 quickly (1–3 min) redistributed to form multiple fluorescent foci throughout the nuclei of H2B–tKR-expressing cells (Figure 3). Such green-light-induced redistribution was not observed in the control cells that were transfected with EYFP–XRCC1 only (Figure 3). Thus we concluded that H2B–tKR mediates massive light-induced damage of genomic DNA and activation of repair machinery. In turn, it causes cell-cycle checkpoint activation and cell-cycle arrest [23]. After successful DNA repair interphase cells can restore normal proliferation.

The influence of H2B–tKR on mitotic cells

We then studied the H2B–tKR-mediated effect on mitotic cells by illuminating individual cells (n = 22) during metaphase (540–580 nm, 0.5 W/cm², 2 min). In all cases, nondisjunction of chromosomes was observed (Figure 4). Notably, most of the illuminated mitotic cells (15 of 22) initiated cytokinesis without significant delay. In seven cells, however, the start of cytokinesis was delayed (Figure 4C). Whatever the case, cells were unable to complete their division normally. The chromosomes remained closely attached, with no anaphase onset observed, and semi-formed cleavage furrows disappeared (or a small part of the cell with no chromosomes was cut off). In the end, the cells returned to an interphase morphology with a decondensed tetraploid chromatin (except for two cells, which died).

Observing cells co-transfected with H2B–tKR and TagGFP2–α-tubulin, we found that the mitotic spindle in the illuminated mitotic cells was formed normally and that illumination had no immediate effect on its structure (Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350065add.htm). Using a EGFP–Securin fusion, we studied further the rate of Securin degradation during mitosis. Securin is one of the key players in the regulation of metaphase–anaphase transition, which maintains sister chromatid cohesion at their centromeres until stable chromosome bi-orientation is established.

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Polyubiquitination and subsequent degradation of Securin is required for the activation of Separase (a protease that triggers chromosome separation) and the onset of anaphase [24, 25]. We observed no significant difference in the rate of green-fluorescence-signal loss in the illuminated H2B–tKR-expressing cells compared with the non-illuminated control cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/435/bj4350065add.htm). Thus we concluded that the illumination of H2B–tKR does not activate the mitotic spindle assembly checkpoint (at least in most cases). The molecular basis for H2B–tKR-mediated chromosomal nondisjunction remains unclear and requires further investigation. One possibility is that KillerRed inactivates chromatin-associated Separase, as a transient inhibition of their proliferation or cell death, and thus to underdevelopment of the illuminated regions of the embryos. A transient inhibition of their proliferation or cell death, and thus to underdevelopment of the illuminated regions of the embryos.

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H2B–tKR action in vivo

To test whether H2B–tKR could be used for cell damage targeting in the whole organism, two sets of experiments were arranged. First, we generated X. laevis embryos ubiquitously expressing H2B–tKR by injecting them with synthetic H2B–tKR mRNA premixed with a FLD tracer at the two blastomere stage. At the midneurula stage, the anterior neural plate regions of ten embryos were individually illuminated for 20 min with green light (540–580 nm, 120 mW/cm²) through the 10× objective of an upright microscope, resulting in a considerable photobleaching of KillerRed (Figures 5A–5A”). Thereafter, the illuminated embryos were kept in the dark until they were at the swimming tadpole stage (stage 43). As a result, a more or less prominent reduction of eyes and forebrain was observed in all tadpoles (n = 56; see a typical tadpole in Figures 5B–5B”). No such effects were seen in the control embryos injected the same way, but kept in the dark from the very beginning (n = 45) (Figures 5C–5C”). This result demonstrates the presence in both tadpoles of H2B-tKR and FLD lineage tracer (A’–A” and B’–B”). Typical tadpoles developed from embryos expressing H2B–tKR and illuminated (B–B”) and not illuminated (C–C”) by green light. Anterior is to the top; dorsal side view. Note overall reduction of the brain and eyes in illuminated non-injected embryos (results not shown). We concluded that, similarly to cultured mammalian cells, illumination of H2B–tKR-expressing Xenopus cells leads to a transient inhibition of their proliferation or cell death, and thus to underdevelopment of the illuminated regions of the embryos.

In the second series of experiments, we generated transgenic X. laevis embryos expressing H2B–tKR under the control of embryonic-tissue-specific promoters. We first used the Xag2 promoter, which specifically induces protein expression in the cement gland, a provisory organ located at the rostral end of the embryonic head. After transfection with the Xag2–H2B–tKR transgene, transgenic tadpoles expressing H2B–tKR under control of the cement gland-specific Xag2 promoter, either illuminated (D–D”) or not illuminated (E–E”) by green light. The anterior is to the top; ventral side view. Scale bars: 100 μm in (A), (D), (E), (F), and (G); 300 μm in (B) and (C).

A) The anterior part of the neural plate of the midneurula embryo injected by H2B-tKR mRNA, as it is seen through a ×10 objective before illumination by green light. The anterior is to the top-left. Borders of the neural plate and presumptive eye rudiments are indicated by broken lines. (A’–A”) The same embryo as (A) is shown as it is seen in the red channel at the beginning (A”) and at the end (A”) of illumination by green light. Note a significant bleaching at the end of the illumination period of H2B–tKR, which is concentrated in cell nuclei. (B–B“) and (C–C”) Typical tadpoles developed from embryos expressing H2B–tKR and illuminated (B–B”) and not illuminated (C–C”) by green light. Anterior is to the top, dorsal side view. Note overall reduction of the brain and eyes in comparison with those in the control tadpole. Photographs made in red (B’ and C’) and green channels (B” and C”) demonstrate the presence in both tadpoles of H2B–tKR and FLD lineage tracer respectively. (D–D“) and (E–E”) Transgenic tadpoles expressing H2B–tKR under control of the cement gland-specific Xag2 promoter. Tadpoles were illuminated (D–D”) or not illuminated (E–E”) by green light. The anterior is to the top; ventral side view. (F and G) Transgenic tadpoles expressing H2B–tKR under the control of the forebrain-specific Xanf1 promoter, either illuminated (F) or non-illuminated (G) by green light. The transgenic status of both tadpoles was confirmed at the late tailbud stage, when a low red signal from H2B-tKR was still visible (results not shown). The anterior is to the top; dorsal side view. Scale bars: 100 μm in (A), (D), (E), (F), and (G); 300 μm in (B) and (C).
construct, one half of these embryos (n = 120) were subjected to a green-light illumination (LED array, 525 nm, 45 mW/cm², 1 h) at stage 13 (the early neurula stage). In the meantime, the other half of the transfected embryos were kept in the dark. At the tadpole stage, all transgenic embryos (n = 15) from the illuminated half demonstrated a clear retardation of cement gland differentiation, with an overall morphology typical for earlier developmental stages. In addition, they lacked the ability to eliminate embryonic pigment granules from the cement gland cells (Figures 5D and 5D'). No such effects were detected in the control illuminated wild-type embryos (results not shown) or in transgenic embryos that were allowed to develop in the dark (n = 13) (Figures 5E and 5E').

Then, we used the tissue-specific promoter of the homeobox gene Xanf1, which is a weak promoter that is specifically expressed only between the middle gastrula and late neurula stages of development. This promoter is active in the cells of the anterior neural fold, in the territory which gives rise to the forebrain (Figures 5E and 5E'). Embryos expressing the Xanf1 promoter-driven H2B–tKR transgene were illuminated at the early midneurula stages with a green LED (570 nm, 15 mW/cm², 1 h). The resulting tadpoles (n = 23) demonstrated various degrees of forebrain retardation accompanied by prominent optic stalk dysplasia, which in extreme cases resulted in a complete cycloptic phenotype (Figure 5F). No such malformations were observed in either the control illuminated wild-type embryos or in the Xanf1–H2B–tKR transgenic embryos developed in the dark (n = 19) (Figure 5G).

Interestingly, the cycloptic phenotype we observed resembled that of tadpoles developed from embryos that had down-regulated Xanf1 expression [27]. In such embryos, the initial continuous eye field remains unsplit along the midline on two separate eye rudiments, which further results in the cyclopic phenotype. These results indicate that the observed abnormalities in illuminated Xanf1–H2B–tKR transgene-expressing embryos could be caused by a retardation of the normal process of eye rudiment splitting, which in turn had resulted from retardation of the histotypic specification of the midline cells due to the inhibitory influence of H2B–tKR on cell-cycle progression. With a retardation of organ differentiation also observed in the case of cement gland-specific expression of H2B–tKR, we suggest that tissue-specific targeting of this chimeraic protein can be a unique tool to investigate the role of correct differentiation timing for normal embryonic patterning. This tool may be of particular interest for the establishment of novel experimental approaches for evolutionary developmental biology, as embryonic heterochrony of differentiation between various regions in the embryo is now recognized as a main source of evolutionary transformation [28].

The results of the present study demonstrate that chromatins targeting of the phototoxic fluorescent protein KillerRed allows transient blocking of cell division using light. We believe that H2B–tKR is a powerful optogenetic tool to study mitosis and meiosis progression per se, and that it can be used to investigate the roles of specific cell populations in development, regeneration and carcinogenesis.

**AUTHOR CONTRIBUTION**

Ekaterina O. Serebrovskaya constructed the vectors and performed the fluorescence microscopy of cells. Tatiana V. Gorodnicheva and Ekaterina O. Serebrovskaya generated the stable cell lines. Galina V. Ermakova, Elena A. Solovieva and Andrey G. Zaraisky performed the experiments on the Xenopus embryos. George V. Sharov and Ekaterina O. Serebrovskaya performed the flow cytometry and cell sorting. Elena V. Zagaynova, Dmitriy M. Chudakov, Sergey Lukyanov and Konstantin A. Lukyanov designed and supervised the project and wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

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Figure S1 Mitotic spindle in a HeLa Kyoto H2B–tKR cell illuminated during metaphase

Time-lapse imaging of a representative cell (n = 7) co-expressing EGFP–α-tubulin (green channel) and H2B–tKR (red channel) after green-light illumination in metaphase. Overlay of the green and red channels is shown in the right-hand panel. Transmitted light (TL) images are shown in the left-hand column. Time after illumination (in min) is designated on the left. Scale bar, 10 μm.

Figure S2 Degradation of EGFP–Securin in mitotic HeLa Kyoto H2B–tKR cells

The normalized level of EGFP–Securin in mitotic HeLa Kyoto H2B–tKR cells illuminated with green light during metaphase (red circles, n = 5) or non-illuminated (open squares, n = 5). Means ± S.D. are shown. For each cell, observation was begun at the stage of metaphase plate (zero time). Cytokinesis started in all cells at 20–25 min.

1 Ekaterina O. Serebrovskaya, Sergey Lukyanov and Konstantin A. Lukyanov are authors of a patent application on H2B–tKR.
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