Photoswitchable gRNAs for spatiotemporally controlled CRISPR-Cas-based genomic regulation.

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We developed a simple approach for light-controlled CRISPR-Cas gene editing based on photocaging of the guide RNA and achieved spatiotemporally restricted gene regulation in living embryos.

Abstract

The recently discovered CRISPR-Cas gene editing system and its derivatives have found numerous applications in fundamental biology research and pharmaceutical sciences. The need for precise external control over the gene editing and regulatory events has driven the development of inducible CRISPR-Cas systems. While most of the light-controllable CRISPR-Cas systems are based on protein engineering, we developed an alternative synthetic approach based on modification of crRNA/tracrRNA duplex (guide RNA or gRNA) with photocaging groups, preventing the gRNA from recognizing its genome target sequence until its deprotection is induced within seconds of illumination. This approach relies on a straightforward solid-phase synthesis of the photocaged gRNAs, with simpler purification and characterization processes in comparison to engineering a light-responsive protein. We have demonstrated the feasibility of photocaging of gRNAs and light-mediated DNA cleavage upon brief exposure to light \textit{in vitro}. We have achieved light-mediated spatiotemporally-resolved gene editing as well as gene activation in cells, whereas photocaged gRNAs showed virtually no detectable gene editing or activation in the absence of light irradiation. Finally, we have applied this system to spatiotemporally control gene editing in zebrafish embryos \textit{in vivo}, enabling the use of this strategy for developmental biology and tissue engineering applications.
Introduction

Genome sequencing has facilitated systematic studies of gene functions and regulation networks, thus providing the basis for understanding organogenesis and complexities of genetic diseases. Various biochemical tools aimed at altering gene expression have been essential for such discoveries. While several therapeutics based on temporal gene overexpression, suppression or splicing correction have reached the market\(^1\), the newer approach of permanent editing of the genomic DNA is being tested in the context of therapeutic intervention in cancer\(^2\), viral infections\(^3\), and monogenic hereditary disorders such as Duchenne muscular dystrophy\(^4\) or Huntington’s disease\(^5\).

Recent transfer of the microbial adaptive immune system CRISPR-Cas to mammalian cells has led to the development of programmable tools for gene editing based on RNA-guided DNA endonucleases (most commonly, Cas9 and its analogues)\(^6\)–\(^9\). Their target specificity is determined by the presence of a nuclease-specific protospacer adjacent motif (PAM) sequence in the target DNA and by the guide RNA (gRNA), which binds through the Watson-Crick base pairing to the complementary sequence adjacent to the PAM. The main advantage of this system is that different genomic targets can be reached by simply altering the sequence of the gRNA. Multiple sites can be targeted simultaneously by using gRNAs with different sequences. Both gRNA and Cas9 can be chemically or genetically modified to improve stability\(^10\) or even add a specific functionality to the system\(^11\)–\(^13\). Thus, nuclease-deficient Cas9 protein (dCas9) can be fluorescently tagged for imaging purposes\(^12,13\) or fused with a transcription factor for specific regulation of target gene expression\(^13\). Examples of gRNA modifications include appending aptamers for protein binding\(^11\) and chemical modifications of the backbone and nucleobases for improved stability and specificity\(^10\).

Given the permanent character of the gene editing, being able to target only a subset of cells at specific time would be a desirable improvement of the CRISPR-Cas systems. Conditional gene editing could potentially reduce off-target gene modifications due to restricted activity of the CRISPR-Cas in the cells\(^14\). Studies of DNA repair pathways would immensely benefit from the higher spatiotemporal control over gene editing events\(^15\). Embryonic, tissue, or cancer development research is another area where precise spatiotemporal regulation of gene expression is of utmost need due to complex and dynamic gene expression patterns\(^16\). This demand has triggered the development of several approaches for externally controlled gene editing and gene expression using small molecules\(^17\), magnetic field\(^18\), or light\(^19\). Among these, light has the advantages of being relatively non-invasive,
having minimal to no molecular footprint on treated cells, having fast on/off response, and having the highest spatial resolution\(^\text{20}\).

Several attempts have been made to render the CRISPR-Cas system light-responsive to enable precise temporal control over gene editing/expression\(^\text{19,21-31}\). Examples of optogenetic approaches to control CRISPR-Cas include light-responsive Cas9 analogues\(^\text{19,21,24-29}\), light-activatable transcription factors\(^\text{30}\), photoswitchable calcium channel-mediated nuclear translocation of modified dCas9\(^\text{31}\), gRNA-blocking oligonucleotides\(^\text{22}\), and light-activatable transfection devices\(^\text{23}\). These systems are designed to respond to far UV (365 nm)\(^\text{19,22}\), blue (450-470 nm)\(^\text{21,24-27,29,31}\), cyan (500 nm)\(^\text{28}\), red (650 nm)\(^\text{26}\), far red (730 nm)\(^\text{30}\) or near-infrared light (980 nm)\(^\text{23}\).

The first demonstration of a light-controlled CRISPR-Cas-mediated gene editing in cancer cells was achieved by incorporation of UV light-sensitive photocaged unnatural amino acid in the structure of the Cas9 protein through expansion of the genetic code of the cells via an engineered pyrrolysyl tRNA/tRNA synthetase pair\(^\text{19}\). As a more biocompatible alternative to in situ synthesis of photocaged Cas9, several systems based on fusion of Cas9 with photosensitive protein domains have been developed to achieve light-controlled gene editing or transcription\(^\text{21,24-29}\). Such systems could be based on split-Cas9\(^\text{25,27}\) or single-chain Cas9 analogues\(^\text{21,24,26-29}\) and be activated either through light-induced dimerization of cryptochrome-based CRY2/CIB1 pairs\(^\text{21,24,26,27}\), pMag/nMag pairs\(^\text{25,27}\), light oxygen voltage-based FKF1/GI pairs\(^\text{26}\), phytochrome-based PHYB/PIF pairs\(^\text{26}\), or dissociation of dimeric green fluorescent protein pdDronpa\(^\text{28}\) or a homodimeric light oxygen voltage domain\(^\text{29}\).

As an alternative to light-mediated assembly of photosensitive protein domains fused to Cas9 fragments, an approach based on light-induced transcription of CRISPR components has been developed\(^\text{30}\). Upon irradiation with far-red light, bacteriophytochrome diguanylate cyclase induces formation of cyclic diguanylate monophosphate (c-di-GMP). This leads to dimerization of c-di-GMP–responsive hybrid transactivator and its binding to an orthogonal promoter, initiating expression of a MS2-transactivator fusion protein. The transactivator can further bind to gRNA fused to MS2 RNA aptamers and induce transcription of the target gene\(^\text{30}\). Another approach to light-controlled regulation of transcription is based on light-mediated nuclear translocation of modified dCas9\(^\text{31}\). The design of this system relies on a photoswitchable Ca\(^2+\) channel Opto-CRAC and a dCas9-VP64 transcription factor protein fused with Ca\(^2+\)-responsive NFAT fragment. Upon blue light activation of the OptoCRAC, Ca\(^2+\) influx activates a Ca\(^2+\)-dependent phosphatase calcineurin, which dephosphorylates NFAT and leads to NFAT-dCas9-VP64 nuclear translocation\(^\text{31}\).
Despite the variety of systems developed, several problems remain such as leakage of activity in the dark, incomplete recovery of Cas9 function, and the requirement for constant illumination during several hours, which could be partially explained by spontaneous self-assembly of split-CRISPR components, context-dependent structural folding, and low light sensitivity of the dimerizing domains, respectively.

An alternative approach to light-activatable protein engineering is to block the gRNA activity by hybridization with a complementary photolysable DNA oligonucleotide. While achieving fast response to the UV light irradiation, this system suffers from unsatisfactory leakage of activity in the dark (7.4% vs. 16.7% of indel in GFP gene before and after UV exposure, respectively), possibly due to complex dissociation inside cells. Another drawback is the relatively high UV dose of 4.0 J/cm² required to degrade the protectors bearing three photolysable linkages and to restore gene editing.

Another example of light-activatable CRISPR-Cas system is based on upconversion nanoparticles, to which Cas9 protein is attached via a photocleavable linker. To assist with the endosomal escape of these particles, a coating with a positively charged polymer PEI was added. Despite complex multistep synthetic process, this delivery system was capable of NIR-mediated gene editing in vitro and in vivo upon intratumoral injection; however, these particles failed to fully exert their effect on tumor tissues due to clearance via mononuclear phagocyte system upon intravenous administration.

In this project, we used a simpler synthetic strategy to directly modify the gRNA of the CRISPR-Cas system with photocaging groups, thus preventing gRNA from recognizing the target genomic DNA sequence until its light-deprotection is induced upon brief illumination. This approach relies on a straightforward solid-phase synthesis of the photocaged gRNA, with simpler purification and characterization processes in comparison to engineering a light-responsive protein or formation of a heteroduplex or engineered nanoparticles. In addition, chemical modification of the gRNA allows for tighter and faster control over Cas9 function, enabling the use of this strategy for developmental biology and tissue engineering applications.
Results and Discussion

Our platform for light-controlled CRISPR-Cas gene editing and regulation is based on chemical modification of the gRNAs with photocaging groups (Fig. 1a). Due to the higher yields and lower costs of shorter RNA molecules obtained via the solid-phase synthesis\textsuperscript{35}, we employed the crRNA/tracrRNA format for the gRNAs (36 and 67 bases long RNAs, respectively) rather than using the single chain gRNAs (100 bases long). The constant part of the duplex (Alt-R® tracrRNA) was chemically stabilized using proprietary modifications (IDT). For the variable part of the duplex (crRNAs), we introduced three phosphorothioate linkages at each terminus to improve their intracellular stability (Fig. 1b). We positioned the photocaging groups in the targeting region of the crRNA so that the Watson-Crick base pairing with the complementary genomic DNA sequence is prevented, thus rendering CRISPR-Cas system inactive. Upon light-mediated deprotection of the photocaged gRNA, its base pairing capability should be restored. It has previously been reported that incorporation of photocaging groups every 5-6 bases of a DNA oligonucleotide completely abolished its hybridization with the complementary strand\textsuperscript{36}. On the other hand, gRNAs of length shorter than 16 bases do not trigger Cas9-mediated DNA cleavage, and the ones shorter than 10 bases are not able to activate gene expression via dCas9-transactivator system\textsuperscript{37}. Therefore, we envisaged that incorporating two photocaging groups within the target recognising region of the gRNA should be sufficient to prevent CRISPR-Cas-mediated gene editing or transcription activation.

Several nucleic acid photocaging groups have been described in the literature, varying in the activation wavelength and the type of the nucleotides they protect\textsuperscript{38,39}. In the present project, we used commercially available nucleic acid photocaging group 6-nitropiperonyloxymethyl (N-POM) dT DNA (Fig.1a), which could be removed upon irradiation with a low intensity of light at 365 nm\textsuperscript{36}. In these conditions, irradiation-induced damage to DNA is negligible, as it usually occurs at wavelengths below 315 nm\textsuperscript{40}. Despite the fact that crRNA naturally does not contain deoxynucleotides in its structure, recent studies demonstrated that virtually any site in the target region could be substituted with a deoxynucleotide, with the requirement that the RNA content is sufficient for preserving an A-form-like helical structure in the crRNA\textsuperscript{41-43}. We designed several crRNAs to target various reporter and functional genes, and their sequences and modifications are presented in Fig. 1b.

First, we confirmed that PC-groups could be removed by light exposure using high performance reverse phase liquid chromatography (Fig. 1c, SI Fig. 1a, b). Complete uncaging of PC-gRNAs irrespective of their sequences could be achieved even with a (1.0±0.15) J/cm\textsuperscript{2} light dose (5 min of (3.3±0.5) mW/cm\textsuperscript{2} 365-nm light
irradiation). Importantly, virtually no single caged by-products were detected above UV light doses of 1.0 J/cm² (SI Fig. 1b), indicating the possibility of full restoration of the gRNA function upon deprotection.

In a cell-free DNA cleavage assay, non-caged crRNA/tracrRNA duplexes were able to guide Cas9 protein to their targets and induce double-stranded breaks for a range of genes (Fig. 1d, e). All photocaged gRNAs tested lost their activity, thereby confirming our hypothesis that positioning photocaging groups within the targeting regions of crRNAs prevents CRISPR-Cas-mediated DNA cleavage. In the dark, virtually no gene editing could be detected. However, exposing the reaction mixtures to the 365-nm light resulted in re-activation of target cleavage. In this assay, complete restoration of gRNA’s function was achieved already after ~1.0 J/cm² light dose across multiple targets (Fig. 1d, e), in accordance with the liquid chromatography assay (Fig. 1c, SI Fig. 1).

Figure 1. Light-induced CRISPR-Cas-mediated target DNA cleavage. (a) Schematic representation of photocaged gRNAs mechanism of action. (b) Sequences and modification strategies of PC-crRNAs. AUGC are RNA bases, P is 6-nitropiperonyloxymethyl (NPOM)-caged T-DNA, asterisks denote phosphorothioate linkages, underlined are targeting sequences. (c) Kinetics of PC-crRNA light-mediated uncaging reaction monitored by reverse-phase high performance liquid chromatography (N = 3, mean ± SD). (d) Biochemical activity assay of
target DNA cleavage by non-caged (NC) or photocaged (PC) gRNAs with 365-nm light illumination (0-2 J/cm²).

(e) Quantification of target DNA cleavage from gel images using Fiji ImageJ software44. Results are expressed as individual data points overlaid with the mean ± SD (N = 3); ***p < 0.001, ns – not significant (p > 0.05) versus NC-gRNA according to one-way ANOVA analysis combined with Tukey’s (Holm-Sidak) post-hoc test.

We then tested our photocaged gRNA approach on HEK293FT cells stably expressing Cas9 (Fig. 2). These cells were derived from a single-cell colony, providing uniform Cas9 expression in the whole cell population. The cells were transfected with PC-gRNAs for 4 h and subsequently irradiated using a 1.0 J/cm² UV light dose. After two days, the gene editing efficiencies were evaluated in a mismatch-based assay. Exposure of cells transfected with regular non-caged gRNAs to the UV light did not affect the gene editing efficiency. Importantly, in the absence of light irradiation, photocaging groups remained attached to the gRNA even in the complex intracellular environment, affording excellent protection from gene editing. However, when cells transfected with photocaged gRNAs were exposed to the UV light, over 70% of the gene editing efficacy was restored (Fig. 2a, b), in accordance with the in vitro results in Fig. 1. Similar results were obtained when we analysed light-controlled ZsGreen knockout at the protein level in HEK-Cas9-ZsGreens cells using fluorescence microscopy and flow cytometry (Fig. 2c, d, SI Fig. 2). Five days after transfection, the level of ZsGreen expression was diminished by 80% when we used uncaged gRNA (the half-life of ZsGreen protein is above 26 h45). With PC-gRNA, we observed complete recovery of gene editing after the light exposure. Despite the relatively high energy of the 365-nm UV light used to activate PC-gRNAs, overall low dose and brief exposure did not affect cell viability (SI Fig. 3). This is in contrast to extended exposure to the blue light necessary to achieve dimerization and significant Cas9 activation in split-Cas9 systems, which often leads to high photocytotoxicity and low cell viability30,46. These experiments demonstrate the utility of photocaging strategy for light-controlled gene editing with a wide range of gRNA sequences. In principle, other types of caging groups can be installed on gRNAs, as it has been previously demonstrated that up to four different photocaging groups could be independently removed from oligonucleotides using light of longer wavelengths with better tissue penetration38, enabling sequential gene editing/regulation at desired locations and times.

After confirming the possibility of light-controlled gene editing using photocaged gRNAs, we sought to define the timeframe of possible activation of the gene editing, which would be determined by intracellular stability of the photocaged gRNA and its dilution upon cell division. In order to answer this question, HEK-Cas9 cells were transfected with the non-caged or photocaged gRNA targeting the HPRT gene and subsequently light-activated after specific time points (4-144 h after starting the transfection; Fig. 2e, f and SI Fig. 4). As expected,
the gene editing activity of the non-caged gRNA remained unchanged with time, due to the permanent character of the mutations and equal division rate of mutated and unmutated cells (SI Fig. 4). Interestingly, the gene editing efficacy of photocaged gRNA was not drastically reduced even when it was activated 48 h after the transfection, which indicates its high intracellular stability. Mutations were still detectable even when the gRNA was activated 4 days after the transfection. Moreover, the prolonged incubation of photocaged gRNA inside the cells did not induce significant gene editing in the absence of light irradiation. Similarly to the on-target activity, photocaged gRNA cleaved predicted off-target sequences only after UV irradiation (SI Fig. 5). In cells, we could not detect gene editing on the predicted off-target sites using the mismatch-based assay, indicating low off-target activity of the chosen gRNA. Overall, these experiments illustrate one of the advantages of our photocaged gRNA approach for light-controlled gene editing over existing systems that suffer from the stronger leakage of the gene editing activity in the absence of light irradiation21,22,25,27–30.
**Figure 2.** Light-induced CRISPR-Cas-mediated gene editing in HEK293FT cells. (a) Representative gel images of mutation detection assay of target genes (*IL1R2, HPRT, and ZsGreen*) in non-treated cells (NT) or in cells transfected with non-caged (NC) or photocaged (PC) gRNAs followed by 365-nm light illumination for 5 min (1.0 J/cm²). (b) Quantification of target gene mutation rates from gel images using Fiji ImageJ software. Results are expressed as individual data points overlaid with the mean ± SD (N = 3 – 4); ***p < 0.001, ns – not significant difference (p > 0.05) between indicated groups according to one-way ANOVA analysis combined with Tukey’s (Holm–Sidak) post-hoc test. (c) Light-induced ZsGreen protein knockout in HEK-Cas9-ZsGreen cells. Merged fluorescence images of ZsGreen signal (green) and Hoechst-stained nuclei (magenta) for cells transfected with non-caged or photocaged gZsGreen in the absence or presence of 1.0 J/cm² of UV light illumination, images were taken 5 days post-irradiation. Scale bar 100 µm. (d) ZsGreen mean fluorescence intensity quantification using flow cytometry analysis 5 days post-irradiation. Results are expressed as individual data points overlaid with the mean ± SD (N = 3). (e) Representative gel image of temporally controlled *HPRT* gene editing using PC-gRNA. HEK-Cas9 cells were transfected for 4 h with PC-gHPRT and were either kept in the dark or exposed to 1.0 J/cm² of UV light at the indicated time points after starting the transfection. (f) Quantification of *HPRT* mutation rates from gel images using Fiji ImageJ software. Results are expressed as individual data points overlaid with the mean ± SD (N = 4).

The photocaged gRNAs could be used not only for gene editing, but also to achieve spatiotemporal control over gene transcription activation (Fig. 3). To illustrate this, we used the same gIL1R2 sequence that was used in the gene editing experiments (targeting a region upstream of the *IL1R2* gene) in transcription activation experiments (Fig. 2a, 3a, b). HEK293FT cells were engineered to constitutively express nuclease-deficient Cas9 fused to previously described V64-p65-Rta tripartite transcription activator (dCas9-VPR) acting as a gRNA-controlled transcription factor. Single transfection of HEK-dCas9-VPR cells with gIL1R2 resulted in a prolonged *IL1R2* mRNA expression (up to 3 days), with the maximum expression at 24 h post-transfection (SI Fig. 6). In contrast, *IL1R2* mRNA was not detected after 40 cycles of qPCR in non-transfected cells or in cells transfected with the negative control gRNA. This indicates a sequence-specific mechanism of *IL1R2* upregulation through CRISPR-Cas activation and confirms the extremely low levels of endogenous *IL1R2* expression in HEK293FT cells. After verifying the sequence-specificity of transcription activation, we next explored the possibility of light-controlled transcriptional activation using photocaged gRNAs. HEK-dCas9-VPR cells were transfected with the non-caged or photocaged gIL1R2, light-activated after specific time points (4-72 h after starting the transfection), and the *IL1R2* mRNA expression levels were analysed one day after the irradiation (Fig. 3a, b). The *IL1R2* mRNA was detected in cells activated up to 48 h post-transfection and remained below the limit of detection for all the samples kept in the dark. Cells treated with the PC-gIL1R2 and exposed to the UV light 72 h post-transfection had C_T values between 35 and 40, which is typically considered to be below reliable
detection threshold. Interestingly, in contrast to the gene editing experiments (Fig. 2c), the efficacy of transcriptional activation by photocaged gRNA was significantly reduced already at 24 h post-transfection, which indicates a higher dependency of transcription activation on the intracellular gRNA concentration (Fig. 3b).

One of the key advantages of the light-controlled gene expression over other stimuli-responsive systems (e.g., based on small molecule regulators) is the possibility of spatial resolution. To illustrate this point, we have used a previously described reporter system based on the expression of tdTomato fluorescent protein under the control of the minimal CMV promoter. Upstream of this cassette a gRNA-binding site based on an AAVS1 sequence was installed. By sequence-specific binding of the gRNA to the AAVS1 sequence the dCas9-VPR fused transcription factor will be brought closer to the transcription start site (TSS) of the tdTomato ORF, thereby enhancing its transcription and protein synthesis (Fig. 3c). Cells were transfected with a mixture of PC-gTomato, tdTomato reporter plasmid, and a plasmid encoding GFP as a transfection control (Fig. 3c, d). After a 4-h transfection, the cells were UV-irradiated through a photomask to expose only a 1.5-mm strip of cells (1.0 J/cm²). One day after the illumination, cells were fixed and imaged using a fluorescence microscope. We observed a confined tdTomato expression pattern, corresponding to the irradiation area. The GFP signal was evenly distributed throughout the well, indicating that the cells were transfected uniformly, and that the tdTomato fluorescence was indeed triggered by light-irradiation. The cells kept in the dark showed virtually no tdTomato expression. We went even further and used a 375-nm programmable laser setup to draw triangular shapes on top of the transfected cells, and similarly observed enhanced tdTomato expression in the irradiated area (SI Fig. 7). The cells uniformly expressed GFP, confirming the successful transfection in the whole well.
Figure 3. Spatiotemporal control over gene expression using PC-gRNA. (a) Schematic representation of the experimental setup for the IL1R2 transcription activation. (b) Light-mediated expression of silent IL1R2 gene in HEK293FT cells. HEK-dCas9-VPR cells were transfected with the NC- or PC-gIL1R2 and were either kept in the dark or exposed to the UV light at 4-72 h post-transfection. The IL1R2 mRNA expression levels were determined 24 h post-irradiation and were normalized to the NC-gIL1R2 treated samples (NC). The GAPDH mRNA expression level served as a reference. N.D. – not detected. (N = 3, mean ± SD). (c) Schematic representation of the experimental setup for visualization of transcription activation. HEK-dCas9-VPR cells were co-transfected with PC-gTomato and a reporter plasmid encoding tdTomato under minimal CMV promoter and containing the gRNA binding site. The uncaging of the PC-gTomato with the UV light leads to its binding to the complementary sequence in the reporter plasmid and enhances the tdTomato expression. (d) HEK-dCas9-VPR cells co-transfected with PC-gTomato, the tdTomato reporter plasmid, and a GFP plasmid as a transfection control, irradiated through a photomask. Transfection in a 24 well plate, tdTomato (red), GFP (green), scale bar 2 mm.

Given the tight control over the gRNA function and rapid uncaging process, we envisioned that our photocaged gRNA approach could be particularly useful for developmental biology research. Indeed, UV-responsive photocaged molecules are widely used in this field\(^4\)-\(^5\); however, the resulting gene regulation is reversible, and therefore light-activatable systems based on CRISPR-Cas would be an important addition to the gene editing toolbox. To this end, we tested a photocaged gRNA targeting the slc45a2 gene (responsible for the pH homoeostasis of melanosomes required for melanin production\(^5\)) in developing zebrafish embryos as a model...
Loss of function mutations in the slc45a2 gene led to albino-like phenotype in the retinal pigment epithelium and in the body of the developing embryo, which can be observed already starting from 24 hours post fertilization (hpf). We used Cas9 protein for the experiments because it has previously demonstrated better gene editing capabilities compared to Cas9 in mRNA format. This was further confirmed by our preliminary test of protein Cas9 versus mRNA Cas9 in zebrafish embryos (SI Fig. 8).

First, we verified that the UV light at a dose of 2.0 J/cm² did not decrease the embryo viability or affect its development (SI Fig. 9), in accordance with previously reported studies. For temporally-resolved gene editing, zebrafish embryos were microinjected with Cas9/PC-gRNA complex at 1-2 cell stage and subjected to the UV light irradiation at various developmental stages. The embryos were imaged 2 days after the irradiation and assessed for slc45a2 gene mutation using a mismatch-based assay (Fig. 4). As expected, the PC-gAlbino/Cas9-microinjected embryos exhibited no detectable slc45a2 gene mutation in the absence of light irradiation neither phenotypically nor on the genomic DNA level. In contrast, UV light exposure of the PC-gAlbino/Cas9-microinjected embryos led to the slc45a2 gene mutations and the albino-like phenotype, while the efficacy of the gene knockout decreased with delayed irradiation (Fig. 4). Automated image analysis could detect the statistically significant difference in total pigment content in embryos irradiated up until 12 somite stage compared to wild-type embryos (Fig. 4b). We could also detect the gene editing phenotypically by the decrease in pigment content in embryos irradiated at up to 26-somite stage using scoring-based system (Fig. 4a), which was also confirmed by the mutation detection PCR-based assay (Fig. 4c). This indicates that the PC-gRNA/Cas9 complex exhibited residual activity inside the zebrafish embryos even at the 26-somite stage after single injection (22 hpf).

We next explored the possibility of spatially restricted gene editing in zebrafish embryos using photocaged gRNAs. The embryos were microinjected at 1-2 cell stage and were left in the dark until they reached the 12-somite stage, at which the head and the tail of the embryo are clearly visible. Subsequently, one eye of the embryo was irradiated using a 405-nm laser in a confocal microscope setup. Two days after the treatment, we observed mosaic pigment expression in the eye of the locally irradiated embryos (Fig. 4d). We analysed mutations in the slc45a2 gene in the head and tail parts of the same embryos separately and could detect the mutations only in the head (Fig. 4e). Globally irradiated embryos showed uniform mutations in both head and tail, and embryos kept in the dark (i.e., without UV irradiation) did not exhibit mutations detectable in our assay, confirming the possibility for localized gene editing using photocaged gRNAs. To the best of our knowledge, this is the first demonstration...
of light-controlled CRISPR-Cas-based system in developing embryos resulting in spatiotemporally-resolved gene editing.

Figure 4. Spatiotemporal control over gene editing in zebrafish embryos. (a) Temporally resolved light-mediated gene editing in zebrafish embryos microinjected with PC-g-Albino and Cas9 protein, and globally exposed to the 365-nm light at indicated developmental stages. The total number of embryos exhibiting each knockout phenotype category was counted for each treatment group (N = 11 - 41). The representative images of each knockout phenotype are shown on the left side. Dark – no UV irradiation, only ambient light. Scale bar 1 mm. (b) Automated image analysis of impaired pigment formation in slc45a2 mutated embryos using Fiji ImageJ software. Results are expressed as individual data points overlaid with the mean ± SD (N = 11 – 49); ***p < 0.001, ns – not significant (p > 0.05) versus wild-type embryos according to one-way ANOVA analysis combined with Tukey’s (Holm-Sidak) post-hoc test. (c) Mismatch-based mutation detection PCR assay of slc45a2 gene in embryos microinjected with Cas9 protein and PC-g-Albino, and globally irradiated with 365-nm UV light at various developmental stages. (N = 5 embryos per sample). (d) Representative photographs of zebrafish embryos microinjected with PC-g-Albino and Cas9 protein and either exposed to 405-nm laser light at 12-somite stage locally in the eye area (bottom image), or kept in the ambient light (dark) as a negative control (upper image). (e) Mismatch-based mutation detection assay of slc45a2 gene in embryos microinjected with Cas9 protein and PC-g-Albino, and locally irradiated in the eye area using 405-nm laser. The assay was run on the head and tail parts separately for each individual fish, 3-5 embryos were combined for each treatment group.
In conclusion, we have developed photoswitchable CRISPR-Cas gene editing system based on modification of the gRNAs with photocaging groups. This approach relies on a straightforward solid-phase synthesis of the photocaged gRNAs, with simpler purification and characterization processes in comparison to engineering a light-responsive protein. We have achieved gene editing as well as transcription activation in cells upon brief exposure to the UV light, whereas photocaged gRNAs showed virtually no detectable activity in the absence of light irradiation. Finally, we have applied this system to spatiotemporally control gene editing in vivo, enabling the use of this strategy for developmental biology and tissue engineering applications\textsuperscript{49-51}.

**Supporting Information:**

**SI Figure 1.** HPLC analysis of uncaging of PC-crRNA.

**SI Figure 2.** ZsGreen protein knockout in HEK-Cas9-ZsGreen cells.

**SI Figure 3.** UV light (365 nm) cytotoxicity assay on HEK-Cas9 cells.

**SI Figure 4.** Time-dependency of HPRT gene editing in HEK-Cas9 cells transfected with NC-gHPRT.

**SI Figure 5.** Biochemical activity assay of off-target DNA cleavage by NC- or PC-gIL1R2 with 365-nm light illumination (0-2 J/cm\textsuperscript{2}).

**SI Figure 6.** Time-dependent expression of the IL1R2 gene in HEK293FT cells.

**SI Figure 7.** Spatial control over gene expression using PC-gRNA.

**SI Figure 8.** Comparison of gene editing efficacy in zebrafish embryos using RNP and mRNA format for Cas9 delivery.

**SI Figure 9.** UV light toxicity on zebrafish embryos.

** Materials and methods**

**The Supporting Information is available free of charge on the ACS Publications website at DOI:xxx.**

**Notes**

Safety Statement: No unexpected or unusually high safety hazards were encountered. The authors declare no competing financial interest.

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Supporting information

Photoswitchable gRNAs for spatiotemporally controlled CRISPR-Cas-based genomic regulation.

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Supporting information Figure 1. HPLC analysis of uncaging of PC-crRNA. (a) HPLC traces of PC-crRNA oligos before and after UV light (365 nm) exposure (2.0 J/cm², 10 min exposure). (b) HPLC traces of NC- and PC-crTomato before and after exposure to increasing doses of UV light.

Supporting information Figure 2. ZsGreen protein knockout in HEK-Cas9-ZsGreen cells. Flow cytometry profiles of HEK-Cas9-ZsGreen cells transfected with non-caged or photocaged gZsGreen in the absence or presence of 1.0 J/cm² of UV light illumination, cells analysed 5 days post-irradiation.
Supporting information Figure 3. UV light (365 nm) cytotoxicity assay on HEK-Cas9 cells. Results are expressed as individual data points overlaid with the mean ± SD (N = 3); ***p < 0.001 versus cells kept in the dark according to one-way ANOVA analysis combined with Tukey’s (Holm-Sidak) post-hoc test.

Supporting information Figure 4. Time-dependency of HPRT gene editing in HEK-Cas9 cells transfected with NC-gHPRT. Cells were transfected for 4 h and analysed for mutations at the indicated time points using the mismatch-based assay.
**Supporting information Figure 5.** Representative gel images of off-target DNA cleavage by NC- or PC-gIL1R2 with 365-nm light illumination (0-2 J/cm²) in vitro. NT – non-treated amplicon (Cas9 only).

**Supporting information Figure 6.** Time-dependent expression of the *IL1R2* gene in HEK293FT cells. HEK-dCas9-VPR cells were transfected with gIL1R2 or negative control gTomato, and the *IL1R2* mRNA levels were analysed by qPCR at 6-96 h post-transfection. The *GAPDH* mRNA expression level served as a reference, (N = 3, mean ± SD, normalized to 6-h expression level). Only gIL1R2 was able to induce detectable *IL1R2* gene expression. N.D. – not detected.
Supporting information Figure 7. Spatial control over gene expression using PC-gRNA. HEK-dCas9-VPR cells co-transfected with PC-gTomato, the tdTomato reporter plasmid, and a GFP plasmid as a transfection control. A four-triangle pattern was drawn on top of the transfected HEK-dCas9-VPR cells using a 375-nm programmable laser (in a 35-mm dish). Red: tdTomato, green: GFP, scale bar 2 mm. The base of the triangles was 1 mm.

Supporting information Figure 8. Comparison of gene editing efficacy in zebrafish embryos using RNP and mRNA format for Cas9 delivery. (a) Automated image analysis of impaired pigment formation in slc45a2 knockout embryos using Fiji ImageJ software. Results are expressed as individual data points overlaid with the mean ± SD (N = 12 – 49); ***p < 0.001, ns – not significant (p > 0.05) versus wild-type embryos according to one-way ANOVA analysis combined with Tukey’s (Holm-Sidak) post-hoc test. Dark – no UV irradiation, only ambient light. (b) Phenotype scoring of zebrafish embryos microinjected with PC-gAlbino and Cas9 protein or mRNA, and globally exposed to the 365-nm UV light (2.0 J/cm²) immediately after microinjection. The total number of embryos exhibiting each knockout phenotype category was counted for each treatment group (N = 12 – 41). The representative images of each knockout phenotype are shown in the Fig. 4a of the main text. Dark – no UV irradiation, only ambient light. (c) Mismatch-based mutation detection assay of slc45a2 gene in embryos microinjected with PC-gAlbino and Cas9 protein or mRNA, and globally irradiated with 365-nm UV light (2.0 J/cm²) immediately after microinjection. (N = 5 embryos per sample). Dark – no UV irradiation, only ambient light.
Supporting information Figure 9. UV light toxicity on zebrafish embryos. Wild-type (WT) or RNP-microinjected zebrafish embryos were either kept in the dark or exposed to 2.0 J/cm² of 365-nm UV light immediately after microinjection and their viability was assessed at 48 hpf. Viable embryos are in blue, dead in red. Dark – no UV irradiation, only ambient light.
Materials and methods

Materials

HEK293FT cells, Dulbecco’s modified essential medium (DMEM) supplemented with 4.5 g/L glucose and GlutaMAX™, Opti-MEM® medium, Dulbecco’s phosphate buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin solution, 0.05 % Trypsin-EDTA, nuclease-free water, Lipofectamine® RNAiMAX, Lipofectamine® 2000, Proteinase K, alamarBlue™ cell viability reagent, PCR primers, high-capacity cDNA reverse transcription kit, PowerUp™ SYBR® Green master mix, E-gel® 2 % and 4 % precast agarose gels were purchased from Thermo Fisher Scientific (Walthman, MA). Non-caged CRISPR RNAs (NC-crRNAs) and trans-activating CRISPR RNA (Alt-R® tracrRNA) were purchased from Integrated DNA Technologies (IDT, Coralville, IA). NC-crRNAs were composed of AUGC and synthesized using proprietary Alt-R® modifications. All photocaged CRISPR RNAs (PC-crRNAs) were custom synthesized from commercially available building blocks and are readily accessible from Microsynth (Balgach, Switzerland). Edit-R® Cas9 protein, Edit-R® lentiviral Blast-Cas9-CAG nuclease plasmid and Edit-R® lentiviral hEF1a-Blast-dCas9-VPR particles were obtained from Horizon Discovery Group (Cambridge, UK). Cas9-encoding mRNA was purchased from Oz Biosciences (Marseille, France). PMD2.G and psPAX2 lentiviral packaging plasmids were a gift from Didier Trono (Addgene plasmids # 12259 and # 12260, respectively). ZsGreen lentiviral particles and Guide-it™ mutation detection kit were obtained from Takara Bio (Kusatsu, Japan). Reporter-gT1 plasmid for tdTomato was a gift from George Church (Addgene plasmid # 47320). Pmax-GFP plasmid used as a transfection control was from Lonza (Basel, Switzerland). Acetonitrile, HEPES, 0.5 M EDTA pH 8.0, NaCl and MgCl₂ were obtained from Sigma Aldrich (St. Louis, MO). Gel Pilot 1 kb Plus Ladder, QIAquick® PCR purification kit, and RNeasy Mini kit were obtained from QIAGEN (Hilden, Germany). Triethylammonium acetate 1 M pH 7.0 (TEAA) was acquired from ITW Reagents Division (Glenview, IL). Blasticidin was purchased from Invivogen (San Diego, CA). PVDF filters with 0.45-μm pore size and 100 kDa molecular cut-off Amicon® ultracentrifugal filter concentrators were obtained from Millipore (Burlington, MA). Imaging dishes (35 mm) with a glass bottom and an imprinted 50-μm grid for laser-guided photoactivation experiments were obtained from Ibidi (Martinsried, Germany).
Monitoring of UV-light mediated uncaging of PC-crRNAs using liquid chromatography (LC)

Twenty five microliters of PC-crRNA at 20 μM concentration were placed in a 0.2 mL PCR tube and were either kept in the dark or irradiated by UV light of 365 nm wavelength for 0-2 J/cm² (corresponding to 0-10 min of irradiation, respectively), and then diluted with 25 μL of 0.1 M TEAA in nuclease-free water to a final concentration of 10 μM. The UV lamp (UVLMS-38 8-watt, Analytik Jena AG, Jena, Germany) exhibited a power density of (3.3±0.5) mW/cm² as measured by UVA/B Light Meter 850009 (Sper Scientific, Scottsdale, AZ). The HPLC analysis was performed on an Agilent 1260 Infinity instrument (Agilent Technologies, Santa Clara, CA) equipped with a variable wavelength detector (VWD) using a Phenomenex Gemini®-NX 5 μm C18 110 Å 150 × 4.6 mm column. The detection wavelength λ = 260 nm, column temperature = 323 K, injection volume = 25 μL and flow rate =1 mL/min using the following gradient of 0.1 M TEAA in water (buffer A) and 0.1 M TEAA in 90 % acetonitrile (buffer B): 0-5 min 11 % B, 5-25 min 11-22 % B, 25-30 min 22 % B, 30-30.5 min 22-11 % B, 30.5-35 min 11 % B.

In vitro cleavage assay

Guide RNAs with or without photocaging groups were produced by annealing equimolar amounts of photocaged or non-caged crRNAs with tracrRNA to obtain a final concentration of 25 μM in nuclease-free water. The mixture was heated to 95 °C for 5 min in a heat block, and slowly cooled to room temperature. Non-caged crRNAs and tracrRNA were Alt-R® modified from IDT.

Three-step PCR (98 °C 2 min, 98 °C 10 s, 60 °C 15 s, 68 °C 1 min, 35 cycles) was performed to amplify HPRT, ZsGreen, slc45a2, IL1R2 genes and three off-target sites of gIL1R2 (predicted using CCTop online tool) using Guide-it™ mutation detection kit according to manufacturer’s instructions on a thermocycler (Alpha Laboratories, UK). Primer sequences were as follows (5′ to 3′): HPRT forward TACACGTGTGAACCAACCG, HPRT reverse GTAAGGCCCTCTCCTTATT, ZsGreen forward CTCGAGAAGCTTGATCGCGT, ZsGreen reverse GACAAGATGTCCCTCGGCGAA, IL1R2 forward GACTTGATGCTGGATTTCCACT, IL1R2 reverse GATTTCTAACCCGTTGCTTGT, slc45a2 forward CTGGGAAGTCCAACGCTCAG, slc45a2 reverse CCTATTGTCCACTCCAGCA, IL1R2 off-target 1 forward GTCACGCCTTCCGTTGAATTT, IL1R2 off-target 1 reverse TGGAGCTGAGAAACCGGTC, IL1R2 off-target 2 forward GGAAGGAGGAGGCTCTACCA, IL1R2 off-target 2 reverse AGGGGCCACATTTACCAGTG, IL1R2 off-target 3 forward CACCCTAGCTCTATGCTGCG, IL1R2 off-target 3 reverse TGGTCTTGGCAGCTTTCAT.
PCR products were purified using QIAquick® PCR purification kit and their concentrations were determined using NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Walthman, MA).

In vitro cleavage assay was performed according to manufacturer’s protocol (Alt-R® CRISPR-Cas9 system, IDT). Briefly, equimolar amounts of crRNA:tracrRNA duplex and Cas9 enzyme were incubated for 5 min at room temperature to form the ribonucleoprotein complex (RNP). Purified target DNA amplicon (20 nM final concentration) was mixed with RNP complex together with 1 μL of Cas9 buffer (200 mM HEPES, 1 M NaCl, 50 mM MgCl₂, and 1 mM EDTA, pH 6.5) to achieve RNP:DNA molar ratio of 10:1. The total volume of the mixture was adjusted to 10 μL with nuclease-free water, which was followed by the incubation at 37 °C for 2 hours. After the incubation, 2 μL of Proteinase K solution (20 mg/mL) was added to the reaction mixture and incubated at 56 °C for 10 min to digest Cas9 protein. Samples were resolved on pre-stained 4 % agarose E-gels® and imaged using Invitrogen™ E-Gel® Imager.

Intensities of the bands were quantified using Fiji ImageJ open-source software, and target DNA cleavage efficacy was calculated using the following formula, where \( I_{\text{fragment 1}} \) and \( I_{\text{fragment 2}} \) are intensities of cleaved fragments and \( I_{\text{full length amplicon}} \) is the intensity of uncut DNA amplicon:

\[
\text{DNA cleavage (\%)} = \frac{I_{\text{fragment 1}} + I_{\text{fragment 2}}}{I_{\text{fragment 1}} + I_{\text{fragment 2}} + I_{\text{full length amplicon}}} \times 100 \% .
\]

Cell culture

HEK293FT cells (human embryonic kidney cell line) were maintained in DMEM supplemented with 4.5 g/L glucose, GlutaMAX™, 10 % FBS and 1 % penicillin/streptomycin (complete medium) in 5 % CO₂ at 37 °C, with regular passaging twice a week using 1:5 split ratio. Each freshly thawed cell line was grown for 2–3 passages before transfection. Cells were regularly tested for mycoplasma contamination.

Cell viability assay after UV-light irradiation

The cytotoxicity of 365-nm light to HEK293FT cells was assessed using alamarBlue™ cell viability reagent according to manufacturer’s instructions. The HEK293FT cells were plated in 96-well plate at a seeding density of \( 4 \times 10^4 \) cells per 100 μL of complete medium per well. After 4 h incubation, cells were exposed to increasing doses of UV light (UVLMS-38 lamp, 0-30 min, 0-6 J/cm²), followed by 48 h of further incubation in the dark. The cells were then incubated with 10 % v/v alamarBlue (100 μL/well) in complete medium for 4 h. The supernatants were transferred into new 96-well plate and fluorescence was read by microplate reader SpectraMax.
M5 (Molecular Devices, San Jose, CA) at Ex/Em = 570/585 nm (auto cut-off) from the bottom of the plate. The alamarBlue™ solution in complete medium was used as a negative control. The cell viability was calculated according to the following equation, where $F_{\text{sample}}$ refers to the fluorescence of cells treated with different doses of UV light, $F_{\text{cell only}}$ refers to fluorescence of untreated cells, and $F_{\text{Alamar Blue}}$ refers to the fluorescence of negative control:

$$\text{Cell viability (\%) } = \frac{F_{\text{sample}} - F_{\text{Alamar Blue}}}{F_{\text{cell only}} - F_{\text{Alamar Blue}}} \times 100 \% .$$

**Lentivirus and cell lines generation**

For Cas9 lentiviral particles production, HEK293FT cells were seeded at ~40 % confluency in a T175 flask the day before transfection. Cells were transfected for 4 h with a mixture of plasmids encoding for Cas9 (62.9 μg), pMD2.G (16.9 μg) and psPAX2 (31.3 μg) using Lipofectamine® 2000 according to the manufacturer’s instructions. After medium exchange, cells were incubated for another 48 h. The supernatant containing Cas9 lentiviral particles was filtered through a 0.45-μm PVDF filter, 100-fold concentrated using 100 kDa molecular cut-off Amicon® ultracentrifugal filter concentrator, and aliquots were stored at −80 °C.

For generation of HEK-Cas9 or HEK-dCas9-VPR cell lines, HEK293FT cells were seeded at a $1 \times 10^5$ cell per well of a 24 well plate in complete medium the day before transduction. Cells were transduced with 10-fold serial dilutions of the viral concentrates in complete medium for 24 h, followed by medium exchange. Successfully transduced cells were selected using 10 μg/mL of blasticidin for 7 days, and the samples with the cell viability of less than 30 % were used for further studies to encourage single gene copy insertions. Monoclonal cell lines were obtained by plating cells in a 96-well plate at 0.5 cell/well seeding density and subsequent colony propagation.

For generation of HEK-Cas9-ZsGreen cell line, HEK-Cas9 cells were seeded at a $1 \times 10^5$ cell per well of a 24 well plate the day before transduction. Cells were transduced with 10-fold serial dilutions of the viral concentrate in complete medium for 24 h, followed by medium exchange. The samples with less than 30 % of ZsGreen-positive cells were FACS sorted for ZsGreen expression at the Flow Cytometry Facility of Imperial College London. Monoclonal cell lines were obtained by plating cells in a 96-well plate at 0.5 cell/well seeding density and subsequent colony propagation.
Light-activated gene editing in cells

For the HPRT and IL1R2 gene editing experiments, HEK-Cas9 cells were transfected for 4 h in tissue culture 96-well plates with 12.5 pmol of NC-gRNA or PC-gRNA using Lipofectamine® RNAiMAX as per manufacturer’s instructions. For ZsGreen gene editing experiments, HEK-Cas9-ZsGreen cells were transfected for 4 h in tissue culture 96-well plates with 12.5 pmol of NC-gRNA or PC-gRNA using Lipofectamine® RNAiMAX. After the transfection medium exchange, the cells were either kept in the dark or exposed to 365-nm UV light for 5 min (1.0 J/cm²) at the indicated time points, followed by 2 days of further incubation for gene mutation assay or for 5 days for ZsGreen protein expression evaluation by fluorescence microscopy and flow cytometry.

Mutation detection assay

Mutation detection assay was performed using Guide-it™ mutation detection kit according to manufacturer’s instructions (Takara Bio, Kusatsu, Japan). Target genes were purified from the genomic DNA using the same primers as for the in vitro cleavage assay, followed by amplicon rehybridization and resolvase-mediated mismatched strands cleavage. The products were resolved on 2 or 4 % agarose E-Gels® and imaged by Invitrogen™ E-Gel® Imager. Fiji ImageJ open-source software was used to analyze the intensities of the bands and to calculate the rate of insertions and deletions in the target genes (indel) using the following equations, where

\[ f_{\text{mismatch}} = \frac{N_{\text{mismatched amplicons}}}{N_{\text{all amplicons}}}, \quad f_{\text{mutation}} = \frac{N_{\text{mutated amplicons}}}{N_{\text{all amplicons}}}, \]

\[ N_{\text{ref}} \text{ refers to number of molecules, } I \text{ refers to band intensity:} \]

\[ f_{\text{mismatch}} = \frac{I_{\text{fragment 1}} + I_{\text{fragment 2}}}{I_{\text{full length amplicon}}}, \]

\[ \text{Indel (\%) } = f_{\text{mutation}} \times 100 \% = \left(1 - \sqrt{1 - f_{\text{mismatch}}}\right) \times 100 \%. \]

Spatially controlled transcription activation

For spatially resolved transcriptional activation of a reporter gene using a photomask, HEK-dCas9-VPR cells (2.5 × 10^5 cells per well of a 24-well plate) were transfected for 4 h with 0.75 μg of reporter tdTomato plasmid 48, 0.375 μg of GFP plasmid, and 9.38 pmol of NC-gRNA or PC-gRNA targeting a sequence upstream of tdTomato ORF cassette using Lipofectamine® RNAiMAX. A small portion of the well was illuminated through a tin foil photomask (1.0 J/cm²) using collinear 365-nm light emitting device OmniCure S1500 (Excelitas...
Technologies, Waltham, MA) equipped with a light guide. Fluorescence images were taken 1 day after the irradiation.

For laser-guided activation of gene expression, $6 \times 10^5$ HEK-dCas9-VPR cells were seeded into imaging dishes (35 mm) with a glass bottom and an imprinted 50-μm cell location grid 24 h before the experiment. The next day, cells were transfected for 4 h with 0.5 μg of reporter tdTomato plasmid, 0.25 μg of GFP plasmid, and 6.25 pmol of PC-gRNA targeting a sequence upstream of tdTomato ORF cassette using Lipofectamine® RNAiMAX. Four triangular shapes with the base of 1 mm were scanned on top of the transfected cells using the laser lithography system Dilase 250 (Kloe, Montpellier, France). The 375-nm laser spot size was 5 μm, the power modulation was 2.6 %, and the scanning speed was 1-8 mm/s. After the exposure, the cells were further incubated for 1 day to allow for the tdTomato expression, which was assessed by fluorescence microscopy.

**Temporally controlled transcription activation**

To evaluate the time-dependency and the sequence-specificity of the *IL1R2* gene activation, HEK-dCas9-VPR cells (2.5 × 10^5 cells per well of a 24-well plate) were transfected for 4 h with 1 μg of NC-gRNA targeting a sequence upstream of *IL1R2* gene (crIL1R2) using Lipofectamine® RNAiMAX. The gRNA used for activation of tdTomato cassette served as a negative control. Relative expression of *IL1R2* mRNA was assessed by qRT-PCR 6-96 h post-transfection. Due to undetectable *IL1R2* mRNA expression in the non-transfected HEK-dCas9-VPR cells, cells transfected with the NC-gIL1R2 at the 6 h post-transfection time point were used for normalization.

For temporally resolved endogenous gene activation, HEK-dCas9-VPR cells (2.5 × 10^5 cells per well of a 24-well plate) were transfected for 4 h with 1.5 μg of NC-gRNA or PC-gRNA targeting a sequence upstream of IL1R2 gene (crIL1R2) using Lipofectamine® RNAiMAX. The gRNA used for activation of *tdTomato* cassette served as a negative control. After transfection medium exchange, cells were either kept in the dark or exposed to 365-nm UV light for 5 min (UVLMS-38 lamp, 1.0 J/cm² dose) at the specified time points, followed by 1 day of further incubation. Relative expression levels of the *IL1R2* mRNA were assessed by qRT-PCR, and the cells treated with the NC-gIL1R2 served as a normalization control.

**Quantitative real-time PCR analysis (qRT-PCR)**
For the *IL1R2* mRNA expression analysis, total RNA was isolated using RNeasy Mini kit (A₂₆₀/A₂₃₀ > 1.8). The expression levels of *IL1R2* mRNA relative to the internal control *GAPDH* mRNA were quantified by two-step quantitative real-time PCR. One microgram of total mRNA was reverse transcribed in 20 µL of final reaction volume using high-capacity cDNA reverse transcription kit. Quantitative RT-PCR (50 °C 2 min, 95 °C 10 s, 40 cycles × (95 °C 15 s, 60 °C 1 min)) was performed using PowerUp™ SYBR® Green PCR master mix and specific primers for human *IL1R2* (forward CAGGTGAGCAGCAAACAGG, reverse TGCTCCTGACAACTTCCAGA), and *GAPDH* (forward TGGTATCGTGGAAGGACTCATGA, reverse ATGCCAGTGAGCTTCCGTTCAG) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Delta delta Ct (2⁻ΔΔCt) method was used to calculate relative mRNA expression levels. Results are expressed as the *IL1R2* mRNA expression level change between cells treated for 6 h and the following time points for time-dependent gene activation, and between NC-gRNA treated and PC-gRNA treated cells for light-activation experiments.

**Fluorescence microscopy**

ZsGreen, GFP, and tdTomato protein expression was assessed using fluorescence microscopy on Z1 AxioObserver (Zeiss, Oberkochen, Germany). Cells were fixed in 2 % paraformaldehyde (PFA) in PBS for 10 min, washed with PBS, and the cell nuclei were stained with Hoechst. Images were taken in bright field, Hoechst 33258 (Ex/Em 352/455), Alexa Fluor 488 (Ex/Em 493/517), and tdTomato (Ex/Em 554/581) channels using 20× objective (NA 0.4). Whole well images were processed and stitched using Zen 2 (blue edition) imaging software.

**Flow cytometry analysis**

Flow cytometry analysis of ZsGreen knockout in HEK-Cas9-ZsGreen cells was performed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA). HEK-Cas9-ZsGreen cells transfected as described in gene editing section were trypsinized and resuspended in 0.5 mL of complete medium, centrifuged and washed with PBS. Cells were fixed with 2 % PFA in PBS for 10 min, centrifuged and resuspended in 0.5 mL of PBS containing 5 % FBS as a blocking reagent. Any debris and damaged cells were excluded in SSC-A versus FSC-A plot, and clumps or doublets were gated out in FSC-H versus FSC-A plot. Flow cytometry data was analysed via BD Accuri™ C6 Software.

**Temporally controlled gene editing in zebrafish embryos**
Experiments involving zebrafish were conducted in accordance with UK Home Office requirements (ASPA 1986, project licence P5D71E9B0, institution licence 70/2722 X32FDCFC1). The fish were kept in the CBS facility of the Imperial College London. Wild-type zebrafish used in this study were reared and maintained according to standard practices at 28.5°C on a 14-hour light/10-hour dark cycle. Zebrafish embryos were obtained via natural mating and were maintained in E2-water supplemented with 0.00003 % methylene blue at 28.5°C.

RNPs were prepared to contain 4 μM of Cas9 protein and 20 μM of NC- or PC-gAlbino in nuclease-free water. For comparison, Cas9 protein in the mixture was replaced with 0.2 g/L of Cas9-encoding mRNA. Single-to double-cell stage embryos of wild-type zebrafish were microinjected with 2.5 nL of these solutions using a pressure microinjector Narishige IM300 (Tokyo, Japan) with pulled borosilicate capillary needles with outer diameter 1.0 mm, inner diameter 0.78 mm, length 100 mm (Harvard Apparatus, Holliston, MA), followed by irradiation with 365-nm light for 10 min (UVLMS-38 lamp, 2.0 J/cm²) or incubation in the dark. For time-dependent gene editing activation study, the injected embryos were exposed to the UV light at different developmental stages. Alternatively, for spatially resolved gene editing, the microinjected embryos were left at the ambient light (no UV irradiation) until they reached a 12-somite stage. Then they were dechorionated, embedded into 0.5 % low melting agarose in a glass bottom 35-mm dish, and oriented laterally. After that, the eye of the embryo was irradiated for 1 min at 20× objective (HC PL APO NA 0.7) and 4× zoom using a 405-nm laser at 50 % power in a confocal microscope setup (Leica SP5 inverted, Wetzlar, Germany). After 2 days of growth, the embryos were scored for albino-like phenotype in the retina and skin and photographed using a stereomicroscope (Leica M205 FCA, Wetzlar, Germany).

For the mutation detection assay, total genomic DNA of 3-5 embryos per sample was extracted and analysed using Guide-it™ mutation detection kit. For spatially resolved gene editing, the mutations in the slc45a2 gene were analysed in the head and tail parts of the same embryos separately. Three to five embryos were combined for the analysis. Only embryos that developed normally were assayed for mutations.

Automated image analysis of impaired pigment formation in slc45a2 knockout embryos was performed using Fiji ImageJ software. First, the gaussian blur with a radius of 10 was run on a duplicate image, then the original image was divided by the blurred image to decrease the background variability, and the threshold was set between -3.4e38 and 0.71 in order to create a binary image. Then the dust was cropped out and the relative area
occupied by the black pixels was quantified. These processing steps were performed on each image. Wild-type embryos served as a normalization control.

**Statistical Analysis**

The statistical analysis was performed using OriginPro 2017 software. All experimental groups were compared pairwise using the one-way analysis of variance (ANOVA) followed by Tukey’s (Holm-Sidak) post-hoc test assuming normal data distribution. The differences between treatment groups were considered statistically significant at $p$-values lower than 0.05.