Supporting Information

Spatiotemporal Control of CRISPR/Cas9 Function in Cells and Zebrafish using Light-Activated Guide RNA

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Methods

Caged gRNA synthesis

The NPOM-caged uridine and guanosine phosphoramidites were synthesized according to established protocols.[1] Requests for caged amidites can be sent to deiters@pitt.edu.

Caged gRNAs were synthesized by Horizon Discovery (formerly Dharmacon, Lafayette, CO) and requests for custom, caged gRNAs can be sent to Michael.Delaney@horizondiscovery.com.

The syntheses were conducted using an LGC Bioautomation MerMade 4 DNA/RNA Synthesizer (Irving, TX, USA) employing standard β-cyanoethyl phosphoramidite chemistry. The caged gRNAs were synthesized on a 1 μmole scale using 1000 Å rU derivatized CPG solid phase supports obtained from LGC Biosearch Technologies (Novato, CA, USA) using 5'-DMTr-ON approach. Reagents and non-caged phosphoramidites for automated RNA synthesis were also obtained from LGC Bioautomation. Optimized synthesis cycles with coupling time of 8 mins were employed for both non-caged and caged phosphoramidites at 0.08 M concentration. Coupling efficiency in each cycle was monitored by following the release of dimethoxytrityl (DMTr) cations after each deprotection step. No significant loss of DMTr was noted following the addition of caged-U or caged-G phosphoramidites to the oligonucleotide.

Following synthesis, cleavage from support and deprotection of the oligonucleotides were carried out with 1 mL of AMA (ammonium hydroxide/40% aq. methylamine 1:1 v/v) solution at 65 °C for 20 min. The TBDMS group (*tert*-butyldimethylsilyl) was deprotected using 65 μL of TEA (triethylamine) and 75 μL of TEA-3HF (triethylamine trihydrofluoride) in 110 μL of DMSO at 65 °C for 2 h.

Caged gRNA purification

Purification was performed on a Waters Autopurification system using a Waters X-Bridge BEH C18 column (10 mm x 100 mm, 130 Å, 5 μm). The buffers used were 50 mM TEAA (triethylammonium acetate) with 5% MeOH (methanol) as buffer A and 100% MeOH as buffer B. The oligonucleotides were purified using a gradient of 15-60% buffer B over 60 mins at a flow rate of 4 mL/min. Fractions were analyzed by UPLC and the highest purity fractions were pooled together.

Final deprotection of the 5'-DMTr group was accomplished by treating the purified pool with 400 μL of 35 mM NaOAc (sodium acetate) at 55 °C for 2 h. The oligonucleotides were then precipitated using 50 μL of 3 M NaOAc and 1.5 mL of cold ethanol. Following incubation at -80 °C for 30 mins, the oligonucleotides were pelleted by centrifugation, quantified at 260 nm and further analyzed by UPLC and ESI-LCMS.

UPLC and ESI-LCMS analysis

The oligonucleotides were analyzed on a Waters Aquity UPLC system using a Waters BEH C18 column (2.1 mm x 50 mm x 1.7 μm). Samples were prepared by dissolving 0.5 nmol of the oligonucleotide in 75 μL of water and injecting 2 μL of the solution. The buffers used were 50 mM Dimethylhexylammonium acetate with 10% CH_3CN (acetonitrile) as buffer A and 50 mM Dimethylhexylammonium acetate with 75% CH_3CN. The gradient utilized for analysis was 25-75% buffer B over 5 mins with a flow rate of 0.5 mL/min at 60 °C.

ESI-LCMS data for the oligonucleotides were acquired on a Thermo Ultimate 3000-LTQ-XL mass spectrophotometer. Samples were prepared by dissolving 0.5 nmol of the oligonucleotide in 75 μL of water and injecting 10 μL of the solution using a Novatia C18 (HTCS-HTC1-4) trap column. Following injection, sample was eluted into the LTQ-MS with 85% CH_3CN, 50 mM HFIP (hexafluoro-2-propanol), 10 μM EDTA (ethylenediaminetetraacetic acid), 0.35% DIPEA (N,N-diisopropylethylamine) and analyzed for mass (**Figures S1-S4**).

Non-caged gRNA preparation

Template sequences for *in vitro* transcription of gRNAs are listed in **Table S1**. Transcription templates were prepared by mixing 10 µg of the Forward strand, 10 µg of the Reverse strand, 1 µL of 10x TE/Mg^{2+} buffer, and nuclease free water to make up a 10 µL annealing mixture, followed by heating at 95 °C for 3 min and cooling down to 25 °C at a rate of −0.2 °C/sec, using a thermal cycler (Bio-Rad, T100). The T7 or SP6 MEGAscript kits (Fisher) were used for *in vitro* transcription of non-caged gRNAs. In a 20 µL transcription mixture, 1 µg of the annealed double strand template was used following manufacturer's protocol, for 16 hrs at 37 °C. The transcription was stopped by adding 1 µL of Turbo DNase from the MEGAscript kit and kept at 37 °C for 15 min. For extraction of the transcribed gRNA, the reaction mixture was mixed with 15 µL Ammonium Acetate Stop Solution (5 M ammonium acetate, 100 mM EDTA), 115 µL TRIzol reagent (Fisher), and 300 µL chloroform (Fisher). After brief vortexing, the mixture was centrifuged at 13,200 rpm at 4 °C for 10 min, after which the top aqueous layer was transferred into 500 µL 2-propanol (Fisher). After brief vortexing, the tube was frozen at −80 °C for at least 2 hours. The tube was then centrifuged at 13200 rpm at 4 °C for 20 min, after which the pellet was resuspended in 20 µL of nuclease-free water. The concentration of each non-caged gRNA solution was determined by absorption at 260 nm using a NanoDrop spectrophotometer and the purity was examined by 10% 6 M urea denaturing TBE PAGE (25 W, 30 min), followed by SYBR Gold (Invitrogen) staining at room temperature for 30 min (1:10000 dilution in 1x TBE buffer) and imaging using the Bio-Rad ChemiDoc™ MP Imaging System (**Figure S6**).

Table S1. Oligonucleotide sequences.

Name	Sequence (5'-3')
T7-DsRed_F (pRG, mammalian)	TAATACGACTCACTATAGGGAGATCGACTCTAGAGGATCCAC GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
T7-DsRed_R (pRG, mammalian)	AAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTGGATC CTCTAGAGTCGATCTCCCTATAGTGAGTCGTATTA
T7-EGFP_F (pRG, mammalian)	TAATACGACTCACTATAGGGAGATAGCTAGTCTAGGTCGATG CGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
T7-EGFP_R (pRG, mammalian)	AAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCATCGA CCTAGACTAGCTATCTCCCTATAGTGAGTCGTATTA
SP6-CTNNb1_F (*ctnnb1*, mammalian)	ATTTAGGTGACACTATAGACGGCAGCAGACTGCTGGGGTTTT AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
SP6-CTNNb1_R (*ctnnb1*, mammalian)	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGA CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCAG CAGTCTGCTGCCGTCTATAGTGTCACCTAAAT

Table S1. cont.

SP6-EGFP_F (*egfp,* zebrafish)	ATTTAGGTGACACTATAGCTCGCCCTTGCTCACCATGGGTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTAT CAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
SP6-EGFP_R (*egfp,* zebrafish)	AAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCATGGT GAGCAAGGGCGAGCTATAGTGTCACCTAAAT
SP6-SLC24A5_F (*slc24A5,* zebrafish)	ATTTAGGTGACACTATAGATTCTTCACGGTGCAGGAGGTTTT AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC AACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
SP6-SLC24A5_R (*slc24A5,* zebrafish)	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGA CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCTCCT GCACCGTGAAGAATCTATAGTGTCACCTAAAT
Cas9 D10A_F	CAATAGGCTTAGCGATCGGCACAAATAGCGTCGGATG
Cas9 D10A_R	GTGCCGATCGCTAAGCCTATTGAGTATTTCTTATCC
Cas9 H840A_F	GATGTCGATGCGATTGTTCCACAAAGTTTCCTTAAAGACG
Cas9 H840A_R	GTGGAACAATCGCATCGACATCATAATCACTTAAACG
pBAD_Cas9_F	CCCATGGATAAGAAATACTCAATAG
pBAD_Cas9-6xHis_R	GCGCGCCGTTTAAACAAAGCTTTAATGATGATGATGATGATG GTCACCTCCTAGCTGAC
DsRed 55mer target_F [b)]	CGCAAGCTTCCTAGACTAGTCGACTCTAGAGGATCCACCGG TCGCCACCATGGCC
DsRed 55mer target_R [b)]	GGCCATGGTGGCGACCGGTGGATCCTCTAGAGTCGACTAGT CTAGGAAGCTTGCG
55mer non-target_F [c)]	CGCAAGCTTCCTAGACTAGACGGCAGCAGACTGCTGGGCG GTCGCCACCATGGCC
55mer non-target_R [c)]	GGCCATGGTGGCGACCGCCCAGCAGTCTGCTGCCGTCTAG TCTAGGAAGCTTGCG
CTNNb1_F1 [d)]	GATCATACTTGTTGCAGCTTCGACAA
CTNNb1_F2 [d)]	CTAGTGACAAGTGGAACCAGAT
CTNNb1_R2 [d)]	CTACGAAGTTTGGCTCCGAGA
CTNNb1_R1 [d)]	CCCGCTGCACTTAGAGTTTAGTTG
SLC24A5_F1 [d)]	CTCTGTTACTGTCAACTCATTGTGTATTAT
SLC24A5_R1 [d)]	CACTGACGGATCTCTGCACT
SLC24A5_F2 [d)]	GCAGTTCTGAAATGATTTGTGTGTGT
SLC24A5_R2 [d)]	ACGAGCTCTGGAGCCGAACTC

[a)] T7 and SP6 promoters are underlined. [b)] 55-mer target dsDNA strands used for gel shift assay. [c)] 55-mer non-target dsDNA strands used for gel shift assay. [d)] Primers used in nested PCR.

Expression and purification of catalytically dead Cas9

The *S. pyogenes* Cas9 (abbreviated Cas9) gene was a kind gift from the Asokan Lab (North Carolina State University). D10A and H840A mutations were introduced by site-directed mutagenesis with primers mentioned in **Table S1** to render it catalytically inactive (dead Cas9, or dCas9) (**Table S1**).[2] dCas9 was then amplified by the pBAD_Cas9_F and pBAD_Cas9-6xHis_R primers (**Table S1**), digested by NcoI and PmeI restriction enzymes (NEB, 37 °C, overnight), and purified by PCR Cleanup kit (Omega Biotek). The pBAD plasmid was digested by NcoI and PmeI restriction enzymes (NEB, 37 °C, overnight), and purified by 0.8% agarose gel and DNA Gel Extraction Kit (Omega Biotek). T4 ligation of the insert and the vector (1:3 molar ratio) was carried out at 16 °C, overnight, with 1 µL 10x T4 Ligase Buffer and 0.5 µL T4 Ligase (NEB) in a 10 µL reaction mixture. Successful site-directed mutagenesis and construction of the pBAD_dCas9-6xHis expression plasmid were confirmed by Sanger sequencing (Genewiz, USA). The plasmid map is shown in **Figure S9a.**

pBAD_dCas9-6xHis was transformed by heat shock in TOP10 *E. coli* competent cells. Overnight starter culture (containing 12 µg/mL tetracycline) was diluted 1:100 into 25 mL of LB broth (containing 12 µg/mL tetracycline) and let grow until the O.D. 600 reaches 0.6, at which point, the cells were induced by 0.2% L-arabinose and protein expression went on at 16 °C for 16 hours. After 16 hours, the cells were harvested, resuspended in 5 mL of lysis buffer (50 mM Na_2HPO_4, 200 mM NaCl, and 10 mM imidazole, pH 8.0), lysed with 1 mg/mL lysozyme (Fisher) and sonification (Fisher Sonic Dismembrator 550, 15% amplitude, 10 sec ON, 10 sec OFF, 5 min total) in the presence of protease inhibitor cocktail (Sigma) at 4 °C. His-tagged dCas9 was then pulled-down by 80 µL Ni-NTA agarose resin (G-Biosciences), washed twice by 500 µL washing buffer (20 mM Tris-HCl, 200 mM NaCl, and 50 mM imidazole, pH 8.0), eluted by 300 µL elution buffer (20 mM Tris-HCl, 200 mM NaCl, and 300 mM imidazole, pH 8.0), and dialyzed against 1 L Cas9 dialysis buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM TCEP, 10% glycerol) at 4 °C overnight.[2] Dialyzed dCas9 protein was then concentrated to ~ 3 µM using 50 kDa spin columns (Millipore) at 4 °C at 14000 *g* for 20 min.

The purity and concentration of dCas9-6xHis was analyzed by 6% SDS-PAGE (**Figure S5**). The gel was photographed by the Bio-Rad ChemiDoc™ MP Imaging System and quantification was performed by integrating the pixels of each band using the ImageLab 5.0 software.

Decaging assay of caged gRNA

The decaging assay was carried out with 1 µM of DsRed-4U-gRNA and its corresponding non-caged version. The experiment was carried out in duplicates. The caged sample (15 µL) was irradiated with 365 nm on a UV transilluminator (VWR, Dual UV Transilluminator, 80 mW/cm^2) for different durations (30 s, 1, 2, 3, 5 and 10 mins) and then analyzed by HPLC on a Waters X-Bridge Oligonucleotide BEH C18 column (4.6 mm x 50 mm, 130 Å, 2.5 µm) (Figure S7). The buffers used were 0.1 M TEAA as buffer A and CH_3CN as buffer B and the column oven was set to 60 °C. The injected sample (10 µL) was then analyzed using a gradient of 5-32% buffer B over 35 mins at a flow rate of 1 mL/min.

Gel shift assays for dsDNA binding and gRNA binding

Gel shift assay was carried out following a published method, with a ^{32}P-labelled dsDNA target.[3] For the isotopic labelling of the target dsDNA, the following protocol was used. The forward strand ssDNA (1 nmol) and reverse strand ssDNA (1 nmol) were mixed with 10x TE/Mg^{2+} buffer and annealed. Annealing was carried out by heating at 95 °C for 3 min and cooling down to 25 °C at a rate of −0.2 °C/sec. The dsDNA was run on 12% native PAGE (200 V, 1 hr, 4 °C), excised from the gel, and passively diffused into 1x TE/Mg^{2+} buffer (6 hrs, 4 °C). The concentrations of purified dsDNAs (both target and non-target) were determined by a NanoDrop Spectrophotometer (ND-1000) and the dsDNAs were then diluted to 1 µM. For ^{32}P-labelling, 1 pmol dsDNA was mixed

with 1 µL 10x T4 PNK buffer (NEB), 0.6 µL gamma-^{32}P-ATP (PerkinElmer, 3000 Ci/mmol), 0.5 µL T4 PNK (NEB), and nuclease-free water to make a 10 µL labeling mixture, which was incubated at 37 °C for 1 hr, followed by heat inactivation at 65 °C for 20 min. The isotopically labelled dsDNAs were then cleaned-up using Microspin™ G-25 Columns (GE Healthcare) and diluted to 1 nM for gel shift assays, assuming 100% recovery from the G-25 column (**Figure 1d** and **Figure S8**).

Purified dCas9-6xHis (abbreviated as dCas9) was mixed with 10x molar excess of the designated gRNA and incubated at 37 °C for 10 min. Then varying concentrations of the dCas9:gRNA complex (0-375 nM or 0-1000 nM) was mixed with 0.125 nM of ^{32}P-dsDNA and incubated at 37 °C for 1 hr. Afterwards, the mixtures were run on 6% native Tris-Glycine PAGE (in 0.5xTBE + 5 mM $MgCl_2$ running buffer) at 100 V and 4 °C for 2 hrs. Then the native gels were exposed to a phosphor screen for at least 8 hours and visualized by Typhoon FLA7000 IP Phosphoimager (GE Healthcare).

Non-caged DsRed gRNA and DsRed-4U gRNA were 5'-^{32}P-labelled similarly as stated above. The gRNAs were then diluted to a final concentration of 0.25 nM, mixed with 0-500 nM EnGen Cas9 (dual-NLS-Cas9, NEB), and incubated at 37 °C for 1 hr. The mixtures were then run on 12% native Tris-Glycine PAGE and visualized as mentioned above (**Figure S8c**). The Cas9:gRNA complex remained in the well and did not migrate into the native gel due to the high pI of Cas9, which was also observed in other studies.[4]

Mammalian cell culture, transfection, and Cas9:gRNA RNP delivery

HEK293T cells were acquired from ATCC and maintained in DMEM media (Fisher) with 10% FBS (Fisher/USB) without antibiotics. Cells were sequentially transfected twice for the pRG reporter plasmid (**Figure S9b**) and the Cas9:gRNA RNP. For the first transfection of the pRG reporter plasmid, 500 ng DNA was combined with 3.23 µg of linear polyethylenimine (Polysciences) per well of a 12-well plate. Six hours after the transfection of the reporter plasmid, cells were gently lifted with 100 µL TrypLE Express (Gibco), spun down at 1000 rpm at room temperature for 5 min, resuspended in fresh DMEM with 10% FBS (no antibiotics), reverse transfected with Cas9:RNP complexes, and left to grow in 96-well plates at 37 °C, 5% CO_2. The RNP complex was pre-assembled by mixing EnGen Cas9 and gRNA at a 1:1 molar ratio and incubated at 37 °C for 10 minutes. For reverse transfection of one well in a 96-well format, 1.5 pmol total RNP was mixed with 0.75 uL Lipofectamine 3000 (Fisher) in 20 µL Opti-MEM (Gibco) for 10 minutes at room temperature before mixing with 80 µL of HEK293T cells ($5x10^4$ cells) transfected with the pRG dual-fluorescence reporter. For editing of the CTNNb1 gene, transfection of the pRG plasmid was excluded. HEK293T cells were only reverse transfected once with the corresponding RNP complexes.

Light-activation of RNP complexes delivered into mammalian cells

HEK293T cells transfected with Cas9:gRNA RNP complexes were allowed 6 hours to attach to the bottom of the plate. Afterwards, the transfection mixture is replaced with DMEM and the cells were subjected to 5 minutes of 365 nm irradiation by a UV transilluminator (VWR, Dual UV Transilluminator, 80 mW/cm^2). Fluorescent imaging of the cells was performed after 72 hours post irradiation. Media was replaced with clear DMEM-high glucose modified growth media (Thermo Scientific) for microscopy imaging on a Zeiss Observer Z1 microscope (10x objective, NA 0.8 plan-apochromat) with EGFP (38 HE; ex: BP470/40; em: BP525/50) and DsRed (43 HE; ex: BP550/25; em: BP605/70) filter cubes, then processed and quantified in Zen 2 imaging software. For the spatial control experiments, UV irradiations were performed through a tin foil mask to only expose a subset of cells to 365 nm light for 5 minutes. Microscopy imaging was then performed in a tiled grid and stitched using the Zen 2 software. Quantification was carried by first setting a lower threshold with a fixed value to subtract background, which was determined by the fluorescence intensity of non-transfected HEK293T cells in the same plate. Then the fluorescence

intensity for each channel was integrated to represent the expression level of the corresponding fluorescent protein. Editing efficiency was calculated by dividing integrated EGFP fluorescence by integrated DsRed fluorescence of each well and error bars represent standard deviation of three independently transfected and treated wells in parallel.

Genomic PCR and TIDE analysis

HEK293T cells were lysed by heating cell suspension at 95 °C (in 1x PBS) for 10min. Nested PCR was then carried out with cell lysates using the corresponding primers (**Table S1**). Zebrafish embryos were lysed following a published protocol.[5] In short, whole embryos were transferred to 20 µL lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.3% Tween 20, 0.3% NP40. Fresh Proteinase K was added to a final concentration of 1 mg/mL on the day of use) and incubated at 55 °C for 16 hrs. Then the mixture was heated to 95 °C for 15 min to inactivate Proteinase K. Nested PCR was then carried out with zebrafish embryo lysates with the corresponding primers (**Table S1**).

Amplified PCR products were purified by a PCR Cleanup kit (Omega Biotek) and sent to Genewiz, USA for sequencing. TIDE analysis was carried out by an online tool (https://tide.deskgen.com/). Representative Sanger sequencing chromatograms and TIDE analyses results are shown in **Figures S11 and S13**.

Mammalian cell and zebrafish embryo viability analysis

To assess the influence of phototoxicity in mammalian cells, HEK293T cells were seeded equally (10^4 cells/well) in a 96-well plate. After cell confluency was reached (~90%), HEK293T cells were subjected to 365 nm irradiation with a UV transilluminator (VWR, Dual UV Transilluminator, 80 mW/cm^2) for 0, 1, 2, 3, 5, 10, 15, or 30 min and then treated with CellTiter-Glo reagent (Promega) according to the manufacturer's protocol. A TECAN INFINITE M1000 Pro microplate reader was used to detect the luminescence intensity for each well. Relative luminescence units (RLU) were measured and normalized to non-irradiated wells for the calculation of cell viability (**Figure S10**).

To analyze phototoxicity in zebrafish embryos, embryos were irradiated at 1 hpf with a handheld UV lamp (4 W, Analytik Jena, UVL-21) at 365 nm placed on top of a 35 mm petri dish containing the embryos suspended in E3 water for 5, 10, 20, or 30 min, then incubated at 28.5 °C until 48 hpf for imaging and scoring for toxicity. Unfertilized eggs were removed at 6 hpf (**Figure S14**).

Zebrafish embryo injection and irradiation

The zebrafish experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Embryos were collected after natural mating of AB* or *Tg(ubi:loxP-EGFP-loxP-mCherry)*[6] fish lines. This transgenic fish line was a kind gift from the Leonard Zon lab (Harvard Medical School). An injection solution of 10 µM EnGen® Spy Cas9 NLS (dual-NLS Cas9, NEB) and 10 µM gRNA were incubated for 20 minutes at 37 °C. Phenol red was added to a final concentration of 0.05% as a tracer for injection. EGFP-RNP (1 nL) or SLC24A5-RNP (2 nL) was injected underneath the cell within the yolk of the single-cell stage embryo using a World Precision Instruments Pneumatic PicoPump injector. Within the first hour after injection (one hour after fertilization), embryos were irradiated with 365 nm light using a handheld UV lamp (4 W, Analytik Jena, UVL-21) placed on top of a 35 mm petri dish containing the embryos suspended in E3 water. The embryos were then incubated at 28.5 °C, unfertilized embryos were removed at 6 hpf, then assessed for phenotype at 48 hpf. The embryos were imaged with a Leica M205 FA microscope with EGFP filter cube (ex: 470/40 nm, em: 525/50 nm) and the bright field channel (**Figures S12, S14, and S15**). Fluorescence intensity was determined using ImageJ software.

Supporting Figures

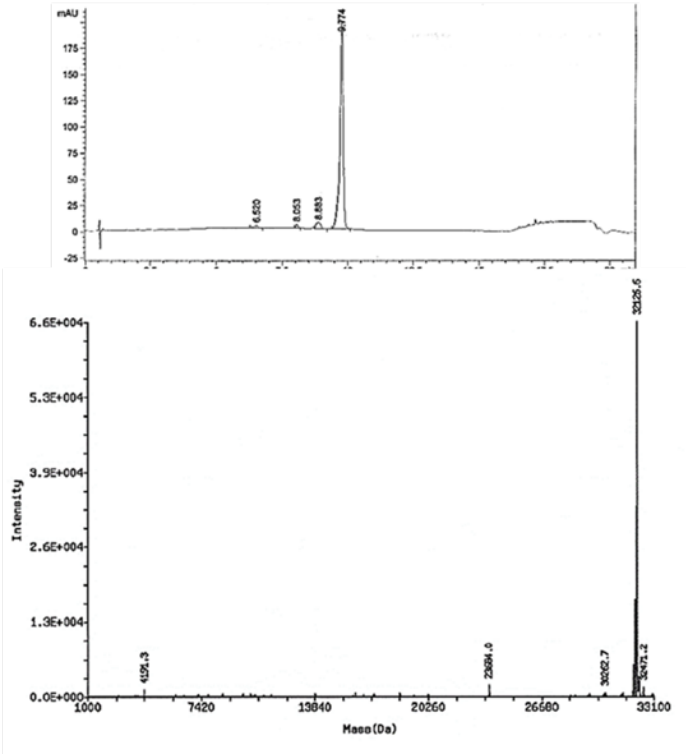

Figure S1. LC chromatogram and ESI-LCMS spectrum of DsRed-4U gRNA, which targets the pRG reporter plasmid in mammalian cells. Calculated mass: 32124 Da; observed mass: 32127 Da.

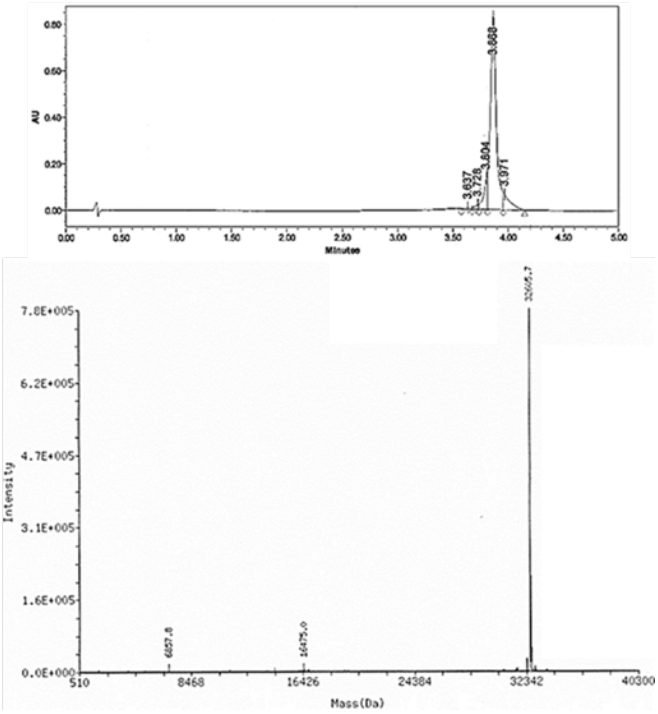

Figure S2. LC chromatogram and ESI-LCMS spectrum of CTNNb1-4G gRNA, which targets the *ctnnb1* gene in mammalian cells. Calculated mass: 32601 Da; observed mass: 32606 Da.

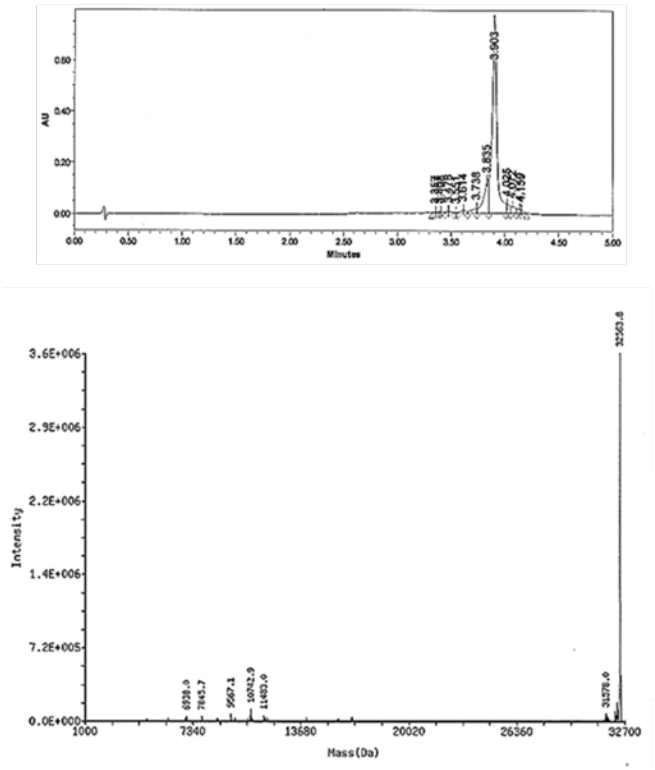

Figure S3. LC chromatogram and ESI-LCMS spectrum of EGFP-4U gRNA, which targets the *EGFP* gene in the *Tg(ubi:loxP-EGFP-loxP-mCherry)* transgenic zebrafish. Calculated mass: 32356 Da; observed mass: 32364 Da.

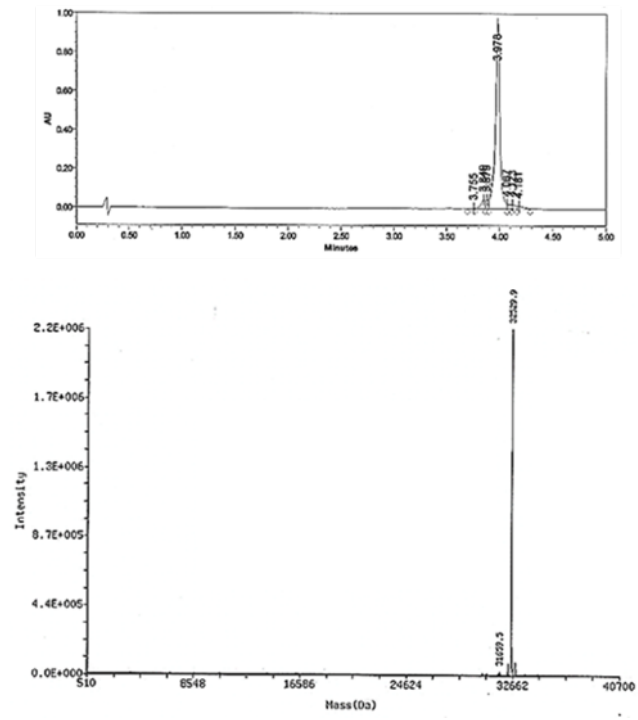

Figure S4. LC chromatogram and ESI-LCMS spectrum of SLC45A-4U gRNA, which targets the *slc24A5* gene in zebrafish. Calculated mass: 32524 Da; observed mass: 32530 Da.

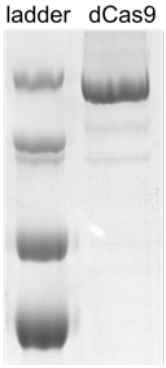

Figure S5. Coomassie-stained SDS-PAGE of dCas9-6xHis with Precision Plus Protein™ Dual Color Standards (Bio-Rad).

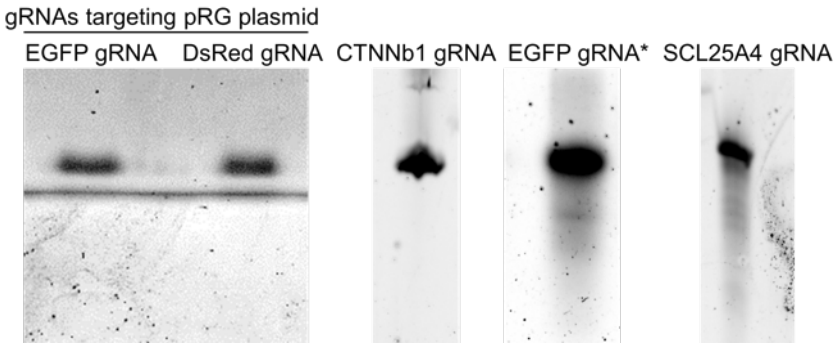

Figure S6. Denaturing 6 M urea TBE PAGE for all the *in vitro* transcribed, non-caged gRNAs (EGFP gRNA* targets the EGFP coding sequence in the *Tg(ubi:loxP-EGFP-loxP-mCherry)* transgenic zebrafish).

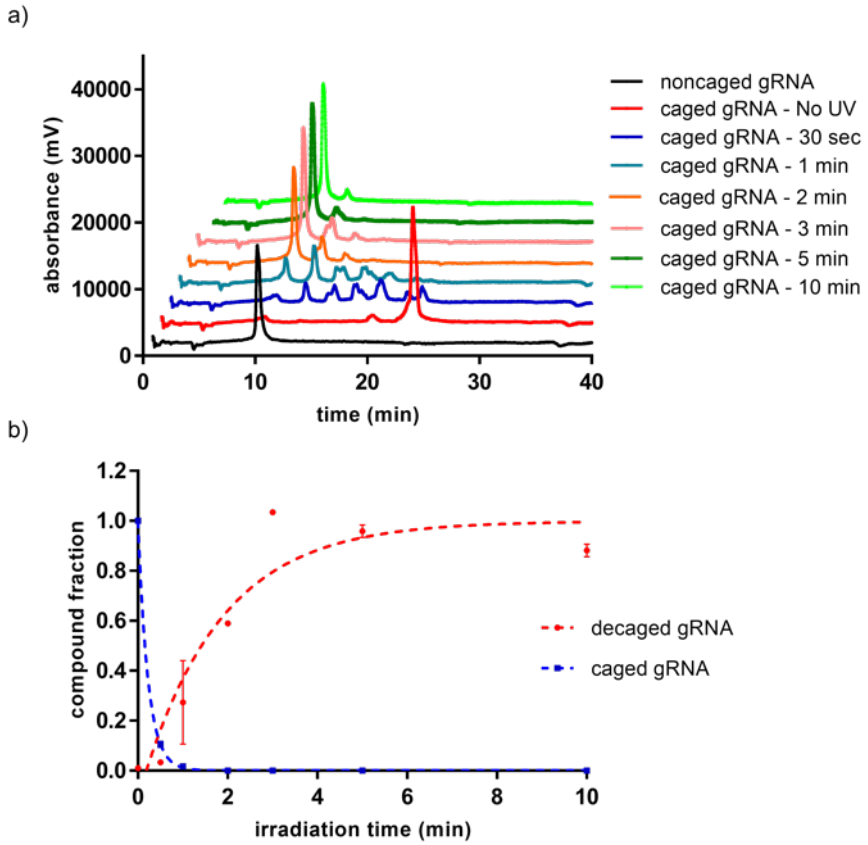

Figure S7. Decaging assay of a representative caged gRNA (DsRed-4U). a) HPLC chromatograms showing the decaging of DsRed-4U gRNA by different durations of 365 nm irradiation. b) Integration of the peaks correspondent to caged and decaged gRNA for each irradiation condition reveals that 3 min of irradiation by a UV transilluminator (VWR, 80 mW/cm^2) is enough for complete decaging of the DsRed-4U gRNA. Error bars are standard deviations from two independent decaging experiments.

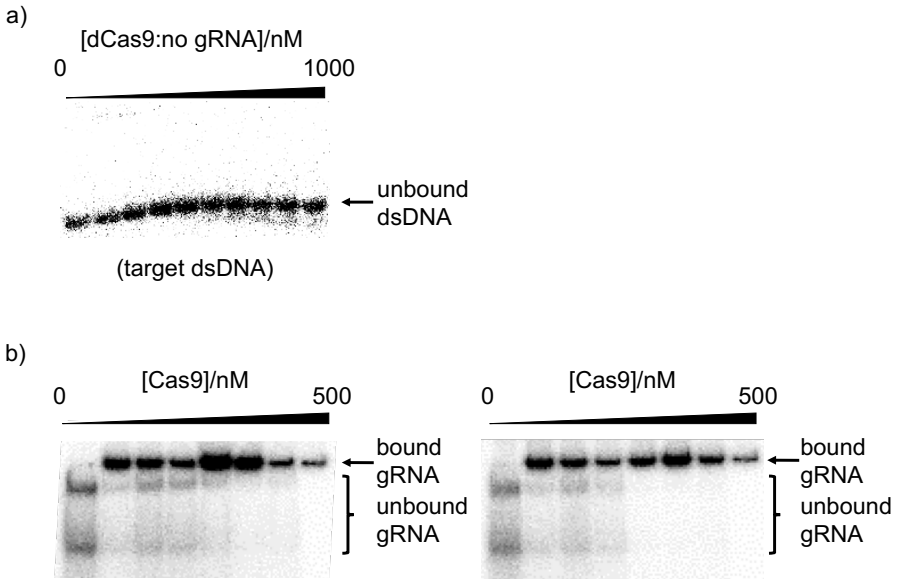

Figure S8. Gel shift assays of dCas9 with ^{32}P-dsDNA and Cas9 with ^{32}P-gRNA (5% native PAGE). a) dCas9 gel shift assay with target dsDNA (1 nM) in the absence of any gRNA. Non-specific binding was not observed. Concentrations of dCas9: 0, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 nM. b) Cas9 with ^{32}P-labeled non-caged DsRed gRNA (left) or DsRed-4U gRNA (right) shows that caging of the protospacer region of gRNA does not alter its binding to Cas9 (12% native PAGE). The concentrations of Cas9: 0, 5, 10, 20, 50, 100, 200, and 500 nM. Two bands for the unbound DsRed gRNA and DsRed-4U gRNA were observed due to their two folding states in the native PAGE.

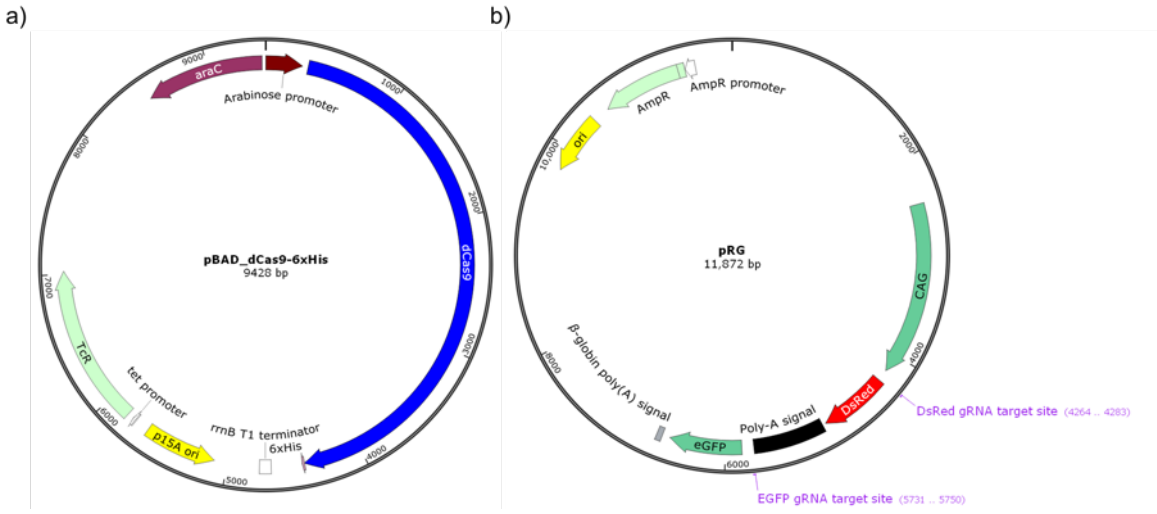

Figure S9. Maps of the plasmids used in this research (generated by SnapGene). a) Plasmid map for pBAD_dCas9-6xHis. b) Plasmid map for the pRG dual-fluorescence reporter plasmid.

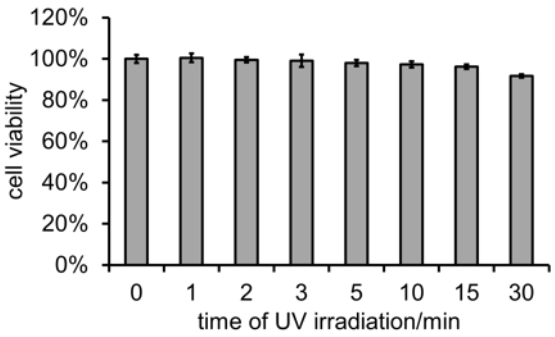

Figure S10. Cell viability assay of HEK293T cells after different durations of 365 nm light irradiation. Data was normalized to no UV irradiation and error bars were generated from three individual wells for each condition.

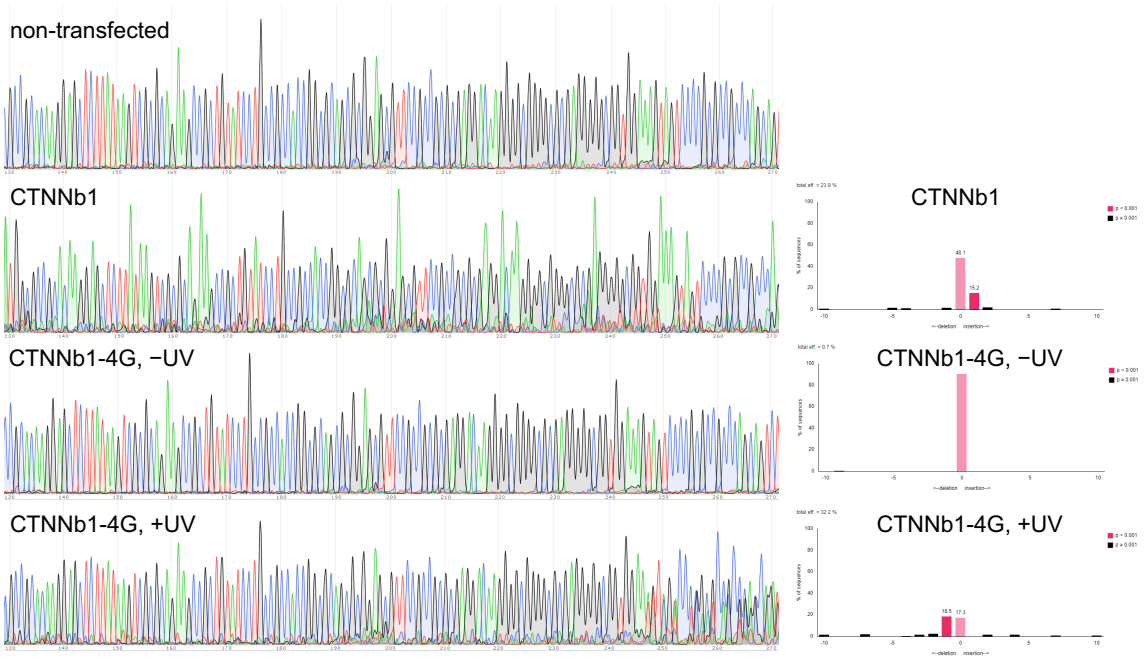

Figure S11. Sanger sequencing chromatograms (left) and TIDE analyses results (right) for genomic PCR products from non-transfected HEK293T cells as well as HEK293T cells transfected with non-caged CTNNb1 gRNA or caged CTNNb1 gRNA (−/+ UV) RNPs.

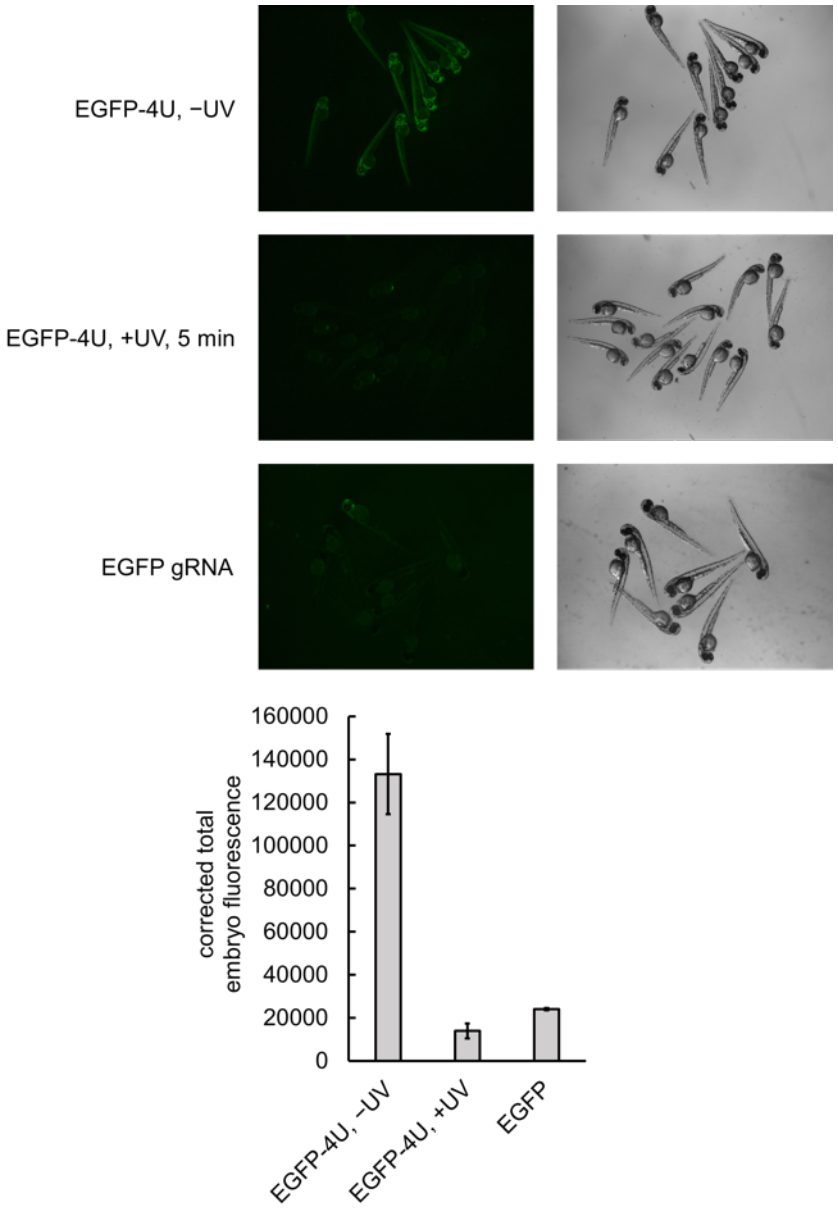

Figure S12. Micrographs of embryos injected with RNP targeting EGFP and irradiated one hour after fertilization, then imaged at 48 hpf. Fluorescence intensity in the EGFP channel of three embryos in each condition was calculated using ImageJ and is presented in the chart below. Bars represent the mean from measurement of three embryos and error bars represent standard deviation.

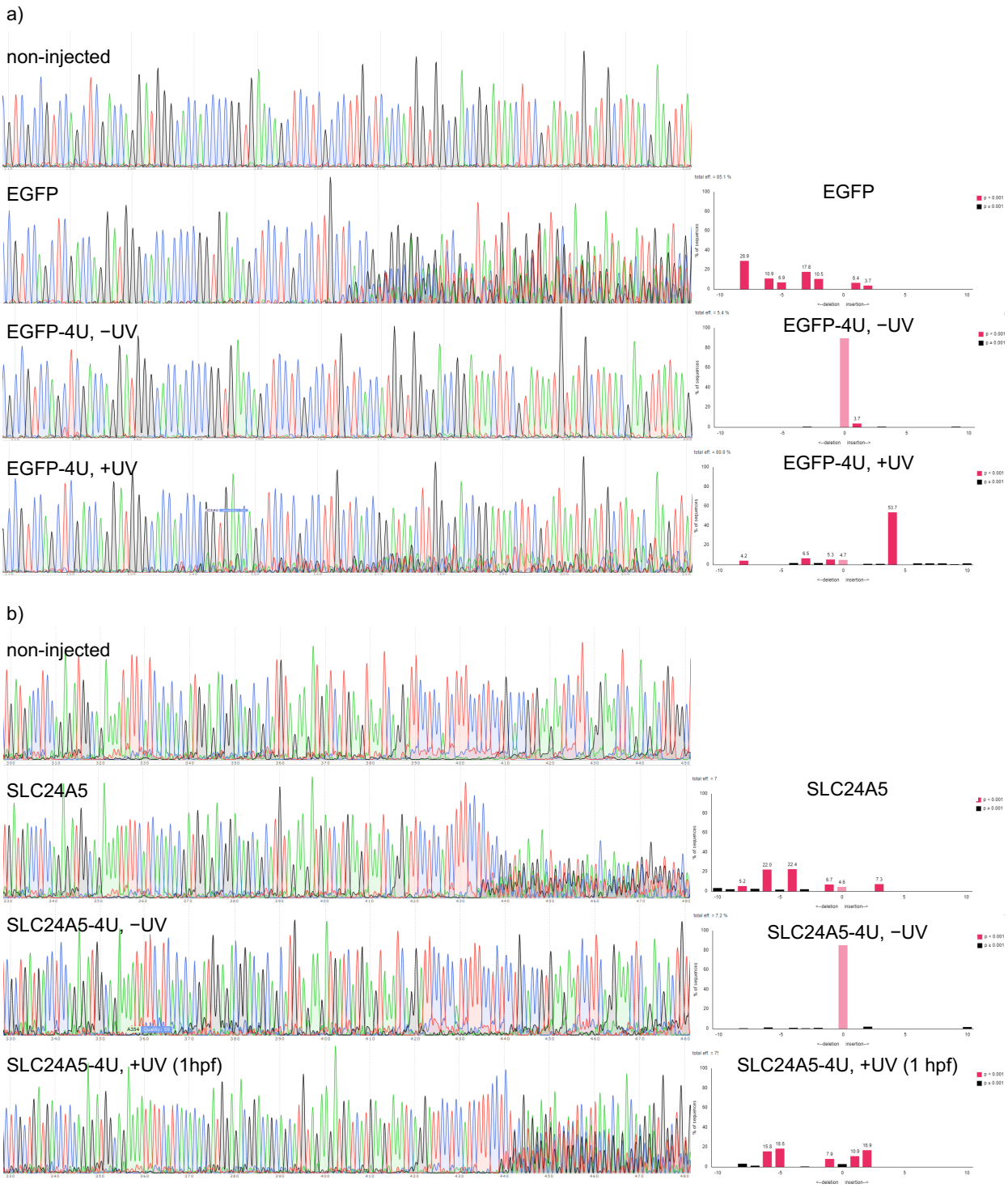

Figure S13. Sanger sequencing chromatograms (left) and TIDE analyses results (right) for zebrafish embryonic PCR products. a) Representative chromatograms and TIDE analysis results from the non-injected zebrafish embryo as well as embryos injected with non-caged EGFP gRNA or caged EGFP gRNA RNPs (−/+ 5 min UV at 1 hpf). b) Representative chromatograms and TIDE analysis results from the non-injected zebrafish embryo as well as embryos injected with non-caged SLC24A5 gRNA or caged SLC24A5 gRNA RNPs (−/+ 5 min UV at 1 hpf).

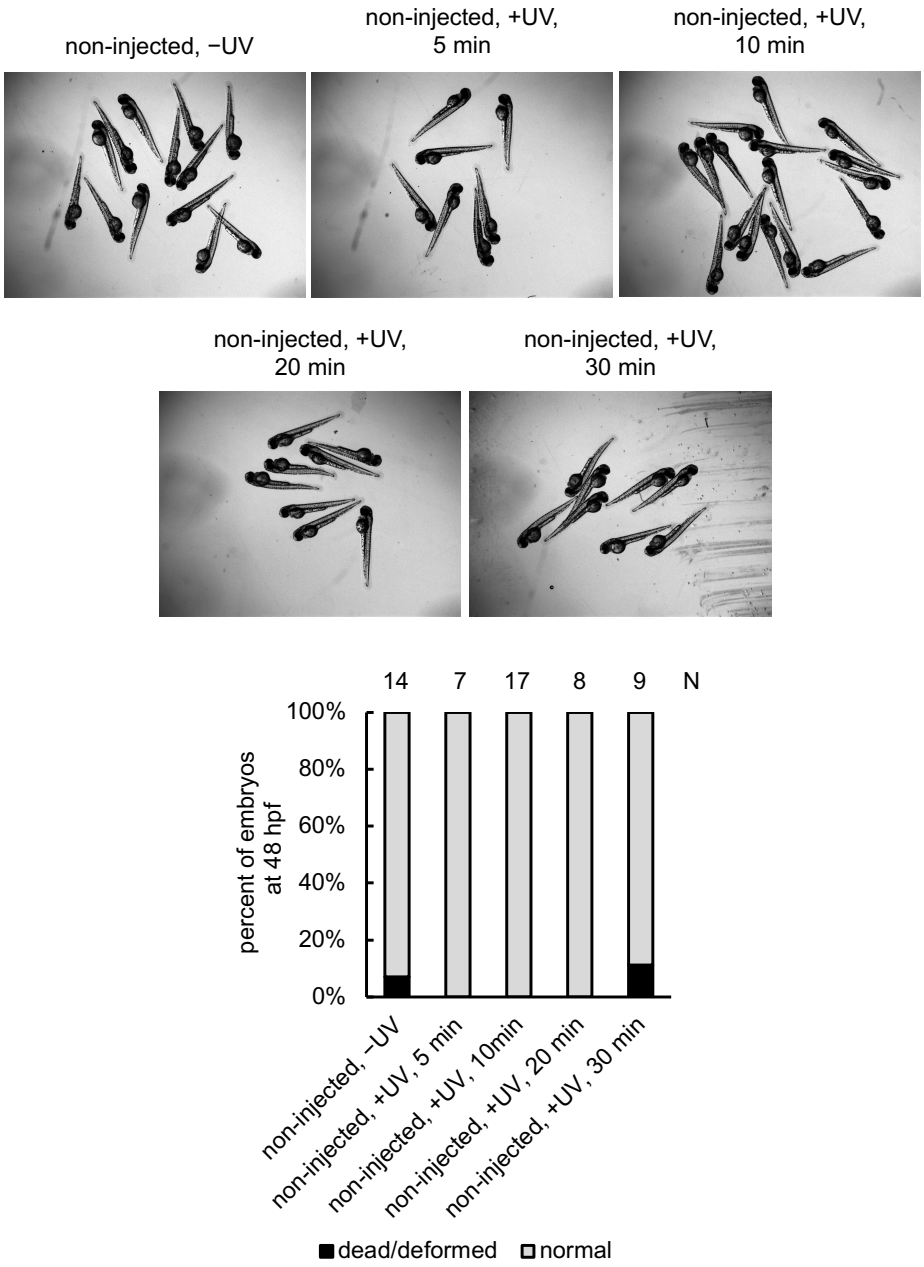

Figure S14. Non-injected embryos were irradiated with 365 nm light for 5, 10, 20, or 30 min at 1 hpf. Embryos were imaged at 48 hpf and analyzed for toxicity. Micrographs of the fish are shown, with a chart of phenotype frequencies underneath.

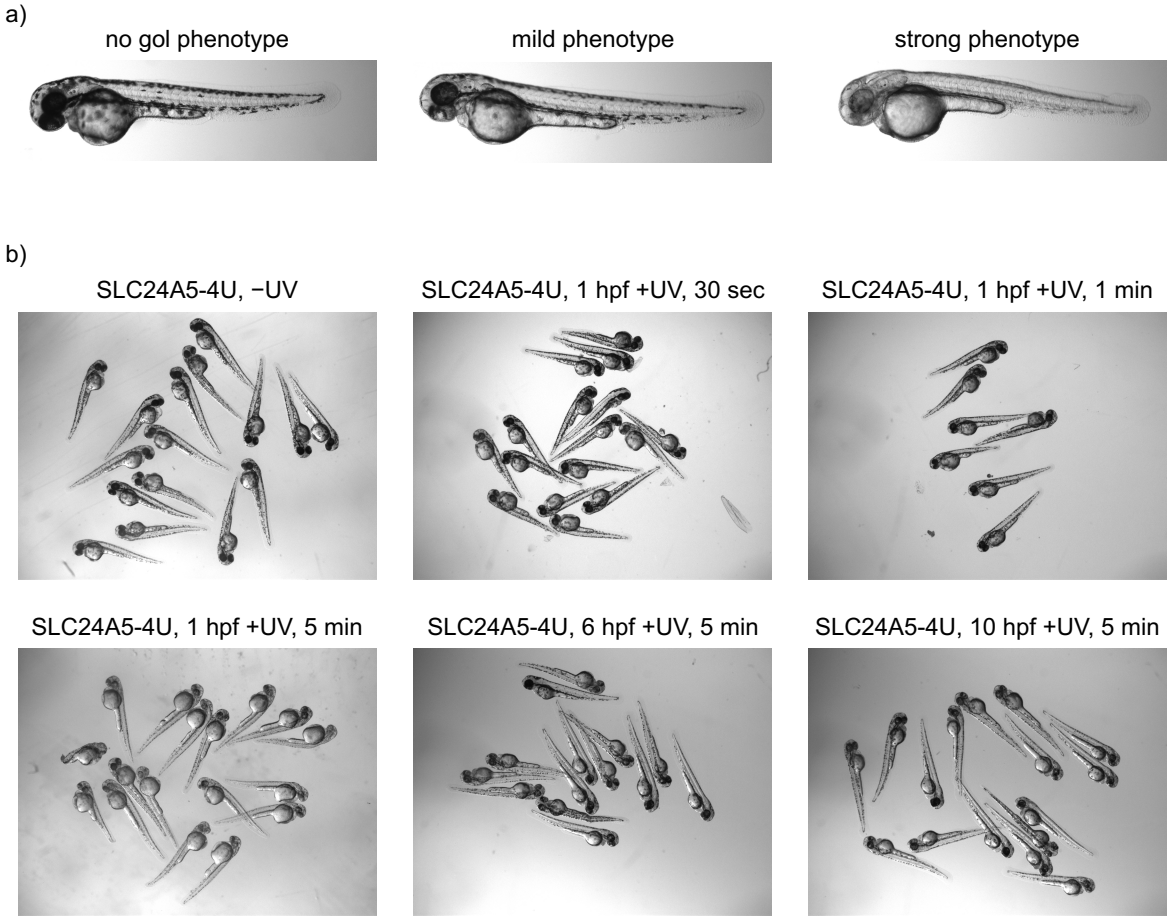

Figure S15. a) Representative whole-animal images of golden phenotypes. b) Images of all embryos injected with the caged RNP and treated with the indicated irradiation conditions.

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