CRISPRoff enables spatio-temporal control of CRISPR editing

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Following introduction of CRISPR-Cas9 components into a cell, genome editing occurs unabated until degradation of its component nucleic acids and proteins by cellular processes. This uncontrolled reaction can lead to unintended consequences including off-target editing and chromosomal translocations. To address this, we develop a method for light-induced degradation of sgRNA termed CRISPRoff. Here we show that light-induced inactivation of ribonucleoprotein attenuates genome editing within cells and allows for titratable levels of editing efficiency and spatial patterning via selective illumination.
CRISPR-Cas9 technology has rapidly revolutionized the genome editing field. However, once introduced into a cell the CRISPR ribonucleoproteins (RNP)s are uncontrollable, capable of forming double strand breaks (DSBs) at locations where the sgRNA binds within the genome. Previous attempts to control CRISPR systems inside the cell have approached this problem on a variety of levels, from controlled expression of integrated Cas9 and sgRNA, sgRNAs that are modulated in response to ligand binding, split Cas9 that dimerizes upon illumination with blue light, to anti-CRISPR proteins, a viral defense against the CRISPR immune system. However, each of these strategies requires an additional physical component of the CRISPR system beyond a typical RNP to be introduced into the cell. These additional engineering steps can also produce challenges to the production of purification of protein Streptococcus pyogenes Cas9 (SpCas9).

To address these challenges, we develop the CRISPRoff system, a synthetic sgRNA that fragments in response to light, preventing formation of new DSBs (Fig. 1a). Here we show that CRISPRoff is effective across multiple genomic targets in multiple cell lines and demonstrate two key uses of this platform: the ability to maximize off-target editing events and the ability to spatially pattern cells in vitro.

Results
CRISPRoff sgRNA synthesis and cleavage. CRISPRoff sgRNAs are chemically synthesized using solid phase synthesis and incorporate photodegradable residues containing a α-nitrobenzyl groups (Supplementary Fig. 1a) at defined positions. This α-nitrobenzyl group undergoes cleavage in response to UV light (Supplementary Fig. 1b), leaving a single phosphate group on the RNA fragment. To develop a universal system, we tested a variety of sgRNA molecules, in which nucleotides at various positions along the backbone have been replaced with photodegradable residues. (Supplementary Fig. 1c) Upon exposure to broad-spectrum light (80 mW cm^-2, Supplementary Fig. 1d), sgRNAs demonstrated fragmentation when analyzed on a fragment analyzer (Supplementary Fig. 1e). However, RNP complexes formed with some of these sgRNAs failed to initiate editing when demonstrated two key uses of this platform: the ability to maximize off-target editing events and the ability to spatially pattern cells in vitro.

CRISPRoff modulates genome editing events in human cells. After determining DBsgRNAs can be effectively cleaved, we next tested the ability of CRISPRoff to modulate genome editing events within human cells. Due to the presence of potentially damaging UVA and UVB wavelengths present within our light source (Supplementary Fig. 1d), we first demonstrated that using a 345 nm long-pass filter did not significantly affect the viability of transfected HEK293 or U2OS cells (Supplementary Fig. 2a) and was used in all subsequent experiments.

Four hours following delivery of RNP sformed with DBsgRNAs targeting DNMT1 into HEK293 cells, samples were illuminated for up to 60 s (Supplementary Fig. 2b) and allowed to recover for an additional 44 h. After harvesting the genomic DNA of these cells and analyzing amplified genomic target regions using Inference of CRISPR Edits (ICE) we found that the degree of editing was significantly reduced in light-exposed samples (Fig. 1e). As a control, we also transfected HEK293s with standard sgRNAs and illuminated samples following the same protocol. Editing in illuminated sgRNA RNPs populations was not significantly different than paired populations left in the dark. The similarity in overall editing efficiency following illumination of standard sgRNA RNPs suggests that DBsgRNAs were effectively cleaved within cells and no longer functional.

We further observed that illumination four hours post transfection retained a small portion of editing events, presumably from DSBs formed prior to external stimulus, including those that had not been repaired. We reasoned that editing levels within populations may be titratable by modulating when the post-transfection timepoint at which samples are irradiated. To test this, we transfected HEK293s with DBsgRNA targeting DNMT1 and illuminated a distinct cell sample, one time each, every two hours for two days. After 48 h, genomic DNA was isolated from all samples and analyzed for insertion/deletion mutations (indels). In alignment with our prediction, we were able to fine tune the level of gene editing within a population using DBsgRNAs (Fig. 1f).

CRISPRoff is effective across cell lines and gene targets. To test the universal effectiveness of the CRISPRoff system, we created a panel of standard sgRNAs and DBsgRNAs targeting a variety of chromosomes and local genomic contexts (Supplementary Table 1). Across all targets, all but two (CAMK1_sg2 and STK3_sg2) intact DBsgRNAs formed DSBs at a similar frequency as standard sgRNAs (p < 0.05, multiple independent t-test with FDR correction) (Fig. 2a–c) and generated a similar indel profile (Supplementary Fig. 2c). The majority of DBsgRNAs also showed a decrease in editing efficiency when illuminated four hours post transfection compared to cells from the same transfection that remained in the dark (Fig. 2a–c). Importantly, irradiation did not decrease editing efficiency of standard sgRNAs suggesting incorporation of photodegradable linkers was wholly responsible for the decrease in efficiency (Fig. 2a–c, right). We also observed that some targets were inactivated to a lesser degree than others, and hypothesized that the decrease in efficiency could be based on the individual editing kinetics at each site.
To test this hypothesis, we identified one sgRNA, FANCF, that does not appear to be inactivated. To confirm that editing at this site can be controlled using CRISPRoff, we ran a high-resolution test of genome editing events where cells transfected with either standard or DBsgRNA RNP were illuminated 15 min post transfection and harvested 15, 30, 60, 90, 120 min as well as longer time points at 4 and 24 h post transfection. In line with our hypothesis, illumination of DBsgRNAs 15 min post transfection completely ablated editing at this site (Supplementary Fig. 2d). Interestingly, at four hours post transfection we observed that nearly 50% of alleles in the standard sgRNA transfection had already experienced a DSB that was repaired through NHEJ, confirming our hypothesis that the editing kinetics at this site is very fast.
CRISPRoff can optimize on/off-target editing events. Within our panel, we included an sgRNA known to be cytotoxic due to having an off-target site in an essential gene. Interestingly, when DBsgRNAs of this guide was used in conjunction with irradiation, a greater proportion of cells survived (Supplementary Fig. 2e), potentially due to an increase in the ratio of on/off-target events, while maintaining editing efficiency. With this in mind, we created sgRNAs that had significant levels of off-target editing at one or two sites within the genome (Supplementary Table 4). Based on previous studies, editing at off-target sites may be slower than editing at the on-target sites18,19, and depend on RNP concentration within the cell20,21. We rationalized that we may be able to maximize the ratio between on/off-target editing (Supplementary Fig. 3a) by illuminating DBsgRNAs at an optimal time point post transfection. We transfected independent pools of cells with 7 unique sgRNAs and exposed the pools to light at 4, 8, 16, 24, or 48 h post transfection. We also harvested genomic DNA from each of these pools at the indicated time point to form a longitudinal editing curve. Following illumination, the degree of editing at many off-target sites plateaued, demonstrating that inactivating DBsgRNAs slowed down off-target editing (Supplementary Fig. 3b). By illuminating DBsgRNAs at discrete times post transfection we found we were able to modulate and maximize the on/off-target cutting ratios (Fig. 3a).

CRISPRoff enables precise spatial patterning. One of the major advantages of using optical as opposed to chemical stimulus is the ability to obtain precise spatial control. This ability enables researchers to study complicated signaling effects such as paracrine vs juxtracrine signaling within a single well or better understand the role of specific genes during differentiation or organoid formation. Further uses in vivo could also help understand the effects of gene knockout in a developing embryo at a 2- or 4-cell state by laser illumination22. As a proof-of-concept, we obtained a GFP-expressing cell line23 and designed sgRNAs to create GFP knockout phenotypes. We used a standard inverted fluorescent microscope which could illuminate a single well at a time. This fluorescent microscope setup contained a 385 nm LED commonly used for illumination, and that is right on the edge of the reactivity of the PC microscope setup. Within this setup, our microscope was able to maximize the on/off-target editing at many off-target sites plateaued, demonstrating that inactivating DBsgRNAs slowed down off-target editing (Supplementary Fig. 3a). By illuminating DBsgRNAs at discrete times post transfection we found we were able to modulate and maximize the on/off-target cutting ratios (Fig. 3a).

![Fig. 2 CRISPRoff sgRNAs are effective across cell lines and genomic targets. a–c Panel of DBsgRNAs compared to standard sgRNAs in (a) HEK293 b U2OS, and c Hep3B cells. When left unstimulated, DBsgRNAs (blue, left) formed indels at rates similar to standard sgRNAs (black, right). In the cells exposed to light 4 h post transfection, editing was ablated for DBsgRNAs (green) but was unchanged using standard sgRNAs (gray) (n = 3 paired experimental replicates, data are presented as mean ±1 SD). Source data are provided as a Source data file.](https://doi.org/10.1038/s41467-020-18853-3)
selectively masking the bottom of the well, we created distinct spatial patterns by knocking out GFP in defined regions. (Fig. 3d).

**Discussion**

Taken together, CRISPRoff allows for tight control of editing from both a spatial and temporal perspective, expanding the toolbox of optogenetic gene editing. We have successfully demonstrated this technology in multiple human cell lines across multiple genomic loci and expect this technology to be turn-key ready with any CRIPSR-based application currently using synthetic or to replace in vitro transcribed sgRNAs. While, at the moment, CRISPRoff is limited in in vivo applications, due to the
low penetrance of UV light through tissues, we are excited by the possibility of further chemistries extending the range of photo- cleavable molecules such as with two-photon cleavage systems. Further, because CRISPRoff makes modifications to the backbone of the sgRNA, it can be compatible with other technologies, such as sgRNA modifications to activate gene editing, or Cas9 modifications to enhance on-target specificity. We anticipate that the CRISPRoff system will be a valuable tool for both in vitro and in vivo control of CRISPR technologies.

**Methods**

**RNA synthesis.** RNA oligonucleotides were synthesized on Synthego solid-phase synthesis platform, using CPG solid support containing a universal linker. 5'-Benzylthio-1H-tetrazole (RTT, 0.25 M solution in acetonitrile) was used for coupling. 3-((Dimethylamino-methylidene)amino)-1H-1,2,4-dithiazole-3-thione (DDTT, 0.1 M solution in pyridine) was used for thiolation, dichloroacetic acid (3-((Dimethylamino-methylidene)amino)-1H-1,2,4-dithiazole-3-thione (DDTT, 0.1 M solution in pyridine) was used for thiolation, dichloroacetic acid (DCA, 3% solution in toluene) for use for deprotection. After synthesis, oligonucleotides were subject to series of deprotection steps, followed by purification by solid phase extraction (SPE). Purified oligonucleotides were analyzed by ESI-MS. All materials for RNA synthesis were obtained from either ChemGenes or Thermo Fisher Scientific.

**CRISPRoff sgRNAs were made with PC Linker phosphoramidite, which was obtained from Glencore (10–4920).**

**Cell culture.** Human embryonic kidney cells (HEK293) and Hep3B were maintained between passage 5–20 in Advanced Modified Eagles Medium (Life Technologies) and 10% v/v FBS. Cells were passaged biweekly at a 1:8 ratio with TrypLE (Life Technologies).

**Electrospray ionization.** RNA samples in TE buffer (3 μM) were analyzed by mass spectrometry (Agilent 1290 Infinity II liquid chromatography system (LC) coupled with Agilent 6530 Q-TOF mass spectrometer (MS)) in a negative ion polarity mode. LC is performed with gradient elution (buffer A: 50 mM HFIP; 15 mM aqueous ammonium acetate, pH 4.5) in a negative ion polarity on an Agilent 1290 InertSustain C18 column (1.8 μm, 2.1 × 5 mm). Electrospray ionization performed with a dual ESI source (gas temp 325 °C, drying gas flow 2 L/min, nebulizer gas 7 psi) and a 3.5 kV spray voltage. The LC is coupled to an Agilent 6530 Q-TOF mass spectrometer (MS) in a negative ion polarity mode. The MS is operated in data-dependent mode, subjecting the MS to full scan followed by MS-MS using a SRM ion of m/z 200-3200 m/z range and deconvoluted in 4000-40000 m/z range.

**Fragment analysis.** Fragment analysis was done using a 5200 Fragment Analyzer System (Agilent) according to manufacturer protocol. DNA analysis was done using DNA small fragment kit (Agilent DNA 470) while RNA was analyzed using the small RNA kit (Agilent DNA 470).

**RNP formation and delivery.** 10 pmol Streptococcus Pyogenes [SV40 NLS]-[SpCas9]-[SV40 NLS] protein (Adevir Cat. #9212) was combined with 30 pmol synthetic sgRNAs (Synthego) in 20 μL total volume and allowed to complex for 10 min. During this incubation, cells were harvested and counted. To the RNP solution 5 μL of cell solution at a concentration of 4 × 10^5 cells/μL was added and gently mixed.

**Cell–RNP solution was transfected using the 4D-Nucleofector system (Lonza) in the 20 μl format. HEK293 transfections were conducted in SF buffer using protocol CM-130. U2OS and Hep3B transfections were conducted in SE buffer using protocol CM-104 and CM-130, respectively. Following transfection, cells were resuspended in culture media and plated into 96-well plates. To create paired replicates, transfactions were split into two pools. One that received light treatment while the other remained in the dark.**

**DBsgRNA inactivation.** CRISPRoff inactivation was performed using a Sunray 600 UV Flood Lamp (Uvitrion International). 345 nm, 6.5° × 6.5° colored glass alternative longpass filters were obtained from Newport.com and mounted using custom 3D-printed containers. Inactivation using an upright microscope was performed using a Zeiss Axios Observer with a Colibri 7 Flexible Light Source and 385 nm LED. Imaging was preformed using a 4x objective lens.

**Genomic analysis.** Genomic DNA was isolated using DNA QuickExtract (Lucigen) following manufacturer protocol. After harvesting, extract solution was incubated at 65 °C for 15 min, 68 °C for 15 min followed by 98 °C for 10 min. Genomic PCR was performed using AmpliTaq Gold 360 Master Mix (Thermo Fisher) using primer sequences found in Supplementary Table 2. Following Sanger sequencing, presence of indels was analyzed via ICE (ice.synthego.com). Raw traces are also available at Zenodo 4009447.

**Digital droplet PCR.** Cellular RNA was extracted using RNA QuickExtract (Lucigen) without DNase. RNA was quantified using RiboGreen (Thermo Fisher) and normalized. Total RNA was reverse transcribed using iScript Advanced cDNA Synthesis Kit (BioRad) with 0.4 μM reverse primer for transcription. Reverse transcription product was amplified using 2x EvaGreen dPCR Mastermix and thermal cycled at 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s and 52.4 °C for 1 min. Signal was then stabilized at 4 °C for 5 min followed by inactivation at 90 °C for 5 min. Droplets were then read by qX200 Droplet Digital PCR System and analyzed with QuantaSoft V1.7 (BioRad).

**Statistics.** All error bars are shown as ±1 SD. p values were computed using Student’s two-tailed t test or one-way ANOVA and deemed significant at α < 0.05. Data was analyzed using Prism 8.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Sanger sequencing data supporting this work are available at Zenodo with accession code 409447. All additional data is also available upon reasonable request. Source data are provided with this paper.

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Author contributions

J.C-S, R.K., I.W., T.M. conceived the study, J.C-S., A.K., and S.I. designed and conducted experiments. N.R. performed bioinformatic analysis. J.C-S. wrote the paper with input from all authors.

Competing interests

All authors declare a potential conflict of interest as employees or stockholders of Synthego Corporation. J.C-S., R.K., A.K., I.W., and T.M. have authored patent application PCT/US2020/015127 regarding CRISPR off sgRNAs which has been filed by Synthego.

Additional information

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