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Development of Light-Activated CRISPR Using Guide RNAs with Photocleavable Protectors

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Abstract: *The ability to remotely trigger CRISPR/Cas9 activity would enable new strategies to study cellular events with greater precision and complexity. In this work, we have developed a method to photocage the activity of the guide RNA called "CRISPR-plus" (CRISPR-precise light-mediated unveiling of sgRNAs). The photoactivation capability of our CRISPR-plus method is compatible with the simultaneous targeting of multiple DNA sequences and supports numerous modifications that can enable guide RNA labeling for use in imaging and mechanistic investigations.*

The RNA-guided CRISPR/Cas9 system is a genome-editing technology with broad biological and therapeutic applications.[1,2] The field's enthusiasm for the potential of this approach has led to a rapidly expanding toolbox,[3,4] which includes an approach for site-specific single-gene editing using photoactivatable CRISPR with a modified Cas9 enzyme that incorporates light-responsive domains or site-specific caging groups.[5–8]

The majority of the light-activated approaches depend on modifications of the Cas9 enzyme, whereas recent efforts have modified the single chimeric guide RNA (sgRNA) as an alternative approach to genome editing.[9,10] In line with this shift, our method, which is called "CRISPR-plus" (CRISPR-precise light-mediated unveiling of sgRNAs), incorporates photocleavable oligonucleotides that complement target regions of the sgRNA in the absence of Cas9 modifications.

Whereas photocleavable nucleotides have been used in other biological systems,[11–15] we believe that our report constitutes the first use of photactivatable oligonucleotides in CRISPR activation. While other approaches genetically modify Cas9,[5–8] our approach does not require any engineering of target cells. Also, these commercially available oligonucleotides are simple to design, chemically synthesize, modify, functionalize, purify, and characterize. Therefore, the photoactivation capability of CRISPR-plus affords simple and convenient control over editing within a genetic sequence, enables indirect and transient labeling of sgRNAs, and can multiplex different sgRNAs. We predict that these features will permit greater mechanistic and causal testing of gene functions and roles in a wide range of cellular systems.

To establish CRISPR-plus, we designed complementary ssDNA oligonucleotides (commercially available from Gene Link, Inc.) or "protectors", of varying lengths and positions along the target region of the guide RNA, and containing photocleavable groups. When the protectors hybridize to the target region of an sgRNA, the resulting complex has a high melting temperature (T_m). The presence of the hybridized protector thus prevents the sgRNA from binding to the target DNA until the protector is photolyzed, releasing it from the sgRNA (Figure 1a; see also Supporting Information, Figure S2 and Table S1c,d). Upon photolysis, the short fragments of the cleaved protector oligonucleotides will have a reduced binding affinity for sgRNAs owing to their lower T_m, rendering the target DNA susceptible to Cas9-mediated cleavage. We tested a range of protectors designed to be complementary to the target regions (T) of sgRNA that contain photocleavable (PC) groups (PCT1–PCT5) spaced six nucleotides (6-nt) apart, as well as corresponding non-photocleavable control protectors (T1–T5) that do not contain PC groups, using an in vitro DNA cleavage assay to determine the efficiency with which they block sgRNAs that target a GFP DNA sequence (Figure 1b and Table S1c,d). In the absence of light, both the PC-containing p-sgRNA (protected guide RNA) and the non-PC control protectors, placed near the 5′-end (T3–T5), eliminated virtually all Cas9-mediated cleavage of GFP target DNA, even at the lowest concentration tested (Figure 1b–d and Figure S3).

However, after only 2–5 s of light exposure (equivalent to 0.4–1.0 J cm^{-2}, using an OmniCure S2000, 365 nm filter, 200 mW cm^{-2}), significant photolysis-mediated cleavage of

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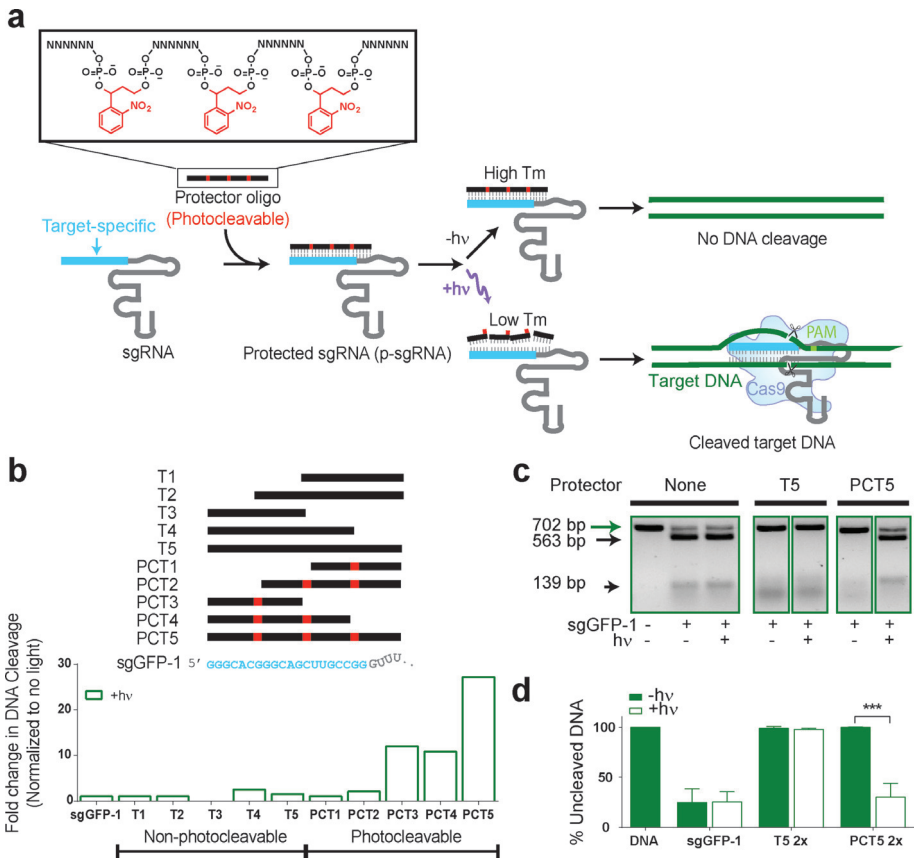

Figure 1. CRISPR-plus achieves photoactivatable blockade of Cas9-mediated DNA targeting. a) The CRISPR-plus concept. b) Fold increase in DNA cleavage following light exposure ($\lambda = 365$ nm, 6.0 J cm^{-2}) with complementary sgGFP1 protectors of different lengths and positions that contain photocleavable (PC) groups spaced 6-nt apart (PCT1-PCT5) or protectors without PC groups (T1–T5), tested using an in vitro cleavage assay ($n = 2$). c) Representative gel electrophoresis data from the in vitro cleavage assay for a 2:1 ratio of protector PCT5/sgRNA. The 702 bp target GFP DNA was cut to yield 563 bp and 139 bp fragments in the presence of sgRNA, but only after p-sgRNA photolysis ($\lambda = 365$ nm, 6.0 J cm^{-2}). All presented samples were run on a single physical gel, and cropped images of marked lanes are shown to streamline figure presentation. Lanes in which 1x concentrations of protectors were added are shown in Figure S3. d) Quantification of replicate in vitro assays from (c), where data is expressed as a fraction of uncleaved DNA, as calculated based on band intensities. Data were normalized to control untreated DNA, and mean values with standard deviation are plotted ($n = 3$). An unpaired Student's t-test was performed for sgRNA with PCT5 in the presence or absence of light exposure, and p values are represented by asterisks, ***$p < 0.001$.

target DNA was observed when using several of the PC-containing protectors, whereas the non-PC protectors retained their complete blocking efficiency even under light irradiation (Figure S4). As an important control, we also confirmed both the purity and photolability of the protectors alone (without sgRNA) using denaturing PAGE gels and/or HPLC (Figure S5). Based on the positioning and length of the most efficient CRISPR-plus protectors for the GFP target sequence (Figure 1 b–d and Figure S3), we designed and tested protectors for additional GFP target regions as well as two endogenous genes, CD71 and CD33 (Figure 2 and Figure S6). Consistent with our initial findings, all six PC protectors afforded protection from DNA cleavage, which was lost after exposure to light. These results suggest applicability to other genomic targets.

The CRISPR-plus method intentionally targets sgRNA to provide distinct advantages over other light-inducible Cas9 methods, yet in doing so, it is important to confirm that CRISPR-plus retains the capacity to target multiple guide RNAs simultaneously.[5–8] To assay for potential multiplex capacity, we performed a series of in vitro cleavage assays in which we combined one, two, or all three PC protectors in the presence of GFP, CD33, and CD71 target DNA and their corresponding sgRNAs (Figure 3; see also Figures S7 and S8). We observed that the light-activated target DNA cleavage response was specific to the presence or absence of the photoactivatable protector sgRNA complexes, even in the context of mixed targets and mixed sgRNA sequences (Figure 3; see also Figures S7 and S8). Notably, we only observed target DNA cleavage after its cognate sgRNA had been unveiled with light-mediated disruption of the protectors. These results suggest that by virtue of its photoactivation capacity, CRIPSR-plus can enable simultaneous, synchronized gene editing of multiple targets.

Having established that photolabile protection is mediated by PC-containing protectors as anticipated in the in vitro cleavage assay, we sought to determine whether this capacity is maintained in a more complex cellular environment. To this end, we generated a Cas9/destabilized GFP (Cas9/d2eGFP) co-expressing reporter line using HeLa cells and tested the efficiency of previously screened ssDNA protectors designed to be complementary to GFP-targeting guide RNA (sgGFP1). To quantify CRISPR activity, we performed FACS analysis of Cas9/d2eGFP HeLa cells to measure GFP protein expression and, as anticipated, observed an increase in the frequency of GFP-negative cells upon addition of sgGFP RNA. We observed a reduced efficiency of CRISPR-blocking activity in cells with shorter protectors (12-nt and 18-nt) relative to their inhibition of Cas9-mediated DNA cleavage in the in vitro cleavage assay, which is possibly due to easier dissociation of shorter protectors inside cells (data not shown). However, when longer ssDNA protectors (24-nt) containing PC groups were used in the cell-based assay, the CRISPR-plus system yielded a population change that was conditionally blocked in the presence of PCT5 p-sgGFP prior to exposure to UV light (Figure 4 a,b and Figure S9). To further support that the light-mediated loss of p-sgRNA-dependent protection of target DNA sequences is mediated

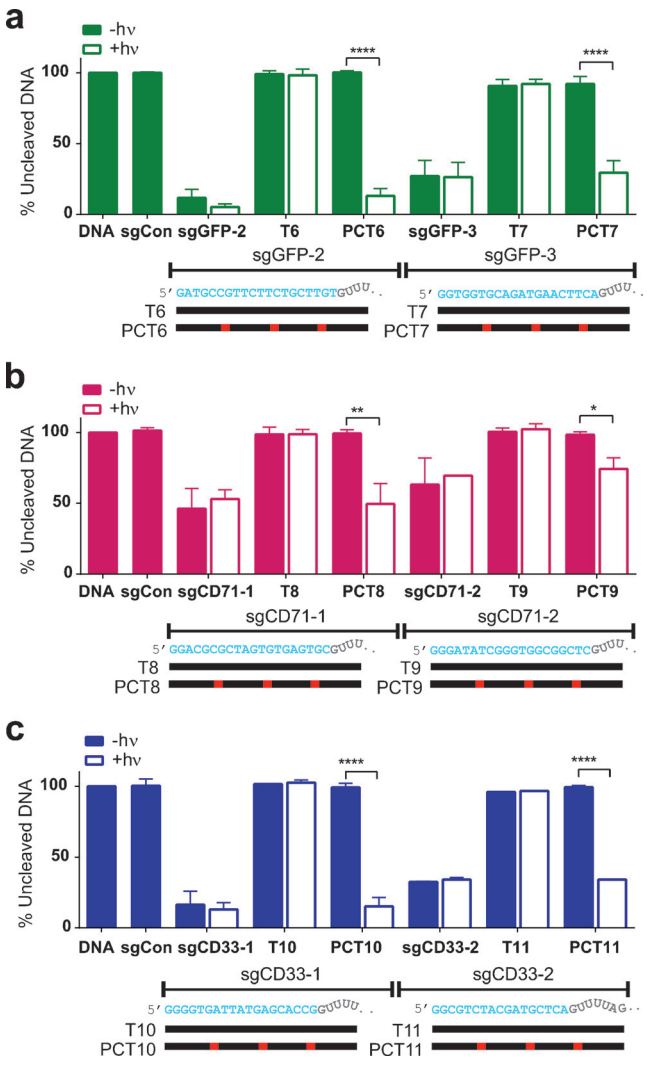

Figure 2. Validation of optimized 24-nt target-specific protectors with other sgRNAs and targets in an in vitro DNA cleavage assay. a–c) PC-containing (PCT6–PCT11) or non-PC (T6–T11) oligonucleotides complementary to six additional sgRNAs targeting three different DNA targets, namely a) GFP (green), b) CD71 (magenta), and c) CD33 (blue), in the absence or presence of light ($\lambda = 365$ nm, 6.0 J cm^{-2}). The percentage of uncleaved DNA was calculated from the band intensities of the gels (Figure S6). Data were normalized to the cleavage of control untreated DNA. Mean values with standard deviation are plotted for multiple repeat experiments (sgGFP-2, sgGFP-3, and sgCD71-1: $n = 4$; sgCD71-2 and sgCD33-1: $n = 2$; sgCD33-3: $n = 2$, with T11: $n = 1$). An unpaired Student's t-test was performed between irradiated and non-irradiated samples, as described in the data analysis section in the Supporting Information, and p values are represented by asterisks, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

by genomic DNA cleavage, we performed a SURVEYOR nuclease assay on intact Cas9/d2eGFP HeLa cells and measured the percentage of indels (insertion/deletion mutations) according to published methods.[16] We observed light-insensitive indel formation with the transfection of GFP- or CD71-targeted sgRNA, whereas the inclusion of the appropriate PC protector RNA for either target reduced indel formation. Furthermore, this protection from Cas9-mediated cleavage was diminished after exposure to UV light (4.0 J cm^{-2} at 365 nm, generated using a CL-1000 UV cross-linker UVP light source with a power density of 4.45 mW cm^{-2}, as measured by an OAI 306 UV power meter) for both GFP and CD71 (Figure 4c,d). It has previously been confirmed in multiple studies that a single exposure to 365 nm UV irradiation of up to 5.0 J cm^{-2} is non-photogenotoxic in the HaCaT (human keratinocyte) cell line,[17] and such radiation has also been used in different tumor models in vivo,[14,18,19] which is consistent with the lack of overt acute photocytotoxicity observed in our approach (Figure S10).

We developed CRISPR-plus as a modular approach that employs a photocleavable complementary oligonucleotide against the target region of sgRNAs to achieve inducible, target-specific editing of any gene(s) of interest. This light-dependent approach allows for the simultaneous targeting of multiple sequences and offers the possibility of achieving temporal precision in the activation of sgRNAs. Using the CRISPR-plus method, we achieved the targeted cleavage of the PCR products of three genomic sequences, including two genes relevant to multiple myeloma and acute myeloid leukemia development.[20–22] This removable protector approach can be immediately extended to numerous Cas nuclease and sgRNA variants with other effector functions.[9,23–29]

Recently, Deiters and co-workers reported a caged Cas9 approach that achieved a robust off/on switch for multiple sgRNAs in cells, minimal leakage of Cas9 activity in the absence of light, and robust recovery of Cas9 activity in a subset of sgRNAs after light exposure.[7] In comparison, our system also showed a robust off/on switch in an in vitro cleavage assay, using multiple guide RNAs individually and in combination, but our dynamic range was lower when tested in cells. We did observe some light-independent cleavage activity after day 5 (data not shown), which is possibly due to complex dissociation inside cells. Despite this time-dependent leakage of activity, an important distinguishing factor is that our approach is based on sgRNA modifications, and thus it opens up new possibilities for modifying, controlling, and improving CRISPR activity by non-genetic methods.

In an attempt to improve the dynamic range and to test whether protectors can tolerate modifications that may enable future functionalization, we 1) changed the backbone of the protectors to RNA or 2′-OMe RNA, 2) decreased the number of PC groups on the ssDNA protector, and 3) modified the 3′-end of ssDNA protectors to sterically block the 5′-end of sgRNA. Each of these modifications was tested in cells, with or without pre-irradiation (Figure S11). In the first case, despite achieving a more stable RNA/sgRNA duplex, the RNA protectors showed very poor blocking of CRISPR activity (Figure S11), whereas 2′-OMe RNA protectors performed similarly to DNA protectors. Second, decreasing the number of PC groups in a 24-nt protector resulted in a comparable blocking of activity in the absence of light, but activation of the CRISPR activity in the presence of light was not as robust (slightly reduced), which is likely due to the higher residual binding of the 8-nt protector fragments compared to 6-nt spaced PC groups. Finally, the addition of a FAM dye to the 3′-end of a ssDNA protector yielded strong

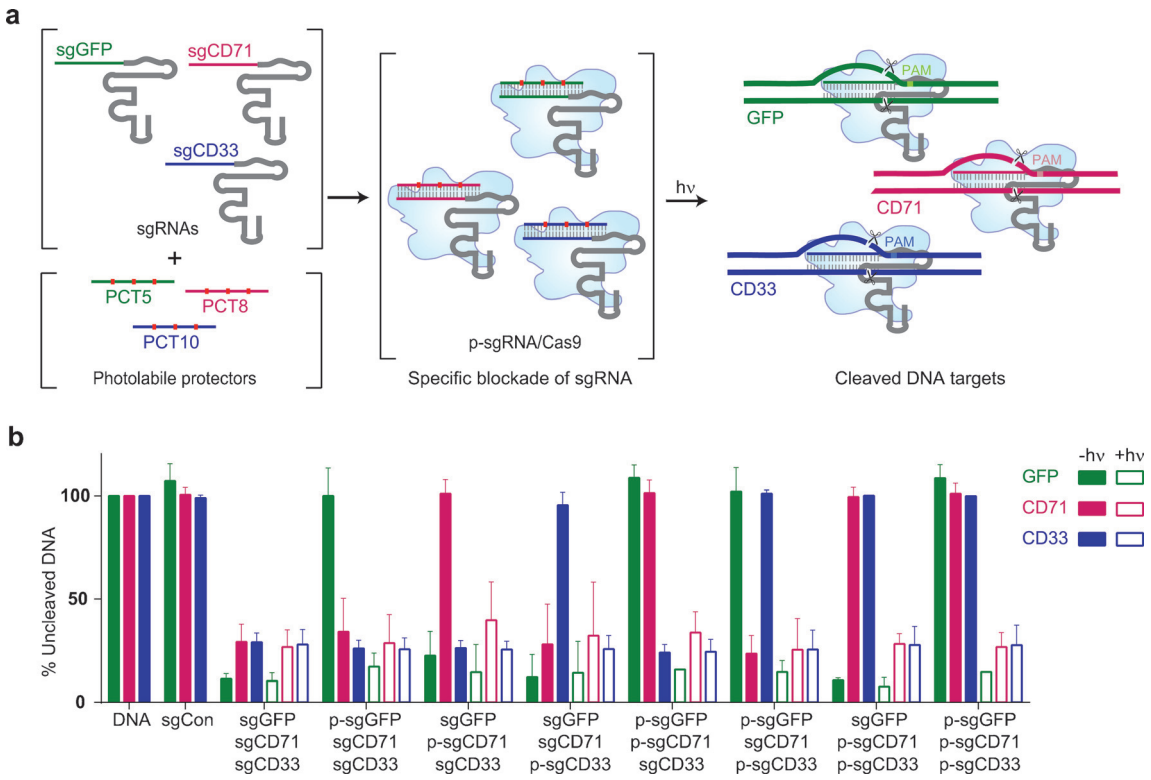

Figure 3. CRISPR-plus enables the simultaneous targeting of multiple genes. a) Schematic representation of combining three different sgRNAs with the respective protectors and their activation with light. b) Simultaneous in vitro targeting of three genes (GFP, CD71, and CD33) by their corresponding sgRNAs (sgGFP (green), sgCD71 (magenta), and sgCD33 (blue)) with (p-sgRNA) or without (sgRNA) their cognate photolabile protectors in the absence (solid bars) or presence (open bars) of light ($\lambda = 365$ nm, 6.0 J cm^{-2}). The proportion of uncleaved DNA is expressed relative to untargeted DNA, averaged over two individual experiments, and sgCon bars represent non-targeting scrambled sgGFP-1. Error bars represent the standard deviation ($n = 2$). DNA cleavage was blocked only in the presence of the corresponding protector, and this protection was overcome such that all the p-sgRNA-treated samples regained target cleavage activity in the presence of light.

inhibition of CRISPR activity in the absence of light and resulted in robust CRIPSR-plus activity, similar to that observed using an unmodified ssDNA protector. This finding emphasizes that protectors tolerate modifications near the 5′-end of sgRNA, and highlights that they are thus amenable for use in indirect labeling of sgRNAs while maintaining their utility as a CRISPR-plus switch.

While we acknowledge that our first-generation CRISPR-plus method lacks a perfect off/on switch, we believe that it still offers an attractive, simple approach to many researchers that they can adapt for their own applications by further modifying the protectors or by conjugating the protector to the sgRNA. Notably, Doudna and co-workers have shown that 10-nt ssDNA can stabilize the Cas9/sgRNA complex and hence is required for target binding with Cas9/sgRNA.[30] Our efforts extend these findings to highlight that the position of ssDNA binding to the sgRNA impacts Cas9-mediated cleavage (Figure 1 b) and may also influence enzyme binding to the complex.

Further studies may provide additional mechanistic insight as well as improved control and specificity. Whereas our current version of genome editing by CRISPR-plus activation of sgRNAs is irreversible and depends on activation with UV light, the activation duration should be controllable by modulating the Cas9/sgRNA persistence, and orthogonal photocleavable groups can be employed to achieve multiplexed activation of protectors in a spatiotemporally controlled manner. Future generations of CRISPR-plus could also incorporate photocleavable oligonucleotides across the sgRNA backbone region to yield universal, off-target, sequence-agnostic protectors or other modified sgRNAs designed to mediate selective blockade and thereby prevent the recruitment of other effector domains.[9] Overall, our CRISPR-plus method provides a rapid and simple approach for the light-mediated control of genome editing.

Acknowledgements

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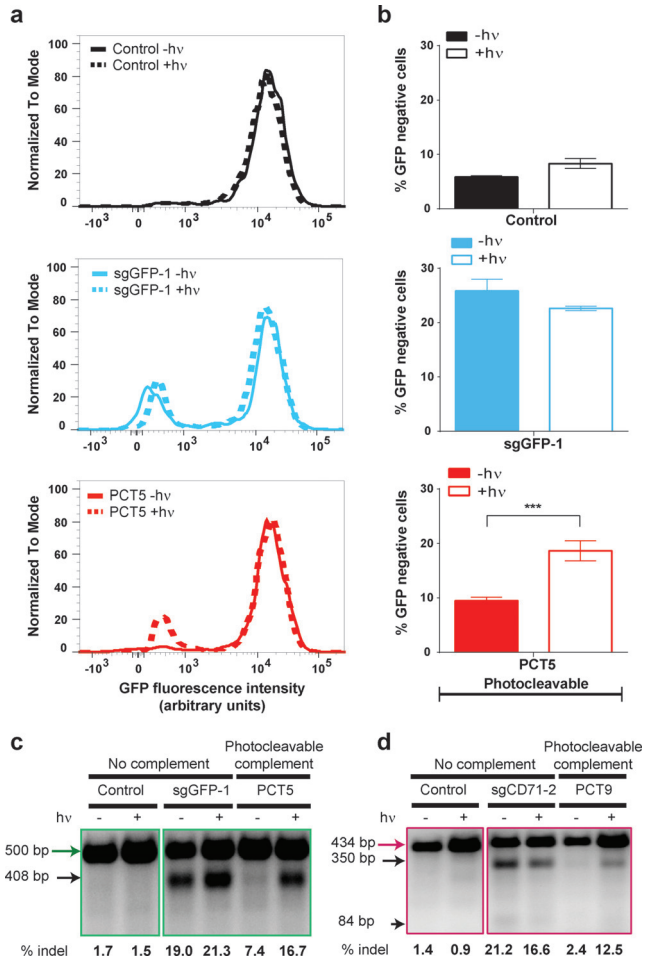

Figure 4. Validation of the CRISPR-plus approach in cells. a) Representative flow cytometry histograms of Cas9/d2eGFP-expressing HeLa cells transfected with sgGFP-1, with or without PCT5. The presence or absence of light ($\lambda = 365$ nm, 4.0 J cm^{-2}) is indicated for each condition, and control traces (top) represent untreated cells. Additional controls are included in Figure S9. b) Quantification of the fraction of GFP negative cells observed in (a), where mean values are plotted with error bars indicating standard deviation ($n = 3$). An unpaired Student's t-test was performed for sgRNA with PCT5 in the presence or absence of light exposure, and p values are represented by asterisks, ***$p < 0.001$. c, d) The SURVEYOR nuclease assay was performed 72 h post-transfection using Cas9/d2eGFP-expressing HeLa cells transfected with sgGFP-1 (c) or sgCD71-2 (d) with PCT5 and PCT8, respectively, in the absence or presence of light. In each case, both control and test sample lanes were run on single physical gels, and cropped images of marked lanes are shown to streamline figure presentation. Uncleaved GFP DNA (green, 500 bp) cut to a shorter fragment (black, 408 bp) and uncleaved CD71 DNA (magenta, 434 bp) with its 350 bp and 84 bp cleavage fragments are indicated ($n = 1$).

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Supporting Information

Development of Light-Activated CRISPR Using Guide RNAs with Photocleavable Protectors

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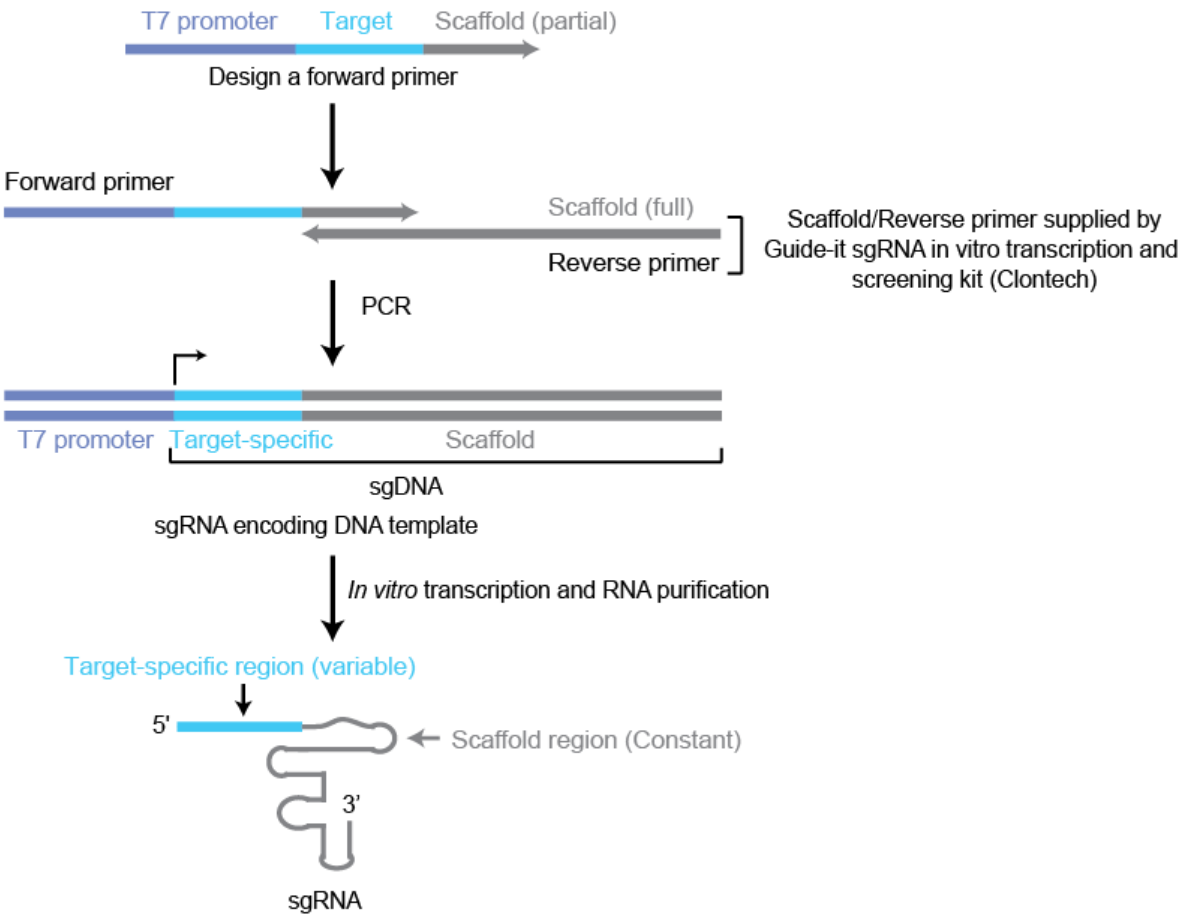
SUPPORTING INFORMATION

EXPERIMENTAL SECTION

Designing and synthesizing sgRNAs

All sgRNA sequences were obtained from published literature. [24, 31-32] We generated sgRNAs by using an *In vitro* Transcription and Screening Kit for sgRNA (Clontech) following manufacturer's protocol with the following changes. To improve the yield, we designed a slightly shorter forward primer sequence (58-62 nt long) by flanking a shorter T7 promoter sequence on the 5' end of target region of sgRNA (See Supplementary Table 1b), PCR amplified to generate sgRNA encoding DNA template and then used 300 ng of PCR fragment and incubated with T7 polymerase mic at 42°C for 3 hrs, instead of 100 ng for 1 hr, as described by the manufacturer.



Supplementary Figure S1: Generation of sgRNAs using *in vitro* transcription kit (Clontech).

Annealing of protectors with sgRNA to create p-sgRNAs

All the PC and non-PC protector DNAs were ordered from Gene Link, Inc and reconstituted in RNase free water. 100 μL of sgRNA (40 ng/uL or approximately 1.24 μM) was annealed with various protector complements by adding either 1x (12.4 μL of 10 μM) or 2x (24.8 μL of 10 μM) of protector complements in the presence of 20 μL of 10x Cas9 buffer (NEB) containing 200 mM HEPES, 1 M NaCl, 50 mM MgCl2, 1 mM EDTA, pH 6.5 and the volume was adjusted to 200 μL with RNase free water. The mixture was heated to 90°C in a heat block for 15 minutes and then the heat block was turned off to allow slow cooling of the mixture to room temperature over ~ 2 hours. Desired amount of p-sgRNAs were irradiated for 30 seconds using a UV laser source, OmniCure S2000, 365 nm, 200 mW/cm^2 at a distance of 4 cm from the source to test activity after photolysis *in vitro*. For photolysis rate experiments, p-sgRNAs were also irradiated for 1s, 2s, 5s, 10s or 15s using the same setup.

Amplification of DNA target amplicons

GFP, CD71 and CD33 genes were amplified from cells using Terra™ PCR Direct Polymerase followed by High Yield EcoDry PCR (Clontech) by following manufacturer's protocol and primer sequences described in Supporting Table 1e.

***In vitro* cleavage assay and multiplexing**

In vitro cleavage assay was performed by using recombinant Cas9 from NEB and PNA Bio. Briefly, 100 ng of DNA amplicon, 60 ng of sgRNA, 1 μg of BSA and 500 ng of Cas9 were incubated with 10 μL of 1x of Cas9 buffer (NEB) in RNase free water for 1 hr at 37°C. Reaction

was quenched by adding SDS containing loading dye (NEB) and loaded onto a 1.5% agarose gel containing 1.5x GelRed dye (Biotium, Inc) for visualization.

Generation of Cas9/d2eGFP cell line

To test CRISPR activity in cells we generated HeLa cells with constitutive Cas9 expression. HeLa-d2eGFP cells were transduced with CAG-h Cas9 (Puro) lentiviral particles (Gentarget Inc.) by spin infection in the presence of 3 µg/ml polybrene. Two days after transduction, 2 µg/ml puromycin was added to the media for one week to select for Cas9 expressing cells. Subsequently, the cells were sorted for GFP+ cells using flow cytometry. (HeLa-d2eGFP, HeLa cells expressing destabilized GFP, were a gift from Prof. Phillip A. Sharp, MIT)

Transfection of cells and mutation detection

To test the transient efficiency of sgGFPs, we cultured the reporter cell line in DMEM medium containing 10% FBS maintained at 37^{o}C and 5% CO_2. The day before transfection cells were plated at a density of 8,000 cells/well in a 96-well plate and then 24 hours later transfected with sgRNA and p-sgRNA at 20 nM concentration using RNAiMAX in OptiMEM by following manufacturer's protocol for RNA delivery. After 6 hours, cells were irradiated with 4.0 J/cm2 (15 min) of light with at a wavelength of 365 nm, using a CL-1000 UV Cross-linker UVP with power density of 4.45 mW/cm^2 (measured by an OAI 306 UV power meter) and then OptiMEM was replaced with the fresh medium. Cells were cultured for another 48 to 72 hours and then GFP levels were analyzed using flow cytometry. Mutations were detected in the genomic DNA using Guide-it Mutation Detection Kit (Clontech) by following manufacturer's protocol and using primer sets described in Table 1f.

Cytotoxicity studies

Cas9/d2eGFP expressing HeLa Cells were plated in a 96-well plate at a density of 8,000 cells/well in 10% FBS/DMEM containing 1% penicillin streptomycin antibiotic media. Similar to all the other cell based experiments, 24 hours later cells were either kept in dark or exposed to 4.0 J/cm^2 (15 min) of light with at a wavelength of 365 nm, using a CL-1000 UV Cross-linker UVP with power density of 4.45 mW/cm^2 as measured by an OAI 306 UV power meter. 72 hours post irradiation, media was replaced with fresh media and cell toxicity was determined using PrestoBlue® Cell Viability Reagent (ThermoFisher) by following manufacturer's protocol. Briefly, wells with cells or without cells (background) were incubated for 45 min in the media containing the reagent and then fluorescence was measured using a fluorescence spectrophotometer (Tecan). Please refer Figure S10 for more details.

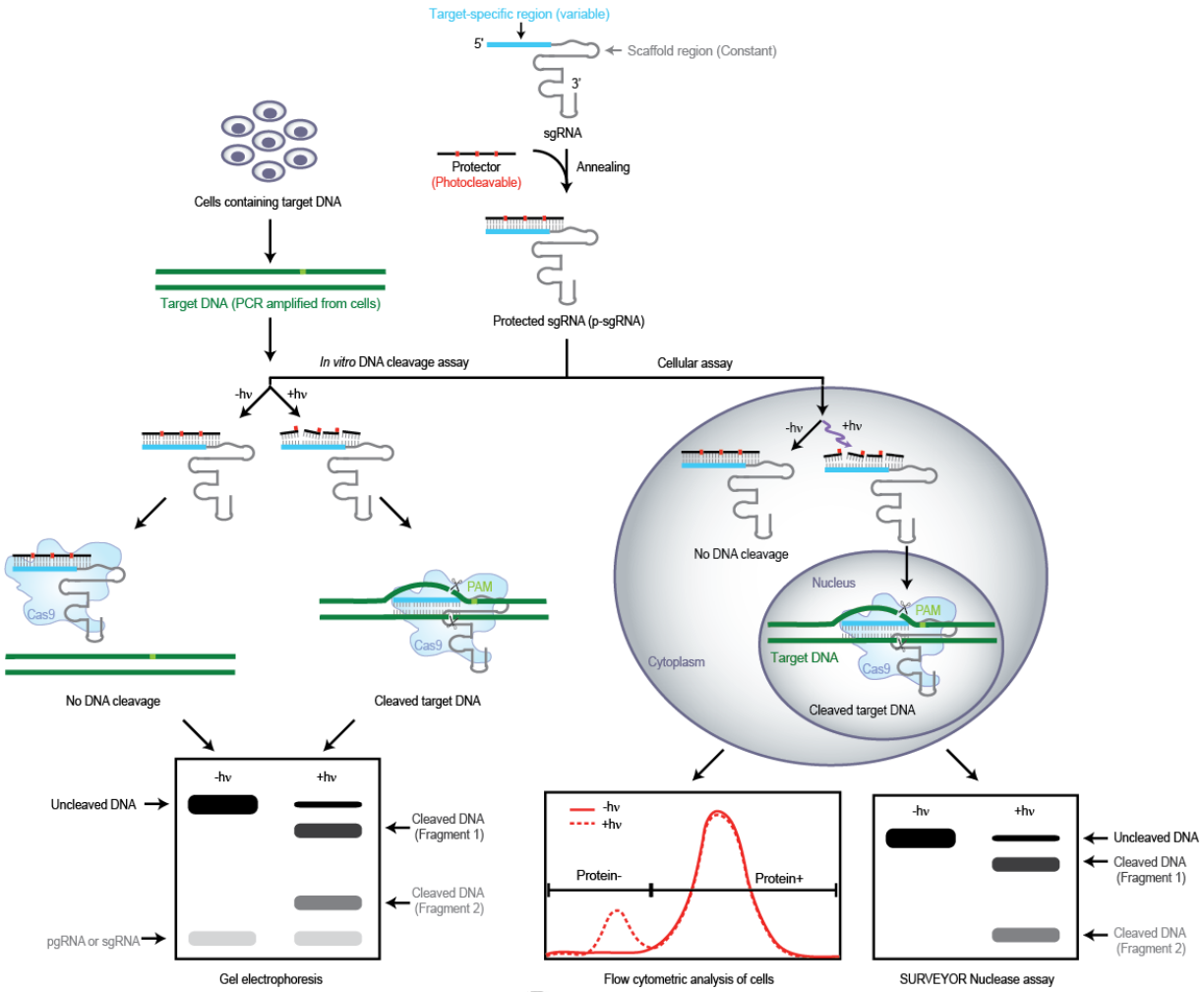
Irradiation of protectors and their characterization by gel and HPLC analysis

Different protectors in RNase free water (50 μM, 10 μL, see figure S5) were either kept in dark or exposed to 30 seconds (or 6 J/cm^2) of light exposure using a UV laser source (OmniCure S2000, 365 nm filter, 200 mW/cm^2). 2 μL of this sample was then mixed 1μL of SDS-containing gel loading dye (NEB B7021S) and were loaded on a denaturing PAGE gel containing 10% TBE urea (Invitrogen) for gel electrophoresis. Following electrophoresis, gel was stained with 2x GelRed staining dye (Biotium) and then analyzed on a gel doc imager. The remaining sample was diluted 5 times with water and then analyzed by HPLC using a modified protocol from the literature.[33] Briefly, a 250 × 4.5 mm C8 column was used with mobile phase containing 0.1 M triethylammmonium acetate buffer (A) and a mixture of 50% acetonitrile and 50% 0.1 M triethylammmonium acetate buffer (B). 40 μL of sample was injected in the HPLC and samples were ran using a gradient: 100% A to 100% B over 30 min at a flow rate of 1 ml min^{-1}.

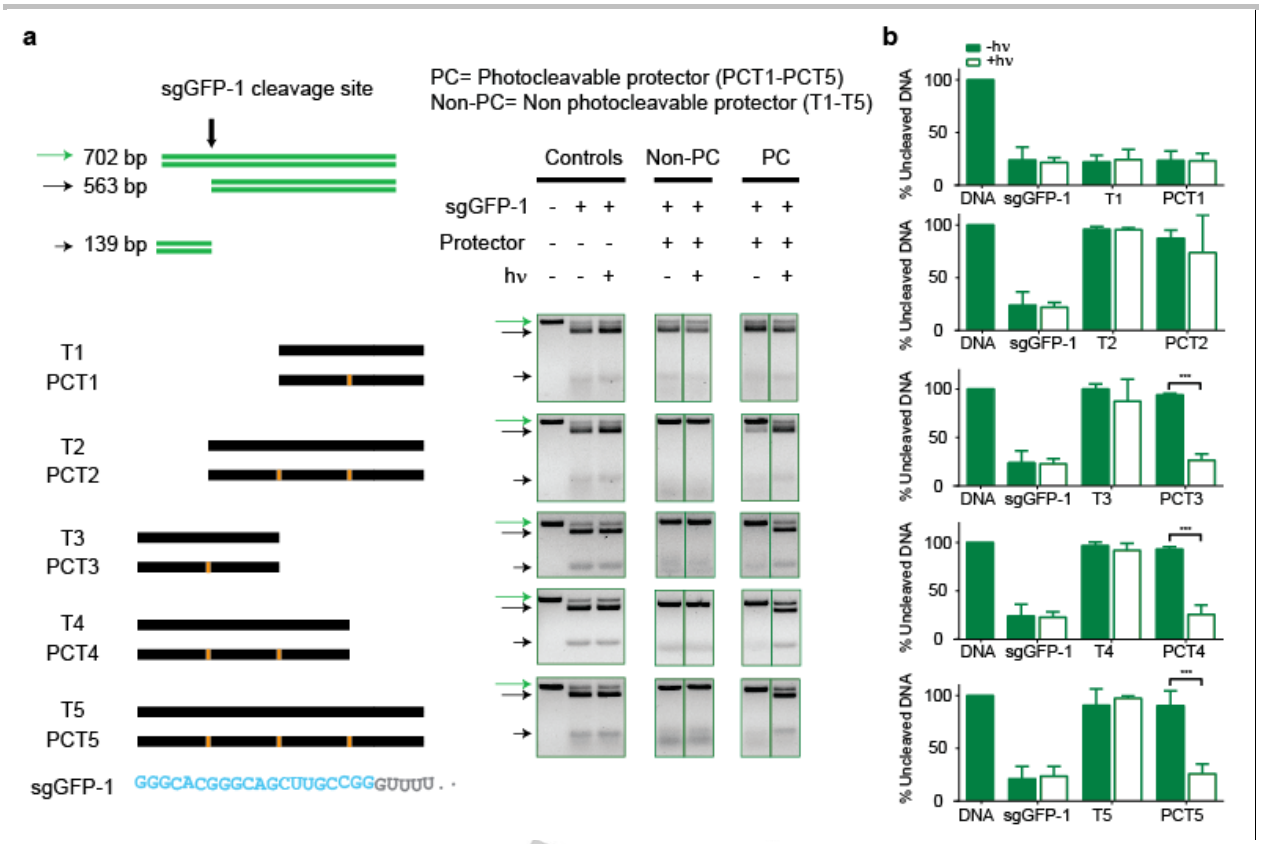
Data analysis

Gels were imaged using a Kodak Gel Logic 200 imaging system and band intensities were analyzed using Fiji (ImageJ) software. Background band intensities were individually calculated for each lane near the band and subtracted from each band individually to correct for non-uniformity in the scanning of the gel. % Uncleaved DNA was calculated by using the formula % Uncleaved DNA= 100 x (a)/(a + b + c), where a is integrated intensity of uncleaved DNA while b and c are integrated intensity of cleavage products after background correction. The data was then normalized to 100% for the control DNA sample without Cas9. % indels were calculated based on the binomial probability distribution of duplex formation using following equation: % Indel = 100 x (1−$\sqrt{(1 - fcut)}$), where $fcut$ = (b + c)/(a + b + c), where a is integrated intensity of uncleaved DNA while b and c are integrated intensity of cleavage products after background correction[11]. Bar graphs with error bars in all the figures indicate mean values +/- s.d or +/- s.e.m. from multiple repeat experiments and value of n indicated in figure legend. Unpaired t-tests with unequal variance were performed to compare -hv and +hv conditions within each sgRNA group, and p-values were corrected for multiple comparisons using the Holm-Sidak method in GraphPad Prism software and p-values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as described in the Supporting data analysis section

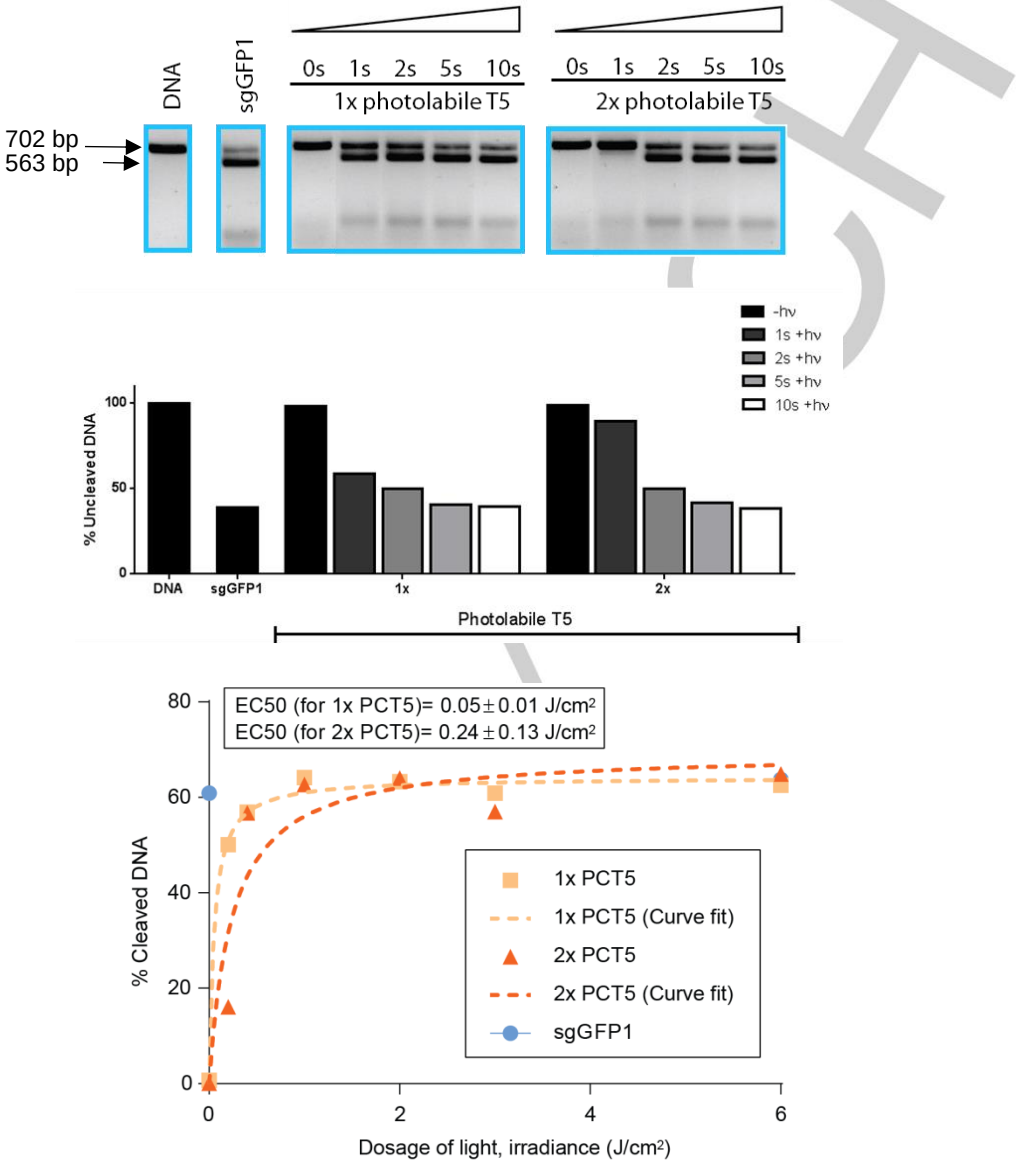
SUPPORTING FIGURES



Supporting Figure S2: Schematic flow of experiments including generation of p-sgRNAs and their testing *in vitro* and in cells. We generated target DNA amplicons from cells using PCR and then tested the cleavage efficiency of DNA by using recombinant Cas9 with sgRNA or p-sgRNA, with or without light irradiation. We then tested them in cells and quantified protein disruption using FACS or DNA cleavage using SURVEYOR assay.



Supporting Figure S3: Design and testing of various PC and non-PC target-specific ssDNA protectors (PCT1-T5 and T1-T5) against sgGFP-1 in order to improve blocking of CRISPR activity in cells. We found that PCT5 protected sgRNA from Cas9-mediated cleavage of DNA both *in vitro* and in cells until irradiated with light (365 nm, UV lamp, 20 minute irradiation of cells). (a) Layout of designed protectors (left), and gel electrophoresis from *in vitro* cleavage assay with or without light tested at 1x concentration of protectors (right). Uncleaved GFP DNA (green arrow, 702 bp) and cleaved fragments of DNA (black arrows, 563 bp and 139 bp) are indicated in each gel. (b) Quantification of the % uncleaved DNA calculated from the band intensities of the gels. Only the protection mediated by PCT3, PCT4 and PCT5 was significantly released following light exposure. Data is normalized to control untreated DNA and mean values with standard deviation are plotted from multiple repeat experiments (n=3 for PCT1, PCT2, PCT3, and PCT4 and n=4 for PCT5) plotted. Please refer to figure 1b for fold increase in DNA cleavage activity with light and figures 1c-d for PCT5 2x data. *** indicates $p<0.0001$. For detailed description of statistical analysis, please refer Supporting data analysis section. Using the sgRNA targeting GFP sequences (sgGFP-1), we found about ~75% cleavage of target DNA using recombinant Cas9 nuclease as analyzed by gel electrophoresis and resulted in the expected product fragments.



Supporting Figure S4: Light based dose dependency of target-specific complement. p-sgRNA dosed at 1x and 2x concentrations of ssDNA protector, with respect to sgRNA, were irradiated for varying exposure times. A short 2-5 seconds (or 0.4-1 J/cm^2) of light exposure using a UV laser source (OmniCure S2000, 365 nm filter, 200 mW/cm^2) was sufficient for both 1x and 2x conditions to completely regain CRISPR activity. (top) *In vitro* cleavage assay showing the cleaved bands, (middle) quantification of upper panels based on band intensities were also graphed as a function of time (bottom) Replotted % cleaved DNA as a function of irradiance of light. EC50 or dosage of light required for 50% of maximum DNA cleavage was calculated by non-linear least square curve fitting with Graphpad Prism software. For 1x concentration of PCT5, EC50 was calculated to be 0.26±0.05 seconds of irradiation (or 0.05±0.01 J/cm^2) and for 2x PCT5, it was calculated to be 1.20 ±0.67 seconds of irradiation (or 0.24±0.13 J/cm^2).

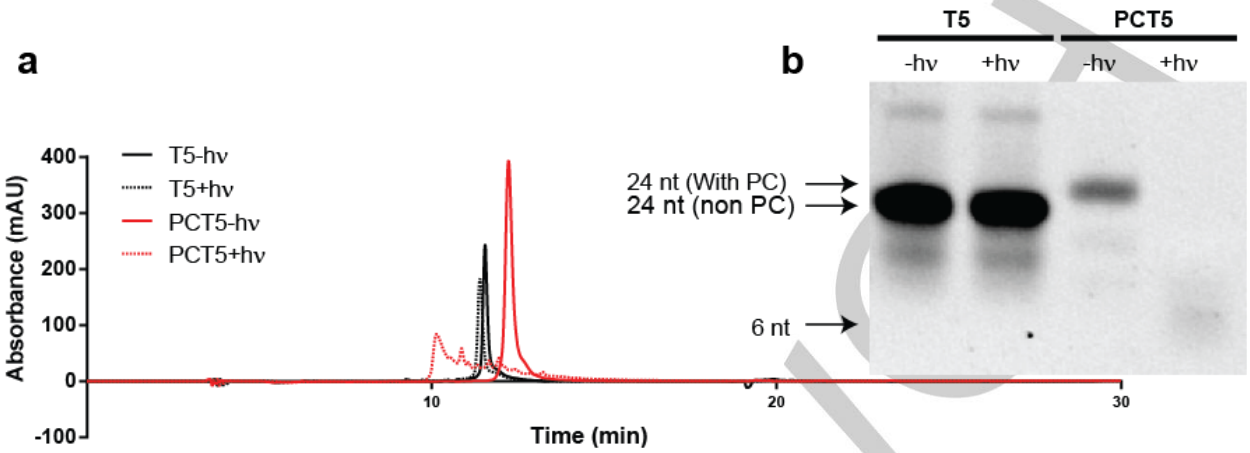
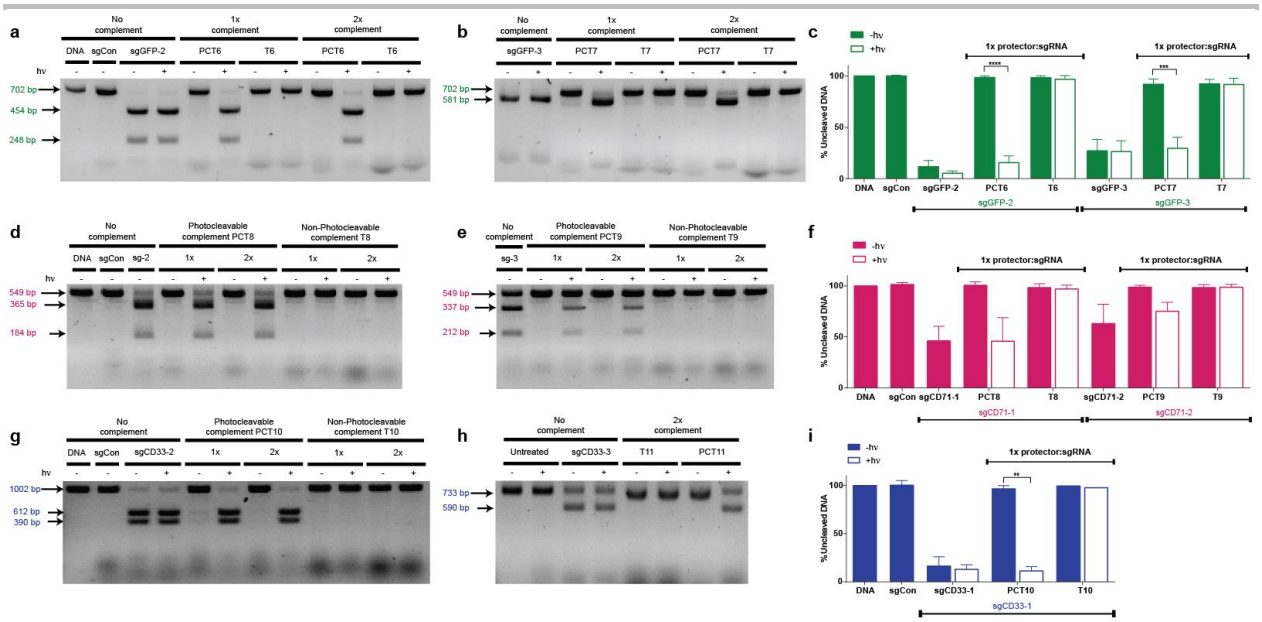
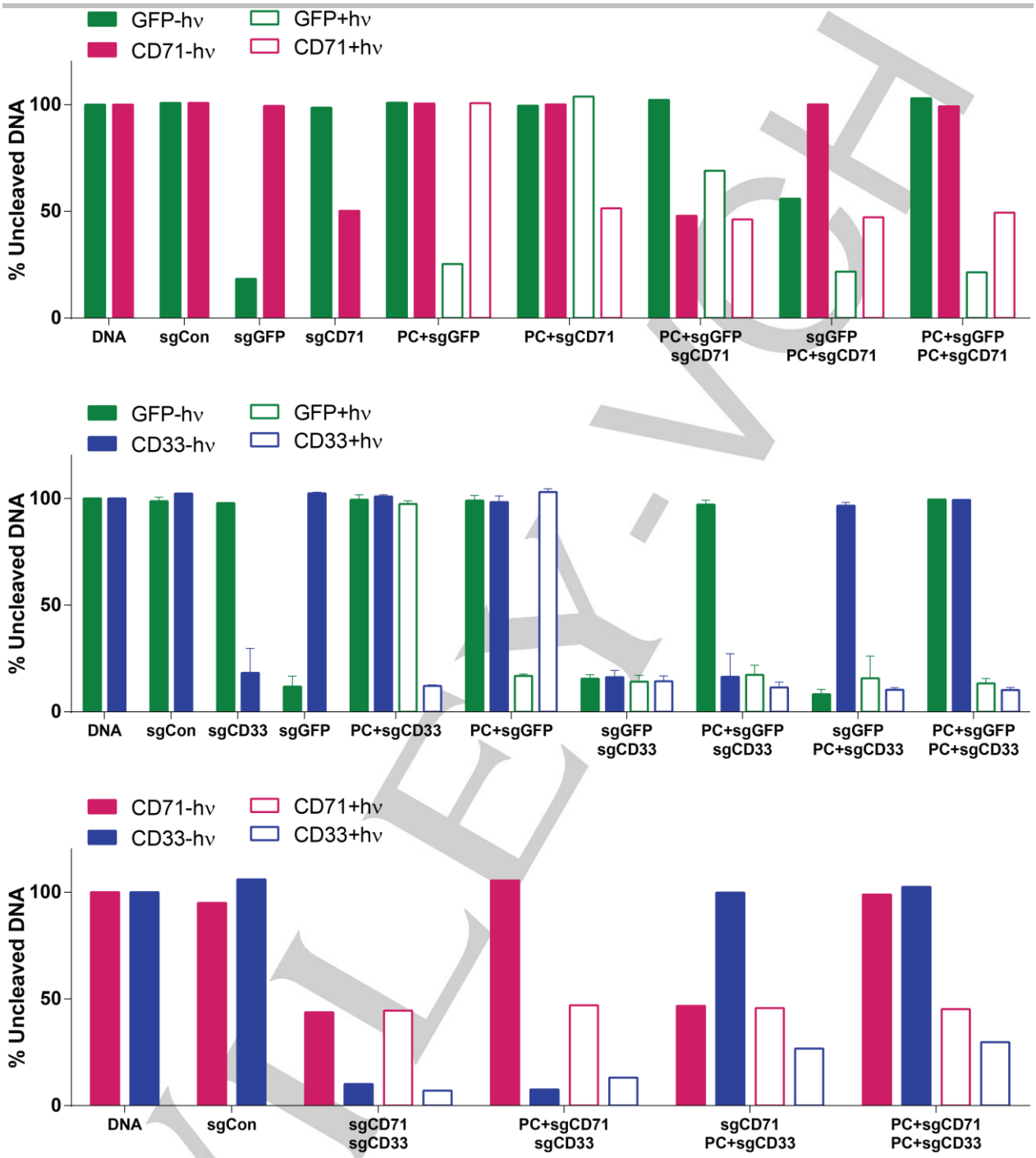


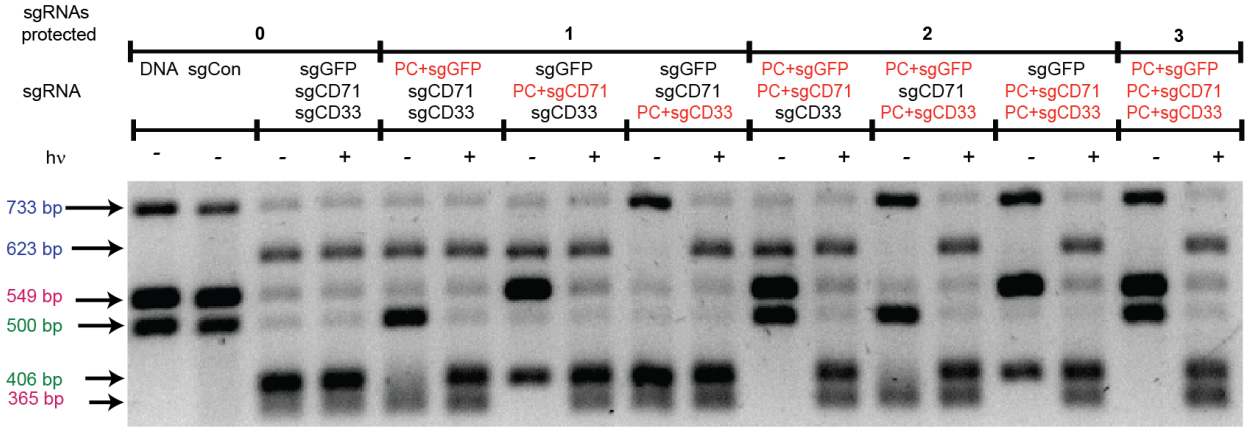
Figure S5: Photolysis of ssDNA photocleavable (PCT5) vs. non-photocleavable (T5) protectors (without any sgRNA) analyzed by HPLC (a) and denaturing PAGE gel (b). 500 pmols (20 μM, 25 μL) of protectors (Gene Link, inc.) in RNase free water was kept in dark or exposed to light using the same irradiation conditions as used with all other p-sgRNA for in vitro cleavage assay (OmniCure S2000, 365 nm filter, 200 mW/cm^2 for 30 seconds or 6.0 J/cm^2). Following exposure, 350 pmols of samples was injected in HPLC using the method described in the methods section, and 100 pmols of protectors was loaded on a denaturing PAGE gel. As expected, T5 protector showed no effect of light while PCT5 showed complete degradation after the irradiation.



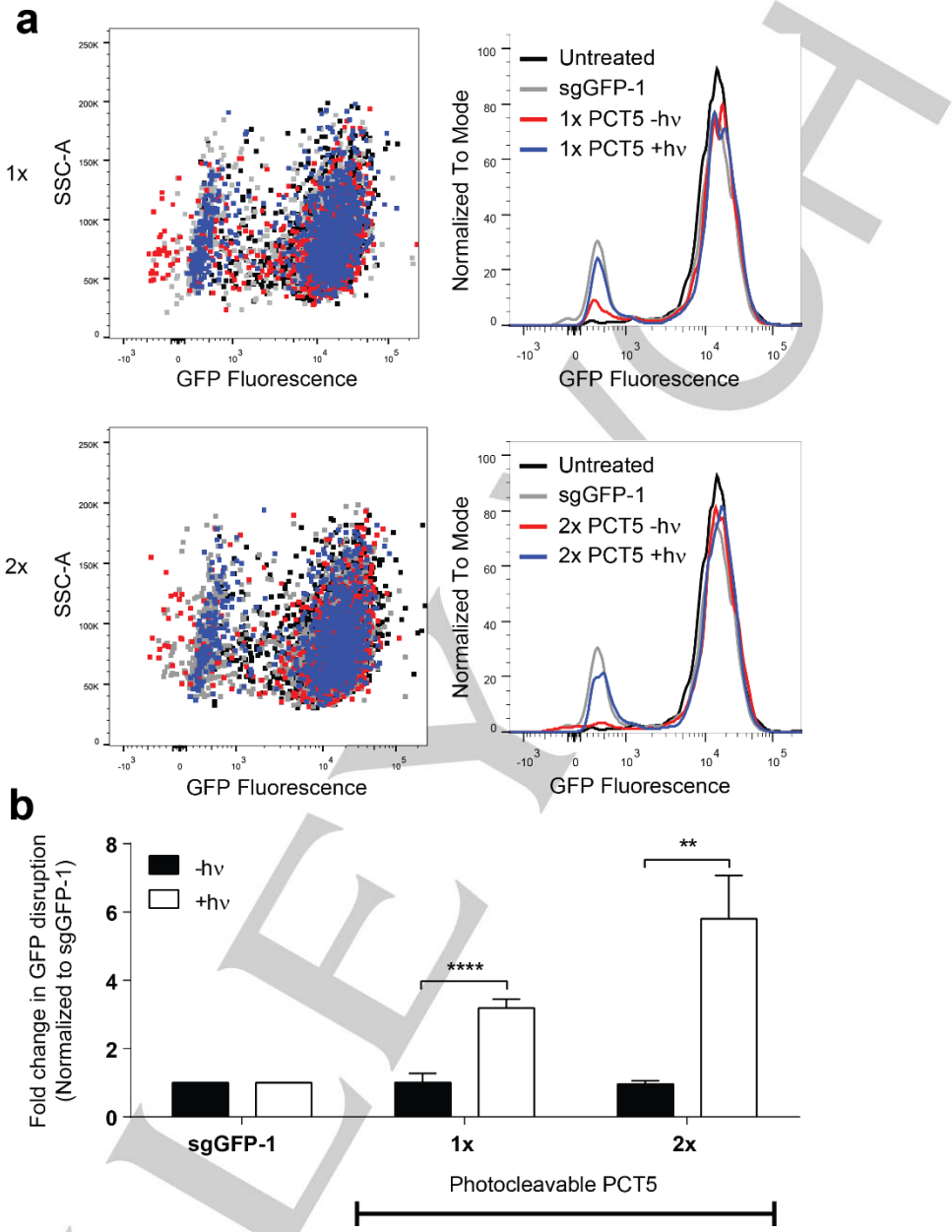
Supporting Figure S6: Gel images of *in vitro* cleavage assay of a 702 bp GFP DNA (green, 702 bp) with (a) sgGFP-2 (yields 454 bp and 248 bp fragments) or with (b) sgGFP-3 (yields a 581 bp fragment); a CD71 DNA (magenta, 549 bp) with (c) sgCD71-1 (yields 365 bp and 184 bp fragments) or with (d) sgCD71-2 (yields 337 bp and 212 bp fragments); a CD33 DNA (blue, 1002 bp) with (e) sgCD33-1 (to yield 612 bp and 390 bp fragments) and a CD33 DNA (blue, 733 bp) cleaved with (f) sgCD33-2 (yields a 590 bp fragment). Different sgRNAs tested with and without PC or non-PC ssDNA protector sequences at 1x and 2x concentrations and the band intensities calculated from replicate experiments (n=2; except for T10, where n=1) are represented in bar graph format on the right for 1x ratio (c, f, i). Bar graph data from 2x ratios are illustrated in the figure 2a-c. Data normalized to control untreated DNA. Mean values with standard deviation plotted with significant difference calculated by student t-test, indicated by asterisk (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). For detailed description of statistical analysis, please refer to the Supporting data analysis section.



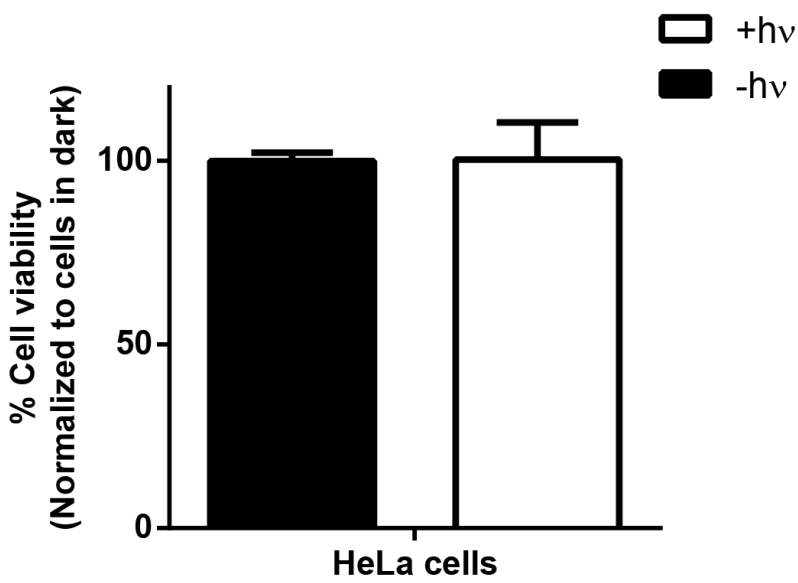
Supporting Figure S7: Multiplexing two genes with CRISPR-plus. Bar graphs quantify the results of *in vitro* cleavage assays performed using mixtures of target DNA sequences with and without the relevant sgRNA and protector oligonucleotides. GFP and CD71 (top), GFP and CD33 (middle), CD71 and CD33 (bottom) combinations all demonstrate light responsive Cas9 activity for specific sgRNA only in the presence of the photocleavable protectors for that sgRNA. Error bars for GFP and CD33 indicate standard deviation from multiple experiments (n=2).



Supporting Figure S8: Multiplexing of three genes with CRISPR-plus. Gel electrophoresis analysis after an *in vitro* cleavage assay using a mixture of three different DNA amplicons: GFP (green, 500 bp; cleaved to 406 bp), CD71 (magenta, 733 bp; cleaved to 623 bp), and CD33 (blue, 549 bp; cleaved to 365 bp). DNA mixture was incubated with Cas9 and sgGFP-1, sgCD71-2 and sgCD33-1 with or without corresponding photocleavable protectors PCT5, PCT9 and PCT10 (labeled as PC) before or after irradiation with light. Length of cleaved DNA is represented in the same color corresponding to the parent uncleaved DNA. Cleavage pattern indicates light responsive Cas9 activity for specific sgRNA only in the presence of the photocleavable protectors for that sgRNA. Please refer to figure 3b for bar graph quantification of the data with repeated experiments.



Supporting Figure S9: Dose dependent concentrations (1x and 2x) of 24-nt ssDNA protector PCT5 (relative to sgRNA) used to assay for GFP negative cells. (a) Various replicate dot plots and FACS histograms of PCT5 at 1x (top) and 2x (bottom). (b) Fold change in GFP disruption, normalized to respective sgGFP-1, with or without light, is indicated with mean ± s.d. values represented in the bar graph and error bars, respectively. Our results indicate that the 24-nt protector (PCT5) blocked the majority of Cas9 activity (Figure 1c, 1d and S3). We observed that the fold increase of GFP negative cells generated after light exposure was higher when a 2x dose of PC-protectors was used. For detailed description of statistical analysis, please refer to the Supporting data analysis section.



Supporting Figure S10: Cytotoxicity assay of HeLa cells in the absence or presence of light. Cas9/d2eGFP expressing HeLa Cells were plated in a 96-well plate at a density of 8,000 cells/well in 10% FBS/DMEM containing 1% penicillin streptomycin antibiotic media. Similar to all the other cell-based experiments, 24 hours later cells were either kept in dark or exposed to 4.0 J/cm^2 (15 min) of light with at a wavelength of 365 nm, using a CL-1000 UV Cross-linker UVP with power density of 4.45 mW/cm^2, as measured by an OAI 306 UV power meter. 72 hours post irradiation, media was replaced with fresh media and cell toxicity was determined using PrestoBlue® Cell Viability Reagent (ThermoFisher) by following manufacturer's protocol. Briefly, wells with and without cells were incubated for 45 min in the media containing the reagent and then fluorescence was measured using a fluorescence spectrophotometer. Background subtracted fluorescence intensity was normalized to cells in dark and mean values are plotted with error bars indicating standard deviation (n=6). No cytotoxicity was observed due to irradiation of cells.

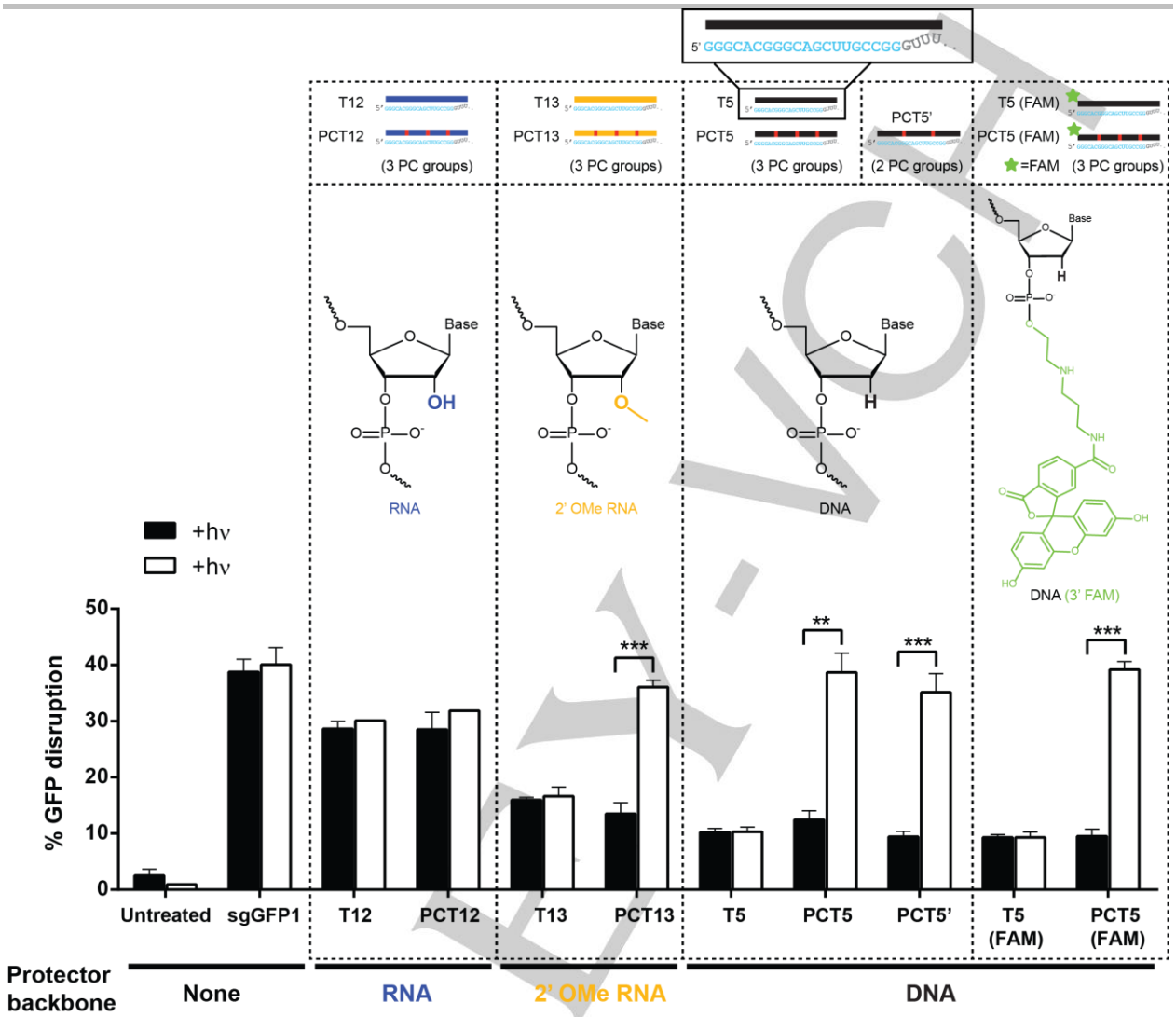


Figure S11: Optimization of CRISPR-plus with modified protectors in cells. Protectors containing different backbone (RNA, 2' OMe RNA or DNA) modifications, number and position of PC groups (2 PC groups separated by 8-nt instead of 3 PC groups separated by 6-nt in a 24-nt DNA protector) or 3' end modifications (3' end of DNA modified with FAM dye) were annealed with sgGFP-1 to yield p-sgRNAs, and then either kept in dark (black bars) or pre-irradiated (white bars) using a UV laser source (OmniCure S2000, 365 nm filter, 200 mW/cm^2) for 30 seconds. Samples were then transfected using Lipofectamine/OptiMEM in d2eGFP HeLa cells expressing Cas9 for 24 hours in DMEM media containing 10% FBS and 1% penicillin-streptomycin. The % GFP disruption was determined 48 hours post-transfection using flow cytometry. Mean values ± s.d. (n=3) are shown. Unpaired student's t-test was performed between the non-irradiated vs. pre-irradiated samples, and p values are represented with asterisks *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPORTING TABLES

Supporting Table 1: Sequences information of primers, sgRNAs and protector complements.

Supporting Table 1a: sgRNA targets

sgRNA	Target DNA (5' to 3')	Length of target region
sgGFP-1	**GGGCACGGGCAGCTTGCCGG**	20
sgGFP-2	**GATGCCGTTCTTCTGCTTGT**	20
sgGFP-3	**GGTGGTGCAGATGAACTTCA**	20
sgCD71-1	**GGACGCGCTAGTGTGAGTGC**	20
sgCD71-2	**GGGATATCGGGTGGCGGCTC**	20
sgCD33-1	**GGGGTGATTATGAGCACCG**	19
sgCD33-2	**GGCGTCTACGATGCTCA**	17

Supporting Table 1b: T7 promoter containing forward primer for generating sgDNA sequences

sgRNA	T7 promoter-**Target region**-Constant region of sgRNA **(5' to 3')**
sgGFP-1	TCTAATACGACTCACTATA**GGGCACGGGCAGCTTGCCGG**GTTTTAGAGCTAGAAATAGCA
sgGFP-2	TCTAATACGACTCACTATAG**GATGCCGTTCTTCTGCTTGT**GTTTTAGAGCTAGAAATAGCA
sgGFP-3	TCTAATACGACTCACTATAG**GGTGGTGCAGATGAACTTCA**GTTTTAGAGCTAGAAATAGCA
sgCD71-1	TAATACGACTCACTATAG**GGACGCGCTAGTGTGAGTGC**GTTTTAGAGCTAGAAATAGCA
sgCD71-2	TCTAATACGACTCACTATA**GGGATATCGGGTGGCGGCTC**GTTTTAGAGCTAGAAATAGCA
sgCD33-1	TCTAATACGACTCACTATA**GGGGTGATTATGAGCACCG**GTTTTAGAGCTAGAAATAGCA
sgCD33-2	TCTAATACGACTCACTATA**GGGCGTCTACGATGCTCA**GTTTTAGAGCTAGAAATAGCA

First nucleotide incorporated into sgRNA is underlined. Note that some sequences have an extra G or GG incorporated into guide RNAs due to use of T7 promoter.

Supporting Table 1c: Photocleavable protector sequences

Name	Target sgRNA	Back-bone	Sequence (*Photocleavable linker, 5' to 3')	Length	Tm [Tm of fragments after photolysis] in °C
PCT1	sgGFP-1	DNA	AAACCC*GGCAAG	12	40.8 [0, 3.1]
PCT2	sgGFP-1	DNA	AAACCC*GGCAAG*CTGCCC	18	59.5 [0, 3.1,9.2]
PCT3	sgGFP-1	DNA	CTGCCC*GTGCCC	12	50.3 [9.2, 10.4]
PCT4	sgGFP-1	DNA	GGCAAG*CTGCCC*GTGCCC	18	63.6 [3.1, 9.2, 10.4]
PCT5	sgGFP-1	DNA	AAACCC*GGCAAG*CTGCCC*GTGCCC	24	68.9 [0, 3.1, 9.2, 10.4]
PCT5'	sgGFP-1	DNA	AAACCCGG*CAAGCTGC*CCGTGCCC	24	68.6 [20.1, 17.1, 41.4]
PCT6	sgGFP-2	DNA	ACACAA*GCAGAA*GAACGG*CATCCC	24	62.4 [0, 0, 3.3, 0]
PCT7	sgGFP-3	DNA	AACTGA*AGTTCA*TCTGCA*CCACCC	24	60.9 [0, 0, 0, 7.2]
PCT8	sgCD71-1	DNA	AACGCA*CTCACA*CTAGCG*CGTCCC	24	65.5 [3.7,0,2.2,9.3]
PCT9	sgCD71-2	DNA	AAACGA*GCCGCC*ACCCGA*TATCCC	24	65.5 [0,21.7, 5.5, 0]
PCT10	sgCD33-1	DNA	AAAACC*GGTGCT*CATAAT*CACCCC	24	59.6 [0, 0, 4.5, 7.2]
PCT11	sgCD33-2	DNA	TAAAAC*TGAGCA*TCGTAG*ACGCCC	24	60.9 [0, 0, 0, 14.4]
PCT12	sgGFP-1	**RNA**	AAACCC*GGCAAG*CUGCCC*GUGCCC	24	68.5 [0, 0, 0, 14.4]
PCT13	sgGFP-1	**2' OMe**	AAACCC*GGCAAG*CTGCCC*GTGCCC	24	68.5 [0, 0, 0, 14.4]
PCT14	sgGFP-1	DNA	AAACCC*GGCAAG*CTGCCC*GTGCCC [6-FAM]	24	68.4 [0, 0, 0, 14.4]

Supporting Table 1d: Non-photocleavable protector sequences

Name	Target sgRNA	Back-bone	Sequence (*Photocleavable linker, 5' to 3')	Length	Tm [Tm of fragments after photolysis] in °C
T1	sgGFP-1	DNA	AAACCCGGCAAG	12	38.8
T2	sgGFP-1	DNA	AAACCCGGCAAGCTGCCC	18	58.9
T3	sgGFP-1	DNA	CTGCCCGTGCCC	12	49.1
T4	sgGFP-1	DNA	GGCAAG CTGCCC GTGCCC	18	63.5
T5	sgGFP-1	DNA	AAACCCGGCAAGCTGCCCGTGCCC	24	68.5
T6	sgGFP-2	DNA	ACACAAGCAGAAGAACGGCATCCC	24	62.1
T7	sgGFP-3	DNA	AACTGAAGTTCATCTGCACCACCC	24	60.4
T8	sgCD71-1	DNA	AACGCACTCACACTAGCGCGTCCC	24	65.5
T9	sgCD71-2	DNA	AAACGAGCCGCCACCCGATATCCC	24	65.5
T10	sgCD33-1	DNA	AAAACCGGTGCTCATAATCACCCC	24	59.6
T11	sgCD33-2	DNA	TAAAACTGAGCATCGTAGACGCCC	24	60.4
T12	sgGFP-1	**RNA**	AAACCCGGCAAGCUGCCCGUGCCC	24	68.9
T13	sgGFP-1	**2' OMe**	AAACCCGGCAAGCTGCCCGTGCCC	24	68.9
T14	sgGFP-1	DNA	AAACCCGGCAAGCTGCCCGTGCCC [6-FAM]	24	68.8

Supporting Table 1e: Primer sequences for DNA template design for *in vitro* cleavage studies and multiplexing

Target	Forward or Reverse	Sequence (5' to 3')	Length of amplicon (bp)	Remarks
GFP	Forward1	GAGGAGCTGTTCACCGGG	702	
	Reverse1	CTTGTACAGCTCGTCCATGC		
GFP	Forward2	GACGTAAACGGCCACAAGTTC	500	
	Reverse2	GGGGTGTTCTGCTGGTAGTG		
CD71	Forward1	AAGGCAGAGAGAAGGGAAGG	998	
	Reverse1	TAACAGCGATGGCAATGAAA		
CD71	Forward2	ACAGCCCCTTCCCCACAG	549	
	Reverse2	ACTGGAGGTTGGTCAAGGTCA		
CD33	Forward1	TCTTTCGGATGGAGAGAGGA	1002	
	Reverse1	GCAAGGGGGAAGTTGCTAGT		
CD33	Forward2	TCATGGTTACTGGTTCCGGGA	733	
	Reverse2	GGTGACGTTGAGCTGGATGG		

Supporting Table 1f: Primer sequences for SURVEYOR assay

Target	Forward or Reverse	Sequence (5' to 3')	Length	Remarks
GFP	Forward2	GACGTAAACGGCCACAAGTTC	500	SURVEYOR & Cleavage
	Reverse2	GGGGTGTTCTGCTGGTAGTG		
CD71	Forward3	AGGCAGAGAGAAGGGAAGGG	434	SURVEYOR
	Reverse3	CGTACGTGCCTCAGGAAGTG		

SUPPORTING REFERENCES

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