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 S1 c,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-photo-cleavable 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 S1 c,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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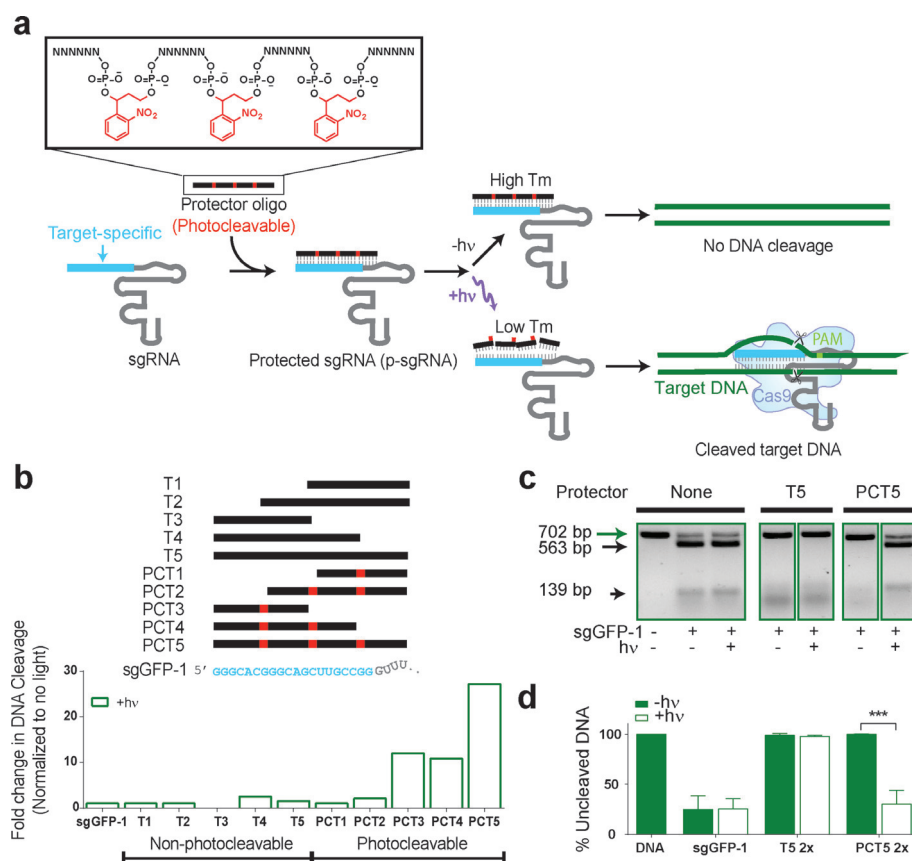Howard Hughes Medical Institute
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Figure 1. CRISPR-plus achieves photoactivatable blockade of Cas9-mediated DNA targeting. a) The CRISPR-plus concept. b) Fold increase in DNA cleavage following light exposure ($\lambda = 365$ nm, 6.0 J cm^{-2}) with 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, ***$p < 0.001$.

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 pro-

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

tectors 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
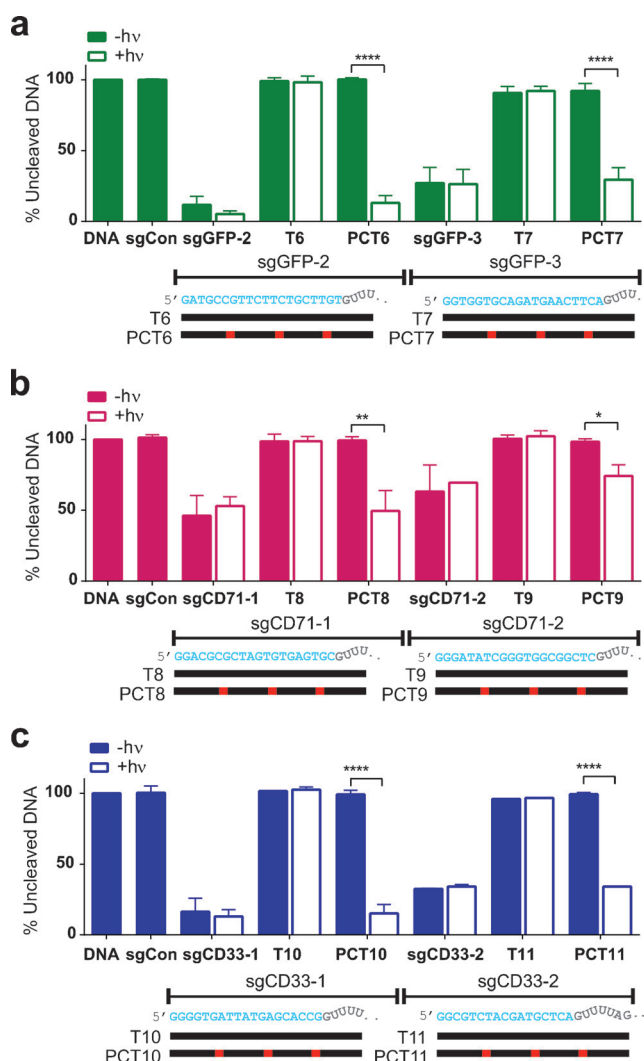
Figure 2. Validation of optimized 24-nt target-specific protectors with other sgRNAs and targets in an in vitro DNA cleavage assay. a–c) PC-containing (PCT6–PCT11) or non-PC (T6–T11) oligonucleotides complementary to six additional sgRNAs targeting three different DNA targets, namely a) GFP (green), 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 4 c, 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
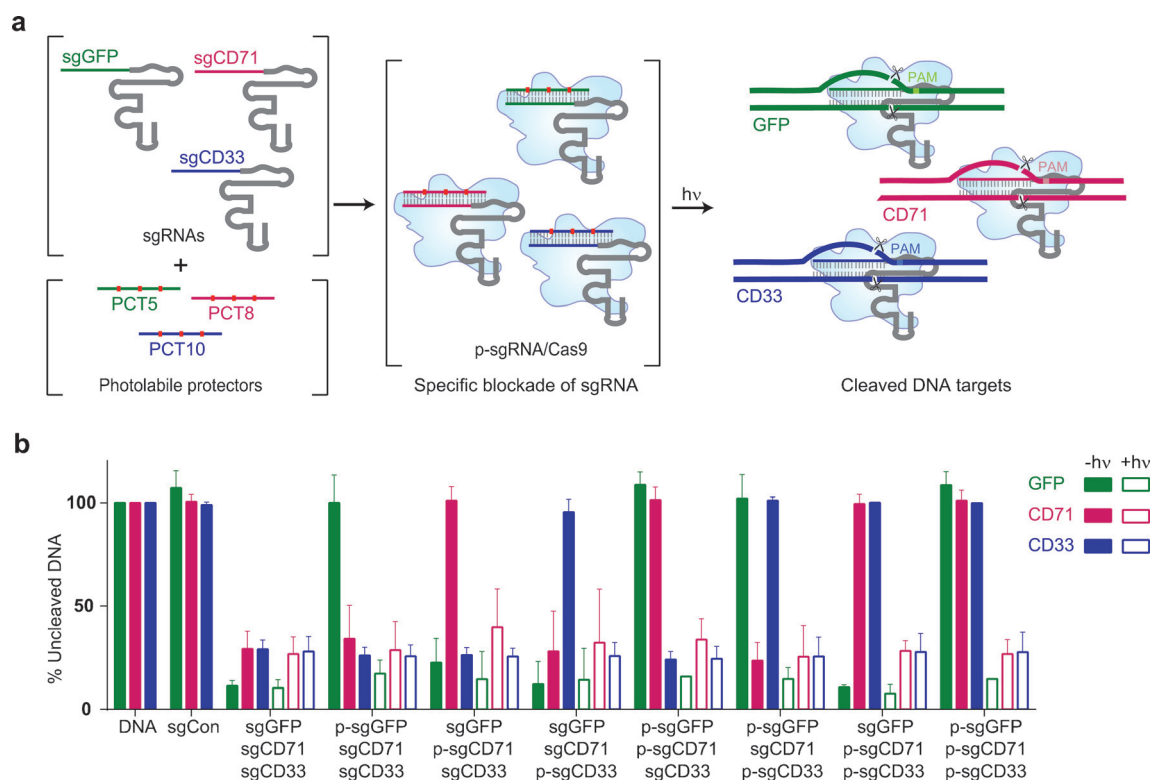
Figure 3. CRISPR-plus enables the simultaneous targeting of multiple genes. a) Schematic representation of combining three different sgRNAs with the respective protectors and their activation with light. b) Simultaneous in vitro targeting of three genes (GFP, CD71, and CD33) by their corresponding sgRNAs (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 1b) 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 control-

lable 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.
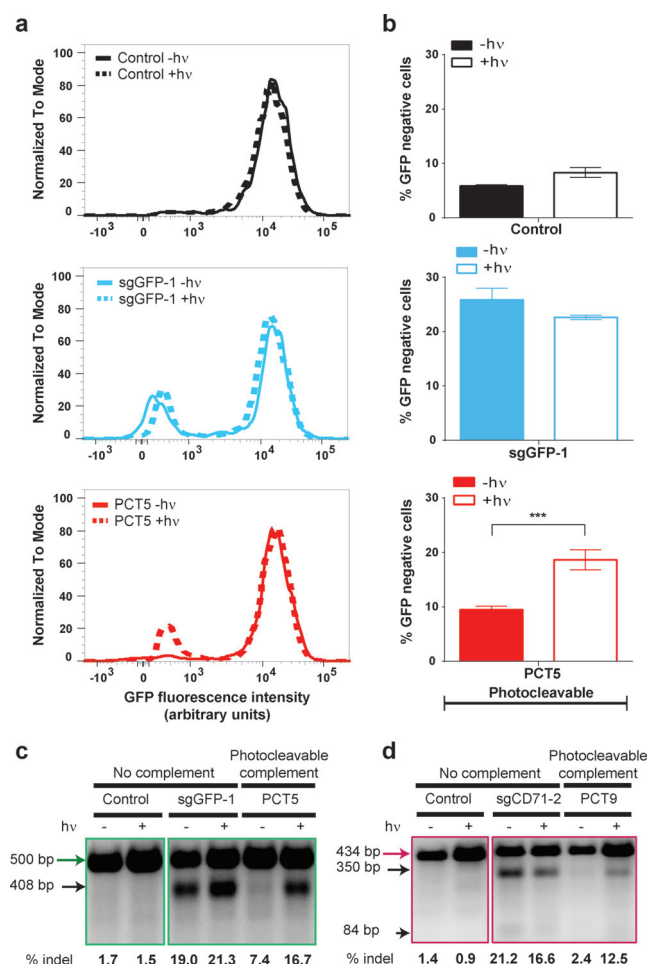
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Figure 4. Validation of the CRISPR-plus approach in cells. a) Representative flow cytometry histograms of Cas9/d2eGFP-expressing HeLa cells transfected with sgGFP-1, with or without PCT5. The presence or absence of light ($\lambda = 365$ nm, 4.0 J 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 ($n = 1$).

Keywords: CRISPR · DNA cleavage · gene technology · nucleic acids · photochemistry

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