A chemical toolbox for labeling and degrading engineered Cas proteins

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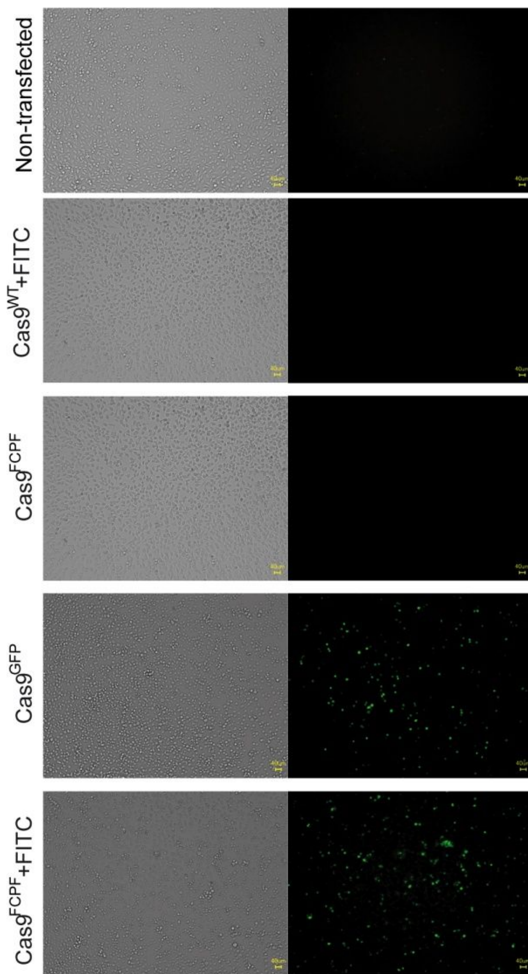
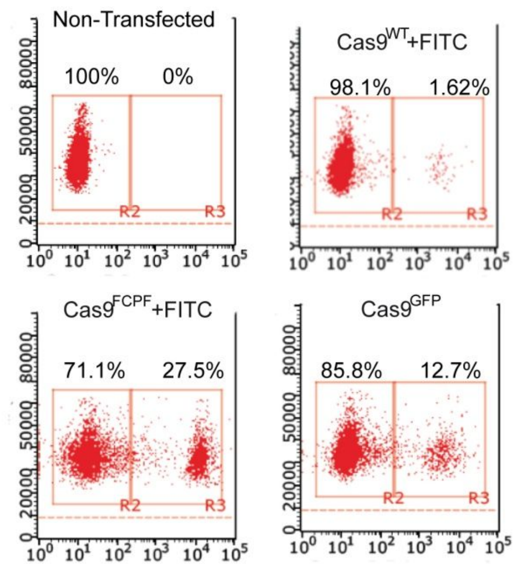
Results





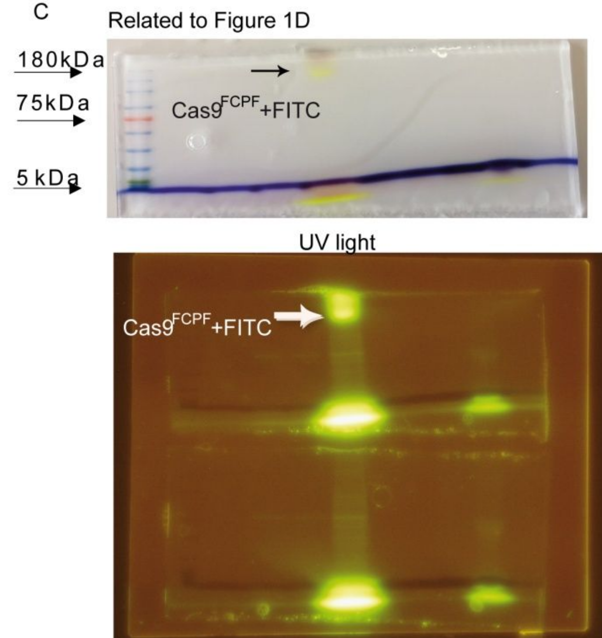


Figure S1: The application of Cas9^{FCPF} for live cell imaging. A) Detection of green fluorescence signaling in FITC-FCPF treated HeLa cells expressing Cas9^{FCPF}. HeLa cells were transfected with Cas9^{FCPF} and treated with FITC-FCPF (10 μM) for 2 h. Non-transfected cells, cells expressing Cas9^{FCPF}, and cells expressing Cas9^{GFP} with or without FITC-FCPF (10 μM) treatment were used as controls. **B)** FACS analysis of non-transfected cells, FITC-FCPF (10 μM, 2 h) treated cells expressing Cas9^{WT}, FITC-FCPF treated HeLa cells expressing Cas9^{FCPF}, and cells expressing Cas9^{GFP}. **C)** The original picture of Figure 1C.

Electrophoresis analysis of samples collected from FITC-FCPF (2 h) treated HeLa cells expressing Cas9^{FCPF} under VIS light and UV light.

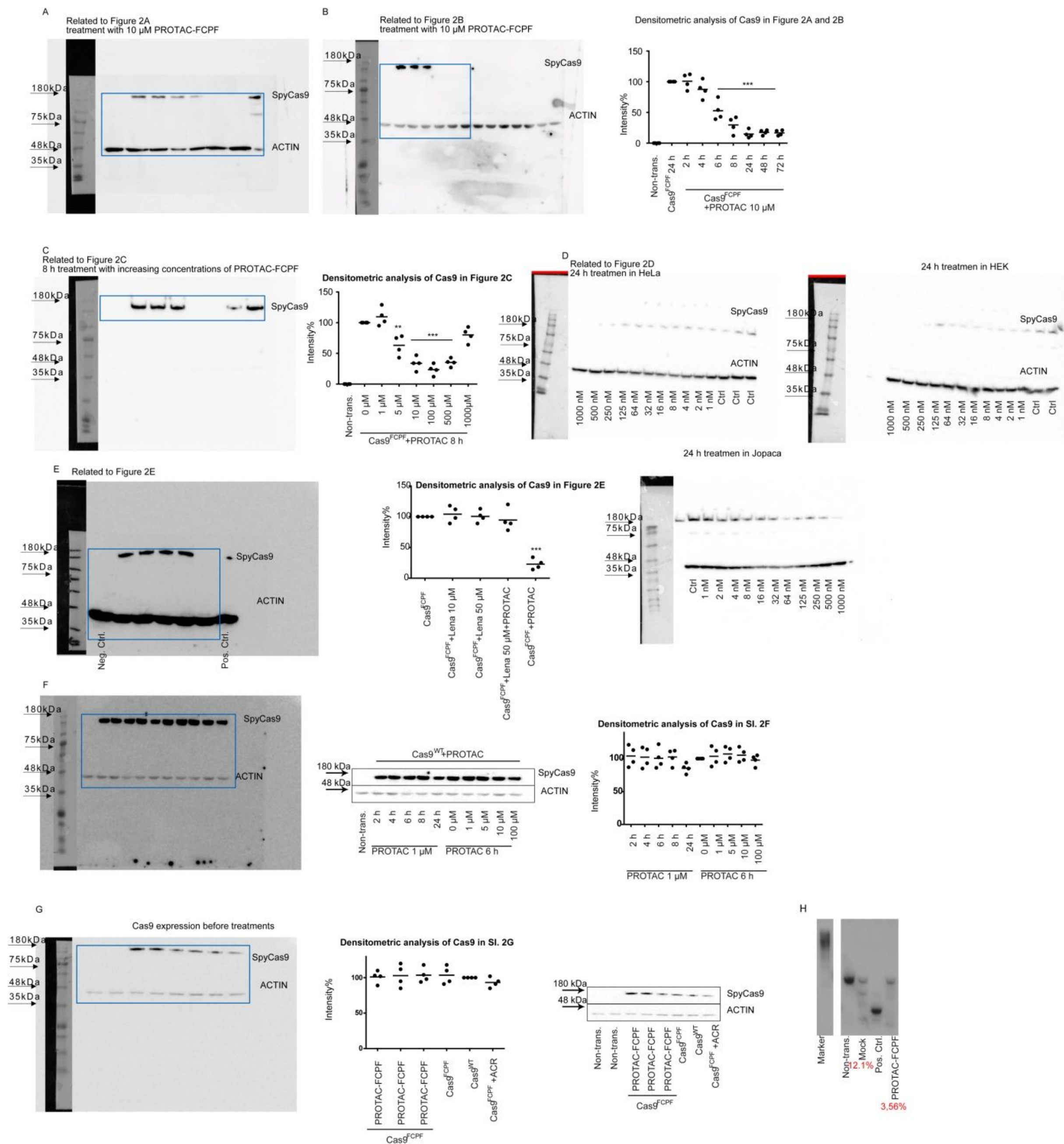


Figure S2: The PROTAC-FCPF inhibits Cas9-mediated genome editing through destabilizing Cas9^{FCPF} protein in cells. A) The original immunoblotting image of Fig. 2A. **B)** The original immunoblotting image of Fig. 2B and the densitometric analysis of protein

expression demonstrated in Fig. 2A and 2B. **C)** The original immunoblotting image of Fig. 2C. PROTAC-FCPF degrades $Cas9^{FCPF}$ in 8 h treatment. Hook effect appears at concentrations higher than 500 μM. The densitometric analysis of protein expression demonstrated in Fig. 2C. **D)** The original immunoblotting images of Fig. 2D. PROTAC-FCPF degrades $Cas9^{FCPF}$ in HeLa, Jopaca-1 and HEK293T cells in 24 h treatment. **E)** The original immunoblotting image of Fig. 2E and its densitometric analysis of protein expression. **F)** PROTAC-FCPF does not degrade $Cas9^{WT}$ protein under various conditions. HeLa cells expressing $Cas9^{FCPF}$ were treated with increasing concentrations of PROTAC-FCPF for 8 h or 1 μM PROTAC-FCPF for indicated time periods. **G)** The comparable Cas9 protein expression for evaluating Cas9 variants-mediated genome editing using T7E1 assay. HeLa cells were transfected with $Cas9^{FCPF}$ or co-transfected with Anti-CRISPR-Cas9 protein AcrIIA4 (ACR). $Cas9^{WT}$ was used as a control. The expression of Cas9 before the treatment of PROTAC-FCPF was analyzed by immunoblotting. **H)** Comparison of off-target effect. HeLa cells were transfected with $Cas9^{FCPF}$ with *AAVS1* sgRNA and treated with DMSO (mock) or PROTAC-FCPF (1 μM) for 24 h. The expected off-target sequence was amplified from genomic DNA and investigated using T7E1 assay. The percentage of off-target effect was highlighted in red. EnGen® mutation detection kit (NEB, #E3321) was used, which includes a positive control. The indicated percentage of off-target effect was an average from three independent experiments The sequences of off-target site can be found in *Materials and Methods*.

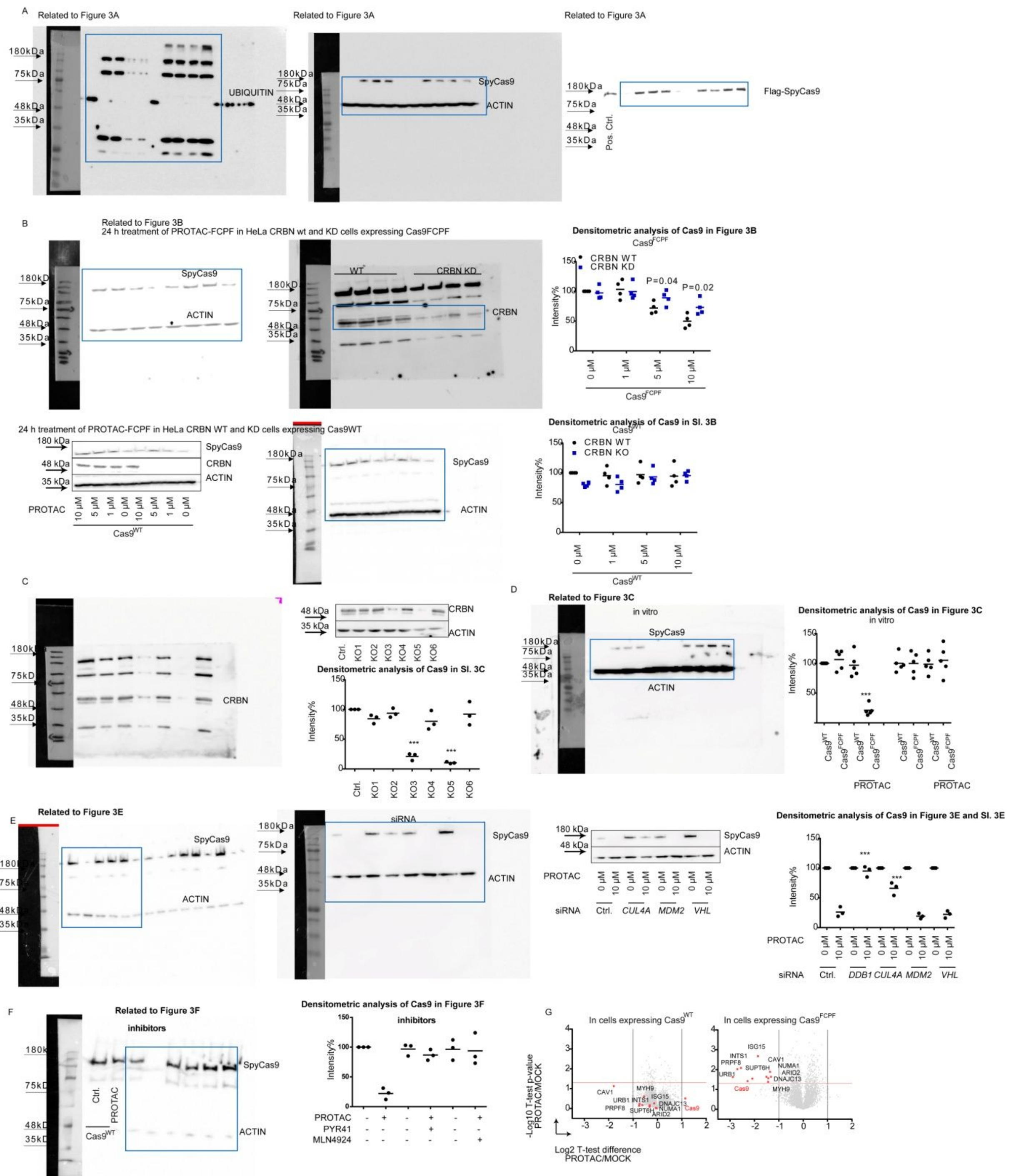


Figure S3: The PROTAC-FCPF-induced Cas9^{FCPF} protein degradation is ubiquitin-dependent. A) The original immunoblotting images of Fig. 3A. **B)** The original immunoblotting images of Fig. 3B and the densitometric analysis of protein expression. PROTAC-FCPF does not affect Cas9^{WT}. CRBN WT or CRBN KD HeLa cells expressing Cas9^{WT} were treated with various concentrations of PROTAC-FCPF for 8 h. **C)** Generation of

CRBN knockout cell lines. HeLa cells were transfected with Cas9 and commercially available CRBN KO sgRNAs (plenti-px330-CRBN-T1-pGK-Pur and plenti-px330-CRBN-T2-pGK-Pur, Addgene: #107382 and #107383). After 7 days antibiotic selection with puromycin (1 μM), single cell expansion was performed to obtain monoclonal CRBN KO cell lines. The KO efficiency was determined by immunoblotting. The CRBN KO5 cell line was used for further experiments. **D)** The original immunoblotting image of Fig. 3C and its densitometric analysis of protein expression. **E)** PROTAC-FCPF mediated $Cas9^{FCPF}$ degradation requires DDB1 and CUL4A and is independent of MDM2 and VHL. HeLa cells were co-transfected with $Cas9^{FCPF}$ and individual siRNAs for 48 h and treated with PROTAC-FCPF (10 μM) for 8 h. The original immunoblotting image of Fig. 3E and the densitometric analysis of protein expression in Fig. 3E and Figure S3E. **F)** The original immunoblotting image of Fig. 3F. G) Evaluation of specificity of PROTAC-FCPF–mediated $Cas9^{FCPF}$ degradation by proteome-wide analysis. HeLa cells expressing either $Cas9^{WT}$ or $Cas9^{FCPF}$ were treated with PROTAC-FCPF (10 μM) for 6 h. DMSO was used as mock. High specificity of PROTAC-FCPF mediated $Cas9^{FCPF}$ degradation was reproducible from three independent experiments. 10 out of total 4436 protein are highlighted due to their significant alternations in cells expressing $Cas9^{FCPF}$ in the presence of PROTAC-FCPF or DMSO (mock) as compared with those in $Cas9^{WT}$ cells. Cas9 was in red.

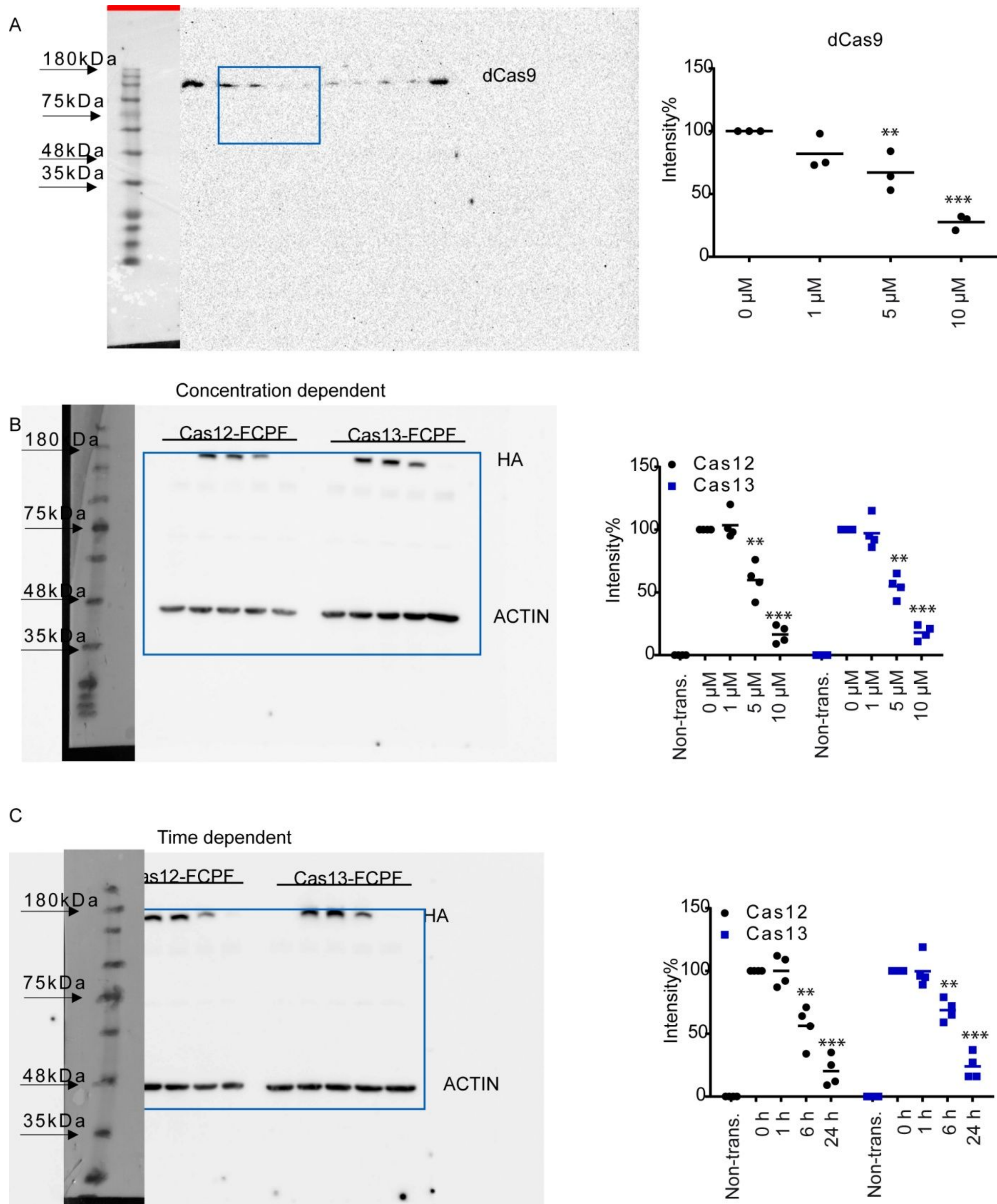


Figure S4: PROTAC-FCPF degrades dCas9^{FCPF}, Cas12^{FCPF} and Cas13^{FCPF}. A)-C) The original immunoblotting images of Fig. 4A-4C and related densitometric analyses of protein

expression. The densitometric analysis of proteins obtained from at least three independent experiments. For each, one of the original pictures was depicted as an example.

Materials and Methods

Chemistry

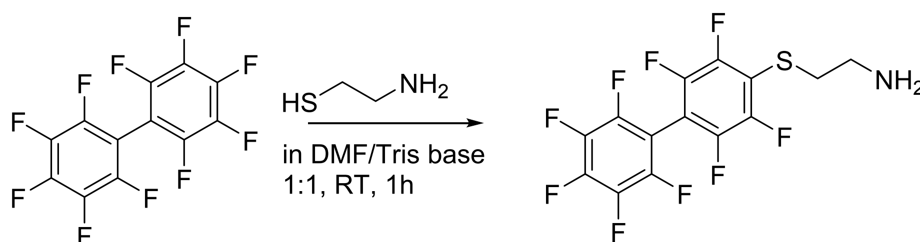
Reagents

Solvents and reagents obtained from commercial suppliers were at least of reagent grade or were purified according to prevailing methods prior to use. TLC was performed to monitor the chemical reactions using Alugram SIL G/UV254 sheets (Macherey & Nagel). Silica column chromatography was conducted using silica gel 60 (Macherey & Nagel, 0.040-0.063 mm). The purity of compounds was determined at least more than 96% by HPLC analysis.

Analytical Methods

^{1}H (300 Hz) and ^{13}C NMR (75 Hz) spectra were recorded on a Varian 300 MHz NMR system. Chemical shifts are reported in ppm. DMSO-d_6 was used as solvent and defined as δ=2.50 for ^{1}H and δ =40.0 for ^{13}C. The following abbreviations were used to explain the multiplicities in NMR spectra: s =singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra (HRMS) were recorded on a Bruker ApexQe hybrid 9.4 T FT-ICR (ESI).

2-((Perfluoro-[1,1'-biphenyl]-4-yl)thio)ethan-1-amine (perfluorobiphenylamine)

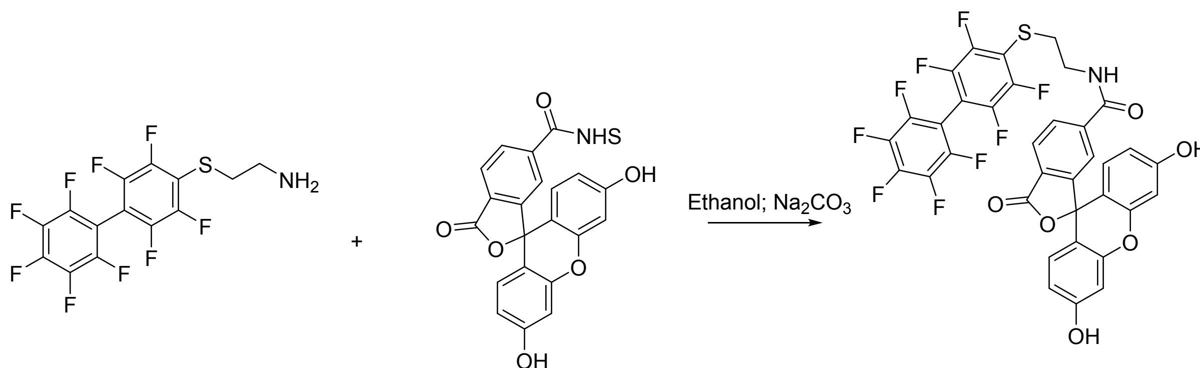


Scheme S1: Synthesis of perfluorobiphenylamine

A reaction mixture of perfluoro-1,1'-biphenyl (1 mmol, 334.1 mg) with β-mercaptoethylamine (1.01 mmol, 77.9 mg) in a solution of 11 mL DMF:Tri base (10:1) was stirred at room temperature for 1 h. HCl (1N, 50 mL) was added. The mixture was extracted with ethyl acetate. The solvent in the organic phase was evaporated. The raw product was purified with a flash chromatography to yield perfluorobiphenylamine (0.65 mmol, 254.6 mg) as a colorless solid (65%).

^{1}H-NMR: 2.89 (t, 2H, J=6), 3.17 (t, 2H, J=6 Hz). ^{13}C-NMR: 21.3, 41.8, 115.8, 136.6, 138.6, 141.2, 142.9, 144.8, 145.9, 147.9. ^{19}F-NMR (-ppm): 132.5-132.6 (m, 2F), 138.1-138.7 (m, 4F), 149.7-149.9 (m, 1F), 160.7-160.9 (m, 2F). HR-MS: $C_{14}H_6F_9NS$ 391.0077 (calculated) 413.9863 $[M+Na]^+$

Synthesis of 6-FITC-FCPF

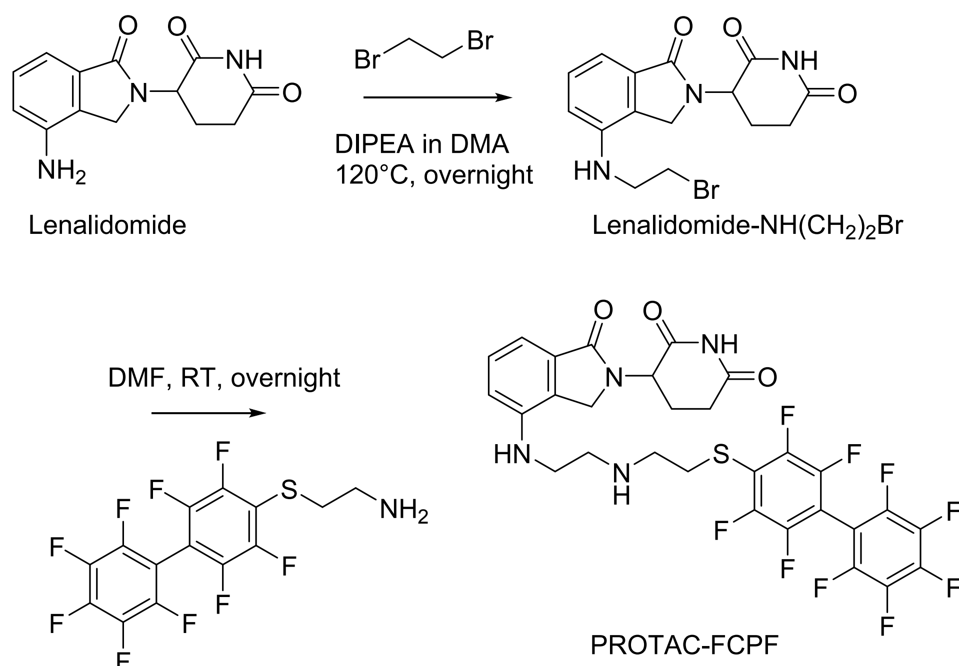


Scheme S2: Synthesis of 6-FITC-FCPF

Synthesis of 6-FITC-FCPF was achieved in a reaction of perfluorobiphenylamine (0.2 mmol, 78.3 mg) and Na_2CO_3 (1 mmol, 106 mg) with commercially available 6-NHS-FITC (0.2 mmol, 100 mg, Lumiprobe, x5120) in 10 mL ethanol. The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was filtered. Ethanol was evaporated. The raw product was purified using a flash chromatography to yield 6-FITC-FCPF (0.10 mmol, 72.1 mg, 50%).

^{1}H-NMR: 2.27-2.28 (m, 1H), 2.73 (m, 1H), 2.81-2.84 (m, 1H), 3.22-3.27 (m, 1H), 6.54-6.57 (m, 4H), 6.69 (m, 2H), 7.63-7.66 (m, 1H), 8.05-8.16 (m, 2H), 8.83-8.85 (m, 1H), 10.15 (m, 2H). ^{13}C-NMR: 26.0, 102.8, 109.2, 113.3, 113.7, 117.6, 126.0, 126.9, 129.9, 131.8, 132.1, 132.3, 136.3, 139.8, 142.7, 146.1, 152.5, 153.2, 158.6, 160.4, 161.4, 167.8, 170.5. ^{19}F-NMR (-ppm): 132.9-132.5 (m, 2H), 138.4-138.7 (m, 4H), 149.6-149.8 (m, 1H), 160.6-160.8 (m, 2H). HR-MS: $C_{36}H_{16}F_9NO_6S$ 749.0555 (calculated), 750.0630 $[M+H]^+$ (found).

Synthesis of PROTAC-FCPF



Scheme S3: Synthesis of PROTAC-FCPF

The synthesis of PROTAC-FCPF was achieved by coupling perfluorobiphenylamine with activated lenalidomide (lenalidomide-NH$(CH_2)_2$Br).

A reaction mixture of lenalidomide (1 mmol, 259.3 mg), dibromoethane (1.1 mmol, 206.7 mg) and DIPEA (3 mmol, 388.2 mg) in dimethylacetamide was stirred at 120 °C overnight. After cooling, the mixture was extracted with ethyl acetate. After removal of the organic solvent, the solid was purified by column chromatography on silica gel (Ethyl acetate with 10% ethanol). The solvent was evaporated and the residue was crystallized in ether to give 3-(4-((2-bromoethyl)amino)-1-oxoisoindolin-2-yl)piperidine-2,6-dione (lenalidomide-NH$(CH_2)_2$Br) as a yellow solid (0.35 mmol, 126.7 mg, 34.6%).

Perfluorobiphenylamine (1 mmol, 391.3 mg) and activated lenalidomide (1 mmol, 366.2 mg) in (10 mL) DMF was stirred overnight at room temperature. Precipitation appeared after adding 50 mL water and was dried to yield PROTAC-FCPF (0.8 mmol, 541.3 mg, 80%).

3-(4-((2-bromoethyl)amino)-1-oxoisoindolin-2-yl)piperidine-2,6-dione (lenalidomide-NH$(CH_2)_2$Br, 34.6%)

^{1}H-NMR: 1.40 (s, 1H), 1.96-2.05 (m, 2H), 2.27-2.33 (m, 1H), 2.58-2.64 (m, 1H), 4.15 (s, J=15, 2H,), 5.10 (dd, J=6, J=12, 2H), 5.41 (s, 2H, NH2+), 6.8 (d, J=6, 1H), 6.91 (d, J=6, 1H), 7.19 (t, J=9, 1H), 10.99 (s, 1H). ^{13}C-NMR: 23.3, 31.7, 46.8, 52.0, 110.0, 110.1, 116.9, 126.1, 129.3, 132.7, 144.1, 169.3, 171.7, 173.4. HR-MS: $C_{15}H_{16}BrN_3O_3$ 365.0375 (calculated), 388.1055 $[M+Na]^+$ (found)

3-(1-oxo-4-((2-((2-((perfluoro-[1,1'-biphenyl]-4-yl)thio)ethyl)amino)ethyl)amino)isoindolin-2-yl)piperidine-2,6-dione (PROTAC-FCPF, 80%)

^{1}H-NMR: 0.83-1.49 (m, 1H), 1.99-2.35 (m, 1H), 2.57-2.64 (m, 2H), 2.72 (s, 2H), 2.88-2.95 (m, 3H), 4.07-4.25 (m, 3H), 4.99-5.37 (m, 2H), 5.37 (s, 1H), 5.71 (s, 1H), 6.80 (d, J=9, 1H), 6.94 (d, J=6, 1H), 7.28 (t, J=9, 1H), 7.94 (s, 1H), 10.99 (s, 1H). ^{13}C-NMR: 23.3, 31.4, 31.7, 32.7, 33.6, 45.1, 46.1, 52.0, 110.9, 116.3, 117.0, 126.2, 129.3, 132.8, 137.1, 139.1 143.4, 144.1, 145.4, 146.3, 148.3, 156.3, 169.4, 171.7, 173.4. ^{19}F (-ppm): 132.7 (m, 2F), 138.4-138.5 (m, 4F), 149.6-149.7 (m, 1F), 160.6-160.8 (m, 2F). HR-MS: $C_{29}H_{21}F_9N_4O_3S$ 676.1191 (calculated); 699.1186 $[M+Na]^+$ (found)

Biology

Sequences of siRNAs

MDM2 (Thermofisher, #122296)

Forward: GCCAUUGCUUUUGAAGUUAtt

Reverse: UAACUUCAAAAGCAAUGGCtt

VHL (Thermofisher, #138745)

Forward: GGAGCGCAUUGCACAUCAAtt

Reverse: UUGAUGUGCAAUGCGCUCCtg

CUL4A (Thermofisher, #139184)

Forward: GCGAGUACAUCAAGACUUUtt

Reverse: AAAGUCUUGAUGUACUCGCtc

DDB1 (Thermofisher, #10596)

Forward: GGUUGGUCUCUCAAGAACCtt

Reverse: GGUUCUUGAGAGACCAACCtc

Electrophoresis and immunoblotting

Cells were trypsinized and washed at least 3x with PBS. Cell pellet (200g, 5 min) were lysed in 6M urea-lysis buffer with protease- and phosphatase inhibitors (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M Urea, 1 mM Na_3VO_4, 10 µg/mL Pepstatin, 100 µM PMSF and 3 µg/mL Aprotinin in PBS). Total protein was resolved on 8% SDS-PAGE gels for Cas9 (~150 kDa) or 10% SDS-PAGE for proteins with MW < 100 kDa. To detect the protein with specific antibody, resolved proteins on SDS-PAGE were transferred onto PVDF membranes (GE Healthcare, Germany) and blotted with 5% milk. Membrane was incubated with primary antibody (1:1000) over night at 4 °C. β-ACTIN (1:5000, Santa Cruz, Germany) was used as

loading control. SuperSignal West Pico Plus Chemiluminescent Substrate (Thermofisher, Germany) and the Fujifilm LAS-3000 imaging system was used to image the membrane. Aida image analysis software was used to quantify the intensity of signals. For intensity normalization, we first normalized the value of investigated protein from sample to that from control and then normalized to actin value as showed below.

$$\frac{\text{Protein}_{sample}}{\text{Protein}_{Control}} / \frac{\text{ACTIN}_{sample}}{\text{ACTIN}_{Control}}$$

Immunoprecipitation

HeLa cells expressing Cas9^{WT} or $\text{Cas9}^{\text{FCPF}}$ were treated with PROTAC-FCPF (10 μM) in the presence or absence of MG132 (5 μM) for 8 h. FLAG Immunoprecipitation kit (Sigma Aldrich, Merck, Germany, #FLAGIPT1-1KT) was used according to the manufacturer's instructions. HeLa cells were seeded in a 6-well plate with density of 500 000 cells/well and transfected with FLAG-Cas9 or FLAG-CRBN plasmids as mentioned in *Materials and Methods* of the main text. Cells can be treated as designed and lysed in lysis buffer (20 mM Tris HCl pH 8, 150 mM NaCl, 1% Nonidet P-40 and 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 10 μg/mL Pepstatin, 100 μM PMSF and 3 μg/mL Aprotinin). Anti-FLAG M2 affinity gel (20 μL) was added into celly lysate (500 μL) and incubated for 24 h at 4°C. Suspension was washed with wash buffer (50 mM Tris HCl and 150 mM NaCl, pH 7.4) and eluted with 2 x SDS loading buffer (125 mM Tris HCL, 4% SDS, 20% glycerol and 0.004% bromphenol blue, pH 6.8) for immunoblotting.

Generation of CRBN KO cell lines

HeLa cells were transfected with plenti-px330-CRBN-T1-pGK-Pur and plenti-px330-CRBN-T2-pGK-Pur plasmids. Lipofectamine 3000 was used to reach the maximal transfection efficiency as described above. Cells were selected by adding puromycin (1 μg/mL). Monoclonal cell lines were achieved by manual single cell expansion described previously.[1]

Proteome-wide analysis

Sample preparation for mass spectrometry

HeLa cells were transfected with $Cas9^{WT}$ or $Cas9^{FCPF}$ for 48 h as described in *Materials and Methods* of the main text. Cells were treated with DMSO (mock) or PROTAC-FCPF (10 μM) for 6 h. The cells were washed with PBS for at least 3 times and lysed with lysis buffer (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M Urea, 1 mM Na_3VO_4, 10 μg/mL Pepstatin, 100 μM PMSF and 3 μg/mL Aprotinin in PBS). The protein concentration was determined with Bradford assay (Sigmal, #B6916-500ML). 40 μg of proteins from cell lysates were loaded on SDS-PAGE gel and ran for approximately 1 cm (80 V). Gel lanes were cut into 7 equally sized bands using a grid-cutter (Gel Company, San Francisco, CA) and gel bands were processed as previously described[2] with minor modifications. Individual gel bands were destained with 40% acetonitrile (ACN) in 50mM Ammonium-bicarbonate (ABC) and proteins were reduced with 10 mM DTT for 30 minutes at 56°C, alkylated with 40 mM Chloroacetamide for 30 minutes at RT under light-protection and digested with 350 ng Trypsin at 37°C overnight, all in the presence of 50 mM ABC. Tryptic peptides were extracted from the gel pieces consecutively with 40% ACN containing 0.5% Formic acid (FA), then 60% ACN containing 1% FA and finally 100% ACN for 30 minutes each. Extraction solutions were combined and dried by vacuum centrifugation before reconstitution

in 200 µl 75% isopropanol, 0.75% Trifluoroacetic acid (TFA) and peptide clean-up using SDB-RPS stage tips (Empore discs, 3M) according to Kulak et al., 2014.[3]

LC-MS analysis

Dried peptides were reconstituted in 2% ACN, 0.1% TFA and analysed on a Q Exactive HF mass spectrometer coupled to an easy nLC 1200 (ThermoFisher Scientific) using a 35 cm long, 75µm ID fused-silica column packed in house with 1.9 µm C18 particles (Reprosil pur , Dr. Maisch), and kept at 50°C using an integrated column oven (Sonation). Peptides were eluted by a non-linear gradient from 4-28% acetonitrile over 45 minutes and directly sprayed into the mass-spectrometer equipped with a nanoFlex ion source (ThermoFisher Scientific). Full scan MS spectra (300-1650 m/z) were acquired in profile mode at a resolution of 60,000 at m/z 200, a maximum injection time of 20 ms and an AGC target value of 3 x 10^6 charges. Up to 15 most intense peptides per full scan were isolated using a 1.4 Th window and fragmented using higher energy collisional dissociation (normalised collision energy of 27). MS/MS spectra were acquired in centroid mode with a resolution of 15,000, a maximum injection time of 25 ms and an AGC target value of 1 x 10^5. Single charged ions, ions with a charge state above 5 and ions with unassigned charge states were not considered for fragmentation and dynamic exclusion was set to 20s to minimize the acquisition of fragment spectra of already acquired precursors.

Mass spectrometry data processing

MS raw data was processed with MaxQuant (v 1.6.14.0) applying default parameters. Acquired spectra were searched against the human “one sequence per gene” database (Taxonomy ID 9606) downloaded from UniProt (12-03-2020; 20531 sequences), the

sequence of the FCPF-mutant of Cas9 as well as a collection of 244 common contaminants ("contaminants.fasta" provided with MaxQuant) using the Andromeda search engine integrated in MaxQuant.[4] Identifications were filtered to obtain false discovery rates (FDR) below 1% for both peptide spectrum matches (PSM; minimum length of 7 amino acids) and proteins using a target-decoy strategy.[5] Protein quantification and data normalization relied on the MaxLFQ algorithm implemented in MaxQuant.[4] The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[6] via the PRIDE partner repository[7] with the dataset identifier PXD022199.

During the review process, raw data can be accessed using the following credentials:

Username: reviewer_pxd022199@ebi.ac.uk

Password: C7Ysv4ek

Satistical analysis

The MaxQuant output ("proteinGroups.txt") was processed in Perseus (v. 1.6.14.0)[4]. First, proteins only identified by a single modified peptide ("only identified by site") or matching to the reversed or contaminants databases were removed. Only well-quantified proteins for which at least 2 peptides were identified and showing no missing value in any of the samples were kept for statistical analysis (4436 proteins). Significantly changing proteins were defined by a Student`s t-test (p-value <0.05) adding a minimum fold-change cut-off (> 2).

T7 Endonuclease I assay

AAVS1

On-target sequence: GGGAGGGAGAGCTTGGCAGG**GGG** (PAM sequence is in bold)

On-target Forward: TGGCTACTGGCCTTATCTCACAGG

On-target Reverse: CTCTCTAGTCTGTGCTAGCTCTTCCAG.

Off-target sequence: GGGAAGGGGAGCTTGGCAGGT**GG** (PAM sequence is in bold and expected mutations in red)

Off-target Forward: ACTCTTCTACCTTGCACGCCTTTGC

Off-target Reverse: CCTGCCTCCCATGCAAACAGTGTC

Statistics and reproducibility

Data were analyzed with the GraphPad Prism software (v7.01, La Jolla, CA, USA). Two-way analysis of variance (ANOVA) was performed. All experiments except for proteomics have been independently repeated at least for three times, from which similar results were obtained.

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