PROTAC molecule-mediated SpCas9 protein degradation for precise genome editing

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Abstract

CRISPR (clustered regularly interspaced short palindromic repeats) technology has revolutionized both fundamental research and genomic medicine. The need for precise control of SpCas9-based CRISPR genome editing has resulted in a number of methods to control the function and activity of Cas9 proteins to minimize off-target editing effects. Proteolysis-targeting chimeras (PROTACs) are an emerging technology using the ubiquitin-proteasome system to selectively degrade the target proteins. Here, we developed CASPROTAC 6, a reversible and cell-permeable degrader directly targeting SpCas9 proteins for degradation. The warhead of CASPROTACs was derived by bridging a known SpCas9 binder BRD7087 with an S-substituted Lenalidomide cereblon (CRBN) ligand via the 6-carbon alkyl linker. Our data showed that CASPROTAC 6 is a broad-spectrum degrader of SpCas9 and SpCas9 variants such as dCas9 and Cas9 nickases. The CASPROTAC 6 degraded SpCas9 and the SpCas9-guide-RNA complex via the proteasome system by ~50%. As a result, the CASPROTAC 6 molecule can reduce the CRISPR/Ca9-mediated genome editing by 30%. The CASPROTAC 6 molecules could be used to regulate the CRISPR/SpCas9-associated nucleases for precise genome editing to reduce off-target effects and strengthen biosafety.

Keywords

PROTAC; CRISPR/SpCas9; SpCas9；Genome editing; Off-target

Introduction

CRISPR (clustered regularly interspaced short palindromic repeats) technology has revolutionized how the genome could be manipulated and opened new venues for basic research and genomic medicine[1, 2]. CRISPR-associated systems (Cas) were initially discovered as the adaptive immune responses in many bacteria and archaea against bacteriophages[3, 4]. Currently, the most widely used CRISPR/Cas system for genome editing is the CRISPR/ Streptococcus pyogenes Cas9 (SpCas9), which produces double-strand breaks (DSBs) via guide RNA (gRNA). In general, the complex of SpCas9 and gRNA, via base-pairing of the target DNA with gRNA, recognizes a 20 bp substrate sequence followed by an NGG protospacer adjacent motif (PAM) region in the genome[5]. Then, the complex will make a DNA double-strand break at 3-bp upstream of the PAM sequence. DNA double-strand breaks can be repaired by either homologous recombination or nonhomologous end-joining repair, which sometimes may lead to unfaithful DNA repair or insertions, resulting in permanent changes in the genome[6]. Based on the CRISPR/SpCas9 system, the CRISPR/dCas9 (catalytic dead SpCas9) has also been developed for genome editing. It does not make DNA double-strand breaks but could still bind to the genomic DNA via the guide RNA. Once the CRISPR/dCas9 is linked to different effector domains, such as VP64 for activation, KRAB for repression, or base editors that could change the base pairs, these systems could also lead to epigenome or permanent genome editing[7].

Although the CRISPR/SpCas9 systems are easy to apply, potential undesirable off-target effects are still the major concerns for basic research and clinical applications[8, 9]. Recent evidence indicates that even the on-target effects of CRISPR/SpCas9 could lead to unwanted genome editing such as chromosomal loss within human embryo cells[10]. To reduce the off-target DNA cleavages, the Acrs (anti-CRISPR proteins) were linked to SpCas9 via genetic fusion, which fine-tuned the SpCas9 activity to desired (lower) levels. As a result, Cas-Acr variants improved genome editing fidelity by keeping on-target editing efficiencies comparable to wild-type SpCas9 and reducing off-target editing[11]. It indicates that the observed off-target effects are mostly likely due to the hyperactivation of SpCas9. To regulate the activity of the SpCas9 protein, small molecule inhibitors have also been developed [12, 13]. However, there are some limitations associated with both methods. It is hard to administer the small inhibitory proteins into cells and bodies, and the activity of these protein-based inhibitors is also dependent on their half-lives. Small molecule inhibitors often work in a dose-dependent manner, and their activity may be associated with side effects[14].

Proteolysis-targeting chimeras (PROTAC) is an emerging technology that takes advantage of the intracellular ubiquitin-proteasome system to selectively degrade the protein of interest (POI) [15]. PROTAC works as a chimera molecule that binds to POI on one end and to an E3 enzyme on the other end. By bringing the target protein in the close vicinity of the E3 enzyme, it will recruit the E2 enzyme to transfer the ubiquitin to the POI. The poly-ubiquitinated POIs will then be degraded by the ubiquitin-proteasome system (UPS) [15-17]. This heterobifunctional small molecule offers a promising opportunity to control the CRISPR/SpCas9 system. It has been shown in computational modeling that the ratio between on-target and off-target cleavage drops during time during genome editing[18]. Therefore, by reducing the half-life of SpCas9 and its variants, it is possible to reduce the off-target effects of the genome editing system. In this study, we developed a series of PROTAC molecules to target SpCas9, dCas9 and nCas9 proteins for degradation for optimal genome editing. We optimized the original designs and identified CASPROTAC 6 as the most optimal molecule that can degrade SpCas9, dCas9, nCas9, and RNP complexes by 50% and reduce the CRISPR editing by 30%. This molecule may be used in genome medicine by controlling the half-life of SpCas9 and its variants to reduce the off-target effects.

Results

The design of CASPROTAC molecules to degrade Cas9 proteins

As shown before by the computational analysis, elevated or prolonged activities of genome editing may further contribute to off-target editing [19-22]. It is possible to regulate the half-life of SpCas9 proteins to reduce the unwanted off-target effects. Thus, firstly, we determined the half-life of SpCas9/dCas9 proteins. We observed that these proteins had a relatively long half-life and remained in cells for 5-7 days (Supplementary Figure 1). To show how the on-target editing and off-target effects accumulate by CRISPR/SpCas9, we designed GFP-Offtarg gRNAs (1 or 2 nucleotide mismatches to the gRNA targeting the GFP sequence, i.e. GFP Ontarg) to mimic the off-target effects of CRISPR/SpCas9 mediated genome editing by targeting the GFP coding sequence. Results showed that the on-target gRNA edited GFP sequences, therefore diminishing the GFP fluorescence quickly, and reached the editing plateau (~47%) on day 3. During the same period, the GFP-Offtarg gRNA also edited the GFP sequence and reached their editing plateau on day 3, with an off-target effect of 16.5% and 1.43%, respectively (Supplementary Figure 2). These results are consistent with previous observation that CRISPR/SpCas9 could recognize genomic sequences with the matching targeting regions[23], leading to unwanted cutting/editing, and

the off-target editing also accumulate during time when CRISPR/SpCas9 complexes are still present.

Therefore, we developed PROTAC molecules that directly target SpCas9 proteins for degradation to reduce the half-life of SpCas9 proteins (Figure 1). We named these molecules CASPROTACs. The warhead (WH) of CASPROTACs was derived from a known SpCas9 binder BRD7087, the binding of which to SpCas9:gRNA has been validated by ^{19}F NMR spectroscopy [24]. A series of CASPROTACs was first synthesized by bridging the warhead and S-substituted Lenalidomide, a novel CRBN ligand with various lengths of alkyl linkers ranging from 1 to 10 carbons (Figure 1, 2 and Supplementary File 1, 2) [25, 26]. Then, the effects of these molecules on degrading SpCas9 proteins were tested in K562 cells. K562 cells were electroporated with 60 pmol of recombinant SpCas9 proteins, the concentration of which is often used in genome editing experiments [27]. One day post electroporation, cells were treated with 1uM of the respective CASPROTACs (1 - 4) and the warhead (as the control) for another 24 hrs, before the level of the recombinant SpCas9 proteins was tested. The results indicated that CASPROTAC 3 and 4 with longer linkers may have better SpCas9 degradation capacity (Figure 2B and C). We further tested the optimal concentration of these CASPROTAC molecules on degrading SpCas9 proteins. K562 cells electroporated with SpCas9 proteins were treated with a serial dilution of CASPROTAC 3 and 4. These two CASPROTACs achieved the best degradation effect at a concentration of 1 µM, with a maximal degradation (Dmax) of 20% and an obvious “hook effect” that has been well-documented for PROTAC molecules (Figure 2D and E).

Optimized CASPROTAC molecules degrade SpCas9 protein efficiently

Data from the initial design of the CASPROTACs suggest that a longer linker may increase the SpCas9 degradation efficiency. Therefore, further optimization was made to shorten or extend the linker length of these CASPROTACs (5 - 10) (Figure 1, 2 and Supplementary File 1, 2). These molecules were tested on K562 cells electroporated with SpCas9 proteins at 1uM for 24 hrs. Two molecules CASPROTAC 6 and CASPROTAC 10 showed a better efficiency, achieving around 50% protein reduction of SpCas9 upon treatment (Figure 3B and C). Then, a serial dilution of the CASPROTAC 6 and CASPROTAC 10 was tested; again these two molecules showed a SpCas9 degradation profile similar to typical PROTAC molecules, with the optimal concentration at 1uM (Figure 3D and E). As CASPROTAC 6 showed highly efficient SpCas9 degradation, we focused on this molecule for further testing. To test the optimal duration to apply CASPROTAC 6, time course experiments were performed. K562 cells electroporated with SpCas9 proteins were treated with 1 µM of CASPROTAC 6, and the results showed that 24 hrs treatment was sufficient to reach max

degradation (Dmax) of ~50% (Figure 3F and G). These data showed that the optimized CASPROTAC 6 degraded SpCas9 protein efficiently at the 24 hrs time point.

The CASPROTAC degrades SpCas9 and the SpCas9-guide-RNA complex via the proteasome system

PROTAC molecules rely on the proteasome degradation system to remove the target proteins [28]. To further confirm that the designed CASPROTAC molecules degrade SpCas9 proteins via the endogenous proteasome systems of the cells, K562 cells electroporated with SpCas9 proteins were co-treated with proteasome inhibitor MG132 (500 nM) and CASPROTAC 6 for 24 hrs. The degradation of SpCas9 proteins was stopped completely when the activity of proteasome was blocked, as shown by the accumulation of poly-ubiquitinated species in the cell lysate (Figure 4 A-C), indicating that CASPROTAC 6 functions as a bona fide PROTAC. As SpCas9 proteins need to assemble with guide RNAs into the ribonucleoprotein complexes (RNP) before it could function, we further tested if CASPROTAC 6 is capable of degrading the RNP complexes. The recombinant SpCas9 proteins were first incubated with synthesized guide RNAs in vitro to form functional RNP complexes. Then K562 cells were electroporated with the respective proteins, before treated with CASPROTAC 6 molecules. As shown in Figure 4D and F, CASPROTAC 6 could degrade the RNP complex as efficiently as SpCas9 proteins, indicating that this molecule could target the SpCas9 proteins in different forms to regulate genome editing.

CASPROTAC 6 degrades CRISPR/SpCas9 variants and reduces CRISPR editing efficiency

Besides SpCas9 proteins that are able to make DNA double-strand breaks for genome editing, many other SpCas9 variants were also developed for various applications, either via generating single-strand breaks or only binding to DNA without making DNA breaks. Catalytic dead SpCas9 proteins (dCas9) that could only bind DNA but not make DNA breaks are broadly used in basic research for regulating the epigenome and gene transcription. From the previous study, the SpCas9 inhibitors (BRD20322 and BRD0048) were shown to be the most potent inhibitors of the transcription activation complex [24]. To test if the CASPROTAC molecules still bind to dCas9 to degrade dCas9 proteins and therefore regulate the activity of dCas9 proteins, the 293T cells that conditionally express dCas9-APEX2 were treated with CASPROTAC 6. These cells express dSpCas9-APEX2 when treated with doxycycline for transcription initiation, and the expression would stop once doxycycline is removed. Using this inducible system to monitor the degradation efficiency of dCas9 proteins by CASPROTAC 6, the results showed that 1uM CASPROTAC 6 degraded dCas9 proteins by 50% after 24 hrs treatment, which is as efficient as degrading SpCas9

proteins (Figure 5 A-B). Furthermore, similar to the effects on SpCas9 proteins, a dose-dependent degradation of dCas9 proteins was also observed with CASPROTAC 6 (Figure 5 A-B).

Recently, prime editors and base editors were also developed and widely used, which use SpCas9 nickase (nCas9) fused to enzymes that mediate genome modification or alter the chemical nature of DNA bases[29]. To further investigate whether the CASPROTAC 6 is a broad-spectrum degrader of SpCas9 and SpCas9 variants, 293T cells that express inducible nCas9-PE2 or nCas9-BE3RA were treated with CASPROTAC 6. The results showed that CASPROTAC 6 also degraded nCas9 with different effectors after 24 hrs with 1uM concentration, which is consistent with previous data (Figure 5 C-F).

To evaluate the effect of CASPROTAC 6 on CRISPR editing, 293T cells were electroporated with the FANCF site 2-RNP, which targets the region Chr:22,625,792-22,625,811 [30]. 6 hrs after electroporation, the transfected cells were treated with CASPROTAC 6 for 24 hrs. The on-target effect of FANCF-RNP was monitored by amplicon sequencing. As shown in Figure 5G, CASPROTAC 6 changed the FANCF-RNP on-target editing from 56.69% to 40,67%, which is about a 30% reduction (Figure 5H). The pattern of genome editing at the targeting site showed that the CRISPR-induced nucleotide deletions were reduced by CASPROTAC 6 effectively (Figure 5I). Thus, optimized CASPROTAC 6 degraded SpCas9 variants protein efficiently and effectively reduced the CRISPR editing efficiency.

Discussion

Genome editing via the CRISPR/SpCas9 system has revolutionized modern genetic research and genome medicine. Despite being a powerful tool, there are also some limitations and concerns. For instance, as a genome medicine tool, whilst CRISPR/SpCas9 system can produce on-target genomic changes, it may also generate undesirable genotoxicity and adverse events via cleaving non-targeted sites[31]. It is estimated that there are 2.98 x 10^8 expected “NGG” PAM sites in the human genome, which is approximately equal to one potential CRISPR/SpCas9 targeting site per 10 bases [32]. In reality, CRISPR/SpCas9 could also recognize some similar genomic sequences with only 1- or 2-nucleotide differences from the matching targeting regions, leading to unwanted cutting/editing[23, 31]. Such events are, in theory, less frequent but will generate unwanted editing. If such off-target editing happens in the coding regions of important genes, it may lead to severe side effects when used as genome medicine. Hence, the need to precisely

control the CRISPR/SpCas9 system has raised an urgent demand for the inhibition of CRISPR activity.

From a high-throughput screening, BRD0539 has been identified as the SpCas9 small-molecular inhibitor, which provides a chemical means to block the SpCas9-induced genome editing [24]. Although BRD0539 showed a stronger inhibitory effect on CRISPR/SpCas9-mediated genome editing, such inhibitors are non-covalently bound to the SpCas9 proteins, therefore the inhibitory effects are reversible. Proteolysis-targeting chimeras (PROTACs) are heterobifunctional small molecules consisting of a chemical warhead and ligand of E3 ligase joined by a linker. The warhead binds the POI and brings an interaction with the E3 enzymes via the ligand, which promotes ubiquitination and proteasomal degradation of the POI[33]. In this study, we developed potential PROTAC molecules based on the SpCas9 binder BRD7087, which we named CASPROTAC (Figure 1 and Table 1). Upon chemical optimization, We developed a molecule, CASPROTAC 6 that was able to degrade SpCas9 proteins by ~50% at 1 μM during 24 hrs treatment (Figure 3 A-B). In contrast, the inhibitory efficiency of BRD0539 to SpCas9 in the eGFP-disruption assay is about ~50% at a higher concentration of more than 10 μM [24]. Thus, CASPROTAC 6 may require a lower dose to regulate CRISPR/SpCas9-mediated editing. The CASPROTAC molecule 6 showed the most effective SpCas9 degradation profile, as well as the well-documented “hook effect” seen with small molecule-based PROTACS[34]. Furthermore, we also tested if CASPROTAC molecule 6 also degrades other SpCas9 variants. SpCas9 variant (dCas9/nCas9)-based technologies, including priming editing, base editing, and transcription activation/inhibition systems, were widely used to introduce the facile introduction of genomic alterations and also broadly used in both basic research and clinical trials[29]. We showed that CASPROTAC 6 could also degrade dCas9/nCas9 proteins as efficiently as degrading SpCas9 proteins (Figure 5), indicating that CASPROTAC 6 could regulate the dCas9/nCas9-Based CRISPR systems. Both SpCas9 and dCas9/nCas9 need to assemble with guide RNAs into the RNP before they can function. CASPROTAC 6 could also degrade the RNP complex efficiently (Figure 4D). These indicate that CASPROTAC 6 is a broad-spectrum degrader of SpCas9 and its variants. The degradation of SpCas9 proteins eventually affected the genome editing efficiency. We showed that the CASPROTAC 6 inhibited the FANCF-RNP-mediated genome-editing at the endogenous locus of the genome(Figure 5D). Partial degradation of SpCas9 and its variants by CASPROTAC 6 may provide a new and alternative method for reducing the off-target activity of SpCas9.

Materials and methods

Chemical synthesis

Reagents and solvents were purchased from commercial sources without further purification unless otherwise indicated. Starting materials of 10 are synthesized according to the patent WO2019196812. The progress of reactions was monitored by thin-layer chromatography (TLC) and/or LC-MS. The final compounds were purified by prep-HPLC and characterized by ^{1}H NMR/^{13}C NMR and HRMS. NMR spectra were recorded on Ascend 400 MHz Bruker spectrometer (operating at 400 MHz for 1H NMR and 126 MHz for 13C NMR), chemical shifts were reported in ppm relative to the residual CH_3OD (3.31 ppm ^{1}H) or d_6-DMSO (2.50 ppm ^{1}H) and coupling constants (*J*) are given in Hz. The detail of the synthesis of the BRD7087 analogue was described in supplemental file 1. Multiplicities of signals are described as follows: s --- singlet, br. s --- broad singlet, d --- doublet, t --- triplet, m --- multiple. High Resolution Mass spectra were recorded on solan X 70 FT-MS spectrometer.

Lentivirus production and MOI determination

For generating the pLenti-TRE3G-BE3RA-PGK-Puro (Addgene 110846) virus, the 293T cells were grown in 6 well plates. When cells reached 50% confluency, they were transfected with the mix of 1 µg of pLenti-TRE3G-BE3RA-PGK-Puro plasmid, 0.6 µg of psPAX2, 0.4 µg of pCMV-VSV-G, and 6 µg PEI (Polyscience, 23966) in 50 µl of serum-free medium. Media supernatant containing the virus particles was collected on the third day after transfection.

Cell culture and cell line

K562 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Biowest), L-Glutamine (Gibco), and 1% Penicillin-Streptomycin (Gibco), at 5% CO_2 in a humidified 37°C incubator. 293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Biowest), L-Glutamine (Gibco), and 1% Penicillin-Streptomycin (Gibco).

For 293T-Tet-On 3G cells generation, the wild-type 293T cell line was grown in 6 well plates (50% confluency) and co-transfected with 1.0 µg Inducible Caspex expression (Addgene 97421) plasmid and 1.0 µg PiggyBac Transposase vector using 6 µg PEI (Polyscience, 23966) in 50 µl of serum-free medium and selected with 3 µg ml^{-1} Puromycin (Biomol) for 2-3 days. Single cells were seeded in 96-well plates and expanded.

For 293T-nCas9-PE2 bulk cells, the wild-type 293T cell line was grown in 6 well plates (50% confluency) and co-transfected with 1.0 µg Xlone-PE2 (Addgene 136463) plasmid and 1.0 µg PiggyBac Transposase vector 6 µg PEI (Polyscience, 23966) in 50 µl of serum-free medium and selected with 3 µg ml^{-1} Blasticidin (Biomol) for 2-3 days.

For 293T-nCas9-BE3RA bulk cells, the wild-type 293T cell line was infected with pLenti-TRE3G-BE3RA-PGK-Puro virus at MOI of 0.3. Post-infection 2 days, cells were selected with 3 μg ml-1 Puromycin (Biomol) for 2-3 days. The selected cells were transfected 1.0 μg XLone-GFP (Addgene 96930) plasmid and 1.0 μg PiggyBac Transposase vector. Post-transfection 2 days, using doxycycline to induce GFP expression, then the GFP positive bulk population were sorted.

Reagents

Doxycycline was obtained from Sanbio B.V. Proteasome inhibitor MG132 was purchased from MedChemExpress (HY-13259). Recombinant Streptococcus pyogenes SpCas9 proteins (with HA tag) were produced by the protein facility of Leiden University Medical Center (LUMC), based on the plasmid pET28a/SpCas9-Cys (Plasmid #53261).

Guide RNA Synthesis and ribonucleoprotein complexes formulation

The guide RNAs targeting EGFP sequence were produced by in vitro transcription using the following primer sequences:

Common-scaffold-primer:

AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC;

gRNA-GFP-ontarg:

TTCTAATACGACTCACTATAGGACCAGGATGGGCACCACCCGTTTTAGAGCTAGA;

gRNA-GFP-ontarg #1:

TTCTAATACGACTCACTATAGGACCAGGATGGGCACCCCCCGTTTTAGAGCTAGA

gRNA-GFP-ontarg #2:

TTCTAATACGACTCACTATAGGACCAGGATGTGTACCACCCGTTTTAGAGCTAGA

FANCF-ontarg:

TTCTAATACGACTCACTATAGGCTGCAGAAGGGATTCCATGGTTTTAGAGCTAGA

These primers were annealed and made into double-strand DNA using NEBNext High-Fidelity 2X PCR Master Mix: 98 °C for 2 mins, 60 °C for 1 min and 72 °C for 3 min. For each reaction, 25 ul of 2X PCR Master Mix, 5 ul of T7 -Guide-EGFP (10uM), 5 ul of Common scaffold primer (10 uM) and 15 ul of Nuclease-free water were pooled. The pooled PCR products were further purified using the QIAGEN MinElute column. The gRNAs were transcribed from the DNA template and purified according to the manufacturer's protocol (Agilent SureGuide gRNA Synthesis Kit, 5190-7714, 5190-7719).

To formulate ribonucleoprotein RNP complexes, Cas9 protein and sgRNA for per sample were mixed and preincubated for 20 min at RT. After 20 min of incubation of Cas9 and sgRNA, the RNP complex solution is used for further experiments.

Western blot

Treated cells were washed with cold phosphate-buffered saline (PBS) and lysed in 1X RIPA buffer (Thermo Scientific) containing the protease inhibitor cocktail (Roche). The cell lysate was sonicated followed by centrifugation. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of proteins were separated via 8% or 12 % SDS-PAGE. The separated proteins were transferred to PVDF membranes, blocked with 5% nonfat milk in PBST for 1 h at room temperature. Membranes were probed with primary antibodies against HA (Cell Signaling Technology H9658, 1:1000), Flag (Sigma F1804, 1:1000), SpCas9 (Cell Signaling Technology 14697S, 1:1000), ubiquitin (Abcam ab19247, 1:1000), and β-actin (Sigma A5441, 1:5000) as the loading control overnight at 4°C. Then, the membranes were washed with PBST three times, incubated with the appropriate secondary antibodies (1:10000 dilution) for 1 h at room temperature, and then washed with PBST three times. The resulting signal was visualized by Bio-Rad ChemiDoc Imaging System using the Pierce enhanced chemiluminescence (ECL) Plus Western Blotting Substrate (Thermo Scientific). The intensity of the staining was quantified using Image Lab software (6.0.1).

Amplicon Sequencing and analysis

293T cells were electroporated with FANCF-RNP using the SF Cell Line 4D-Nucleofector kit (Lonza) following the pulse program of CM-130. Transfected cells were plated at a 6-well plate (Corning), and 6 hrs after electroporation, CASPROTAC 6 was added for another 24 hrs. The cells were harvested, and the genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Amplicon sequencing samples were prepared via two-step PCR following the protocol reported previously[30]. Amplicon sequences were analyzed by CRISPResso 2.

Statistical analyses

Data were analyzed with the GraphPad Prism software (version 8). Statistical tests were performed using the Student's t-test. P values less than 0.05 were considered to indicate a statistically significant difference.

Contributions

B.P. conceived and supervised the study. X.Y. and R.S. designed and synthesized the CASPROTACs. S.S. designed and performed the experiments, and analyzed the data, with the help of M.K. M.T. analyzed the amplicon sequencing data. S.S. and B.P. wrote the manuscript with inputs from all authors.

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Competing interests

The authors have filed a patent on the results of this research.

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Figure 1: **The workflow of CASPROTAC molecules.**

CASPROTAC works by designing a chimera molecule that binds to the protein of interest on the one end (derived from SpCas9 binder BRD7087) and binds to an E3 enzyme on the other end (S-substituted Lenalidomide, a novel cereblon ligand). Then, the E3 enzyme will recruit the E2 enzyme that transfers the ubiquitin to the SpCas9, leading to the degradation of the polyubiquitinated SpCas9 protein.

Figure 2: **The initial design of CASPROTAC molecules exhibited degradation efficacy.**

A. Chemical structures of CASPROTACs #1-4. **B.** Western blotting was used to test the efficacy of the first batch of CASPROTACs in degrading SpCas9 proteins. K562 cells were electroporated with 60 pmol SpCas9 protein, followed by incubation with warhead and CASPROTACs 1-4 (1 μM) for 24 hrs. **C.** Quantification of the intensity of the staining from A. **D.** K562 cells were electroporated with 60 pmol SpCas9 protein, followed by incubation with CASPROTACs 3 and 4 in a dose-dependent manner. Western blotting was used to quantify the efficacy of SpCas9 protein degradation. **E.** Quantification of the intensity of the staining from C. Anti-HA antibody was used to detect the level of recombinant Cas9-HA proteins. Anti-β-actin was used as the loading control. Bars show mean value ± s.e.m. and significance was calculated using Student's t-test (n = 2 or 3). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0001$ (versus the control).

Figure 3: **Optimized CASPROTAC molecules degraded SpCas9 efficiently.**

A. Chemical structures of CASPROTACs #5-10. **B.** Western blotting was used to test the efficacy of the optimized CASPROTAC molecules in degrading SpCas9 proteins. K562 cells were electroporated with 60 pmol SpCas9 protein, followed by incubation with CASPROTACs 5-10 (1 μM) for 24 hrs. **C.** Quantification of the intensity of the staining from A. **D.** Optimized CASPROTAC molecules led to dose-dependent degradation of SpCas9 proteins. Western blotting was used to test the degradation of SpCas9 proteins by CASPROTAC 6 and 10. K562 cells were electroporated with 60 pmol SpCas9 protein, followed by incubation with 6 and 10 in a dose-dependent manner for 24 hrs. **E.** Quantification of the intensity of the staining from C. **F.** Western blotting was used to test the degradation of SpCas9 proteins by CASPROTAC 6 in different time points. K562 cells were electroporated with 60 pmol SpCas9 protein were followed at the respective time points with and without CASPROTAC 6 treatment. **G.** Relative quantification was made between the treated and non-treated cells based on the results from E. Anti-HA antibody was used to detect the level of recombinant Cas9-HA proteins. Anti-β-actin was used as the loading

control. Bars show mean value ± s.e.m. and significance was calculated using Student's t-test (n = 2 or 3). *p < 0.05, **p < 0.005, and ***p < 0.0001 (versus the control).

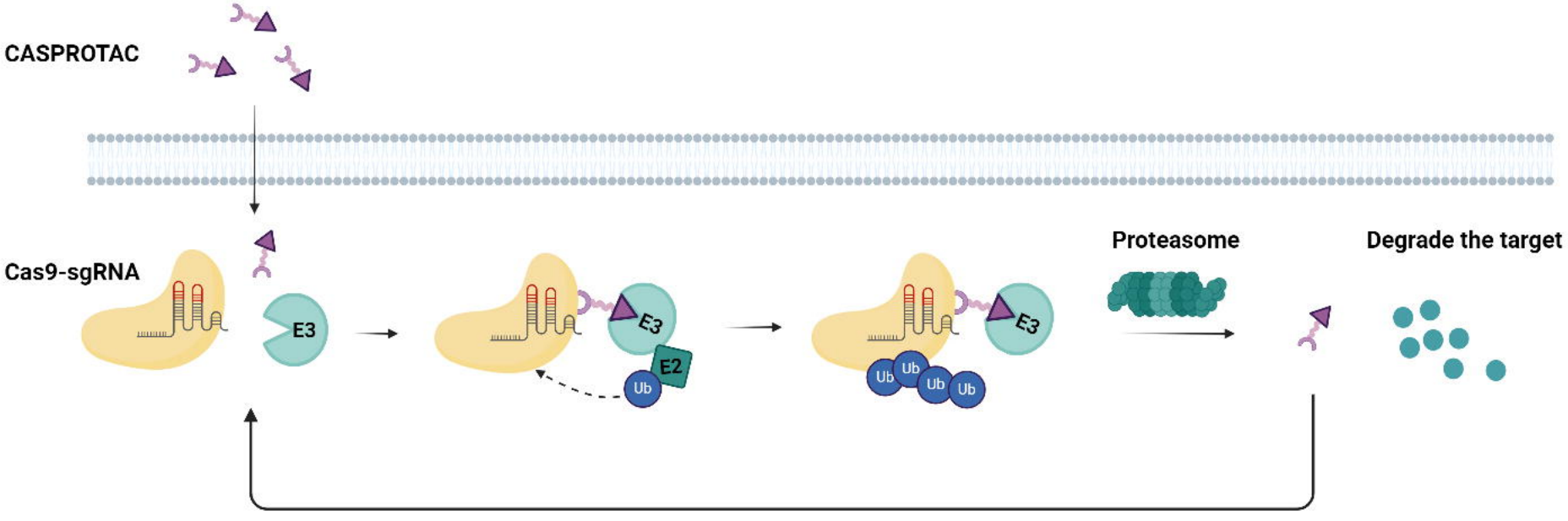
Figure 4: **The optimized CASPROTAC molecule degrades SpCas9 and the Cas9-guide-RNA complex via the proteasome system.**

A. The proteasome inhibitor MG132 blocked the degradation of electroporated SpCas9protein by CASPROTAC 6 in K562 cells. Western blotting was used to test CASPROTAC 6 in K562 cells after electroporated with 60 pmol SpCas9 protein, followed by incubation with CASPROTAC 6 (1 µM) and MG132(500 nM) for 24 hrs. B. The intensity of the SpCas9 protein staining was quantified based on A. **C.** The intensity of the poly-ubiquitinated protein staining was quantified based on A. **D.** CASPROTAC 6 degraded CRISPR ribonucleoprotein complexes efficiently. Mix the EGFP-guide RNA and SpCas9 protein components at a 1:1.2 molar ratio (60 pmol SpCas9+ 72 pmol guide RNA-EGFP for 1 million cells), incubating at room temperature for 20 min to allow the formation of the CRISPR ribonucleoprotein complexes. Western blotting for CASPROTAC 6 in K562 cells after electroporated with 60 pmol SpCas9protein or RNP, followed by incubation with CASPROTAC 6 for 24 hrs; **F.** Quantification of the intensity of the staining from D. Anti-HA antibody was used to detect the level of recombinant Cas9-HA proteins. The anti-β-actin was used as the loading control. The anti-ubiquitin antibody was used to test the general ubiquitin levels of proteins. Bars show mean value ± s.e.m. and significance was calculated using Student's t-test (n = 2 or 3). *p < 0.05, **p < 0.005, and ***p < 0.0001 (versus the control).

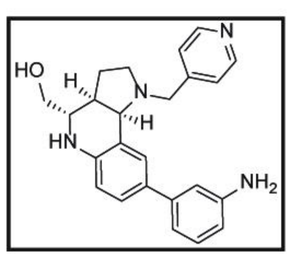
Figure 5: **Optimized CASPROTAC 6 degraded CRISPR/SpCas9 toolbox and reduced CRISPR editing efficiency**

A. Optimized CASPROTAC 6 degraded dCas9 proteins. 293T-Tet-On 3G cells with stable integration of dCas9-APEX2 were pretreated with doxycycline (500 ng/mL) for 9 h to the simultaneous expression of Cas9-APEX2 protein complex, 15 hrs after removing doxycycline, CASPROTAC 6 was added for 24 hrs in a dose manner. The protein level of dCas9-APEX2 was monitored by Western blotting. **B.** Quantification of the intensity of the staining from A. Anti-Flag antibody was used to detect the level of dCas9-APEX-Flag proteins. Anti-β-actin was used as the loading control. **C.** The 293T-nCas9-PE2 bulk cells were pretreated with doxycycline (500 ng/mL) for 24 h, then removing doxycycline, CASPROTAC 6 was added for 24 hrs. The protein level of nCas9-PE2 was monitored by Western blotting**. D.** Quantification of the intensity of the staining from C. Anti-Cas9 antibody was used to detect the level of dCas9-APEX-Flag proteins. Anti-β-actin was used as the loading control. **E.** 293T-nCas9-BE3RA bulk cells were pretreated with doxycycline (500 ng/mL) for 24 h. Then, after removing doxycycline, CASPROTAC 6 was added for another 24 hrs. The protein level of nCas9-BE3RA was monitored by Western blotting. **F.** Quantification of the intensity of the staining from E. Anti-Cas9 antibody was used to detect the level of dCas9-APEX-Flag proteins. Anti-β-actin was used as the loading control. Bars show mean value ± s.e.m. and significance was calculated using Student's t-test (n = 2 or 3). *p < 0.05, **p < 0.005, and ***p < 0.0001 (versus the control). **G.** CASPROTAC 6 reduced CRISPR editing efficiency. FANCF-gRNA and SpCas9 protein were mixed at a 1:1 molar ratio (20 pmol SpCas9+ 20 pmol FANCF-gRNA for 1 million cells) and incubated at room temperature for 20 min to allow the formation of the CRISPR ribonucleoprotein complexes. 293T cells were electroporated with FANCF-RNP. Six hours after

electroporation, cells were incubated with CASPROTAC 6 for 24 hrs. CRISPR editing efficiency was measured by amplicon sequencing. **H.** The reduction of CRISPR/SpCas9-mediated editing based on the amplicon sequencing results. **I.** Batch output mode comparing CRISPR editing of control and CASPROTAC 6-treated cells. Each column represents the base composition of a single nucleotide at the FANCF site 2 locus. The percentage of each base or indel at each nucleotide is shown as a proportion of each row (n=1). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0001$ (versus the control).


CASPROTAC
Cas9-sgRNA
E3
E3
E2
Ub
E3
Ub
Ub
Ub
Ub
Proteasome
Degrade the target

A



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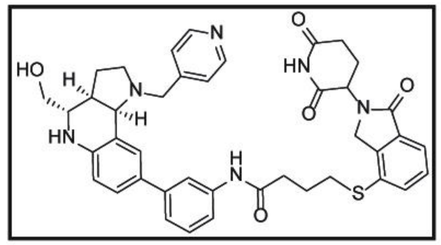

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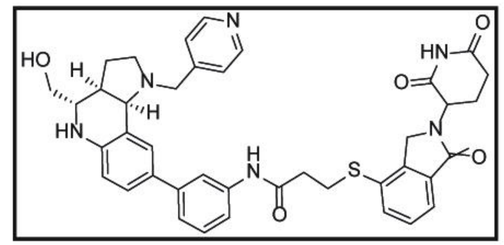

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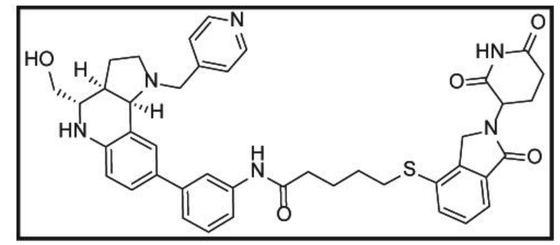

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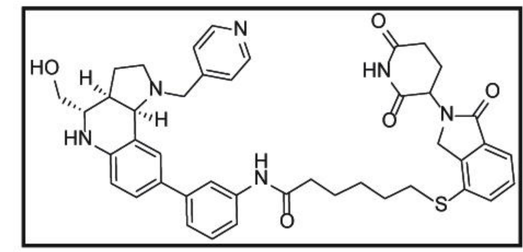

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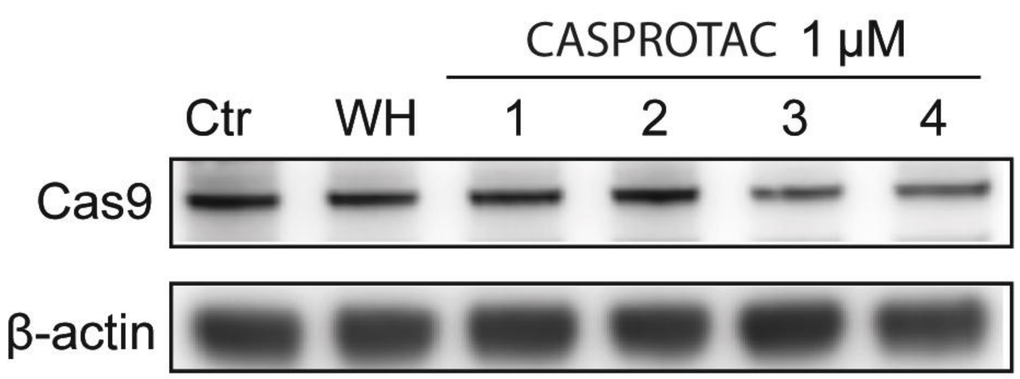

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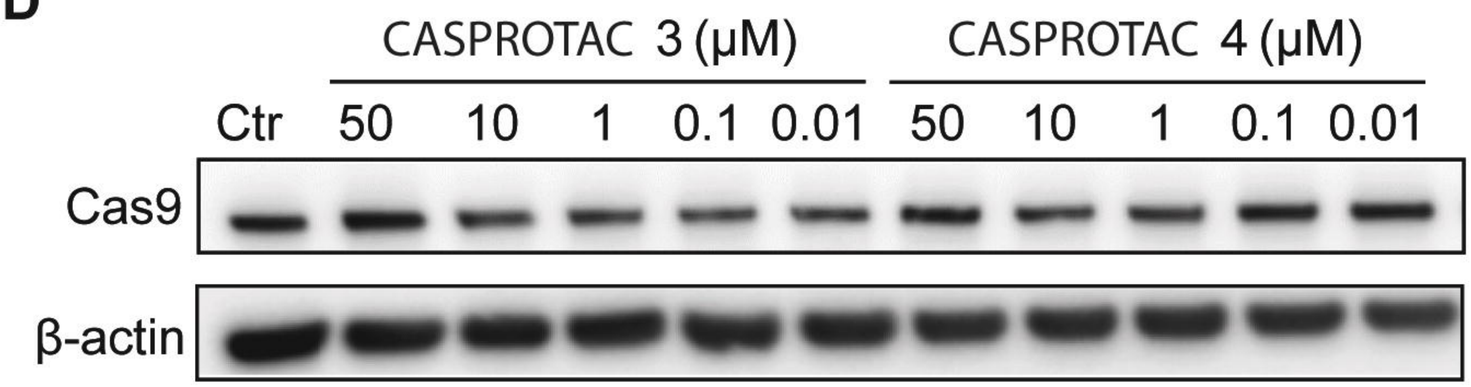

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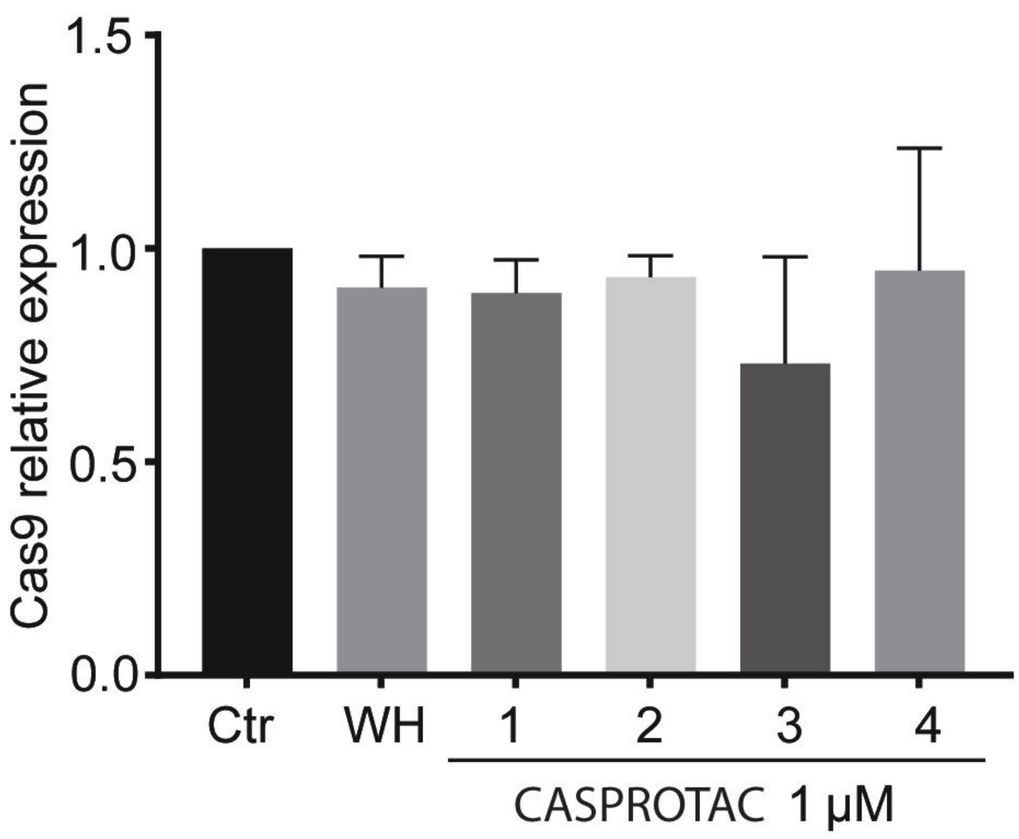

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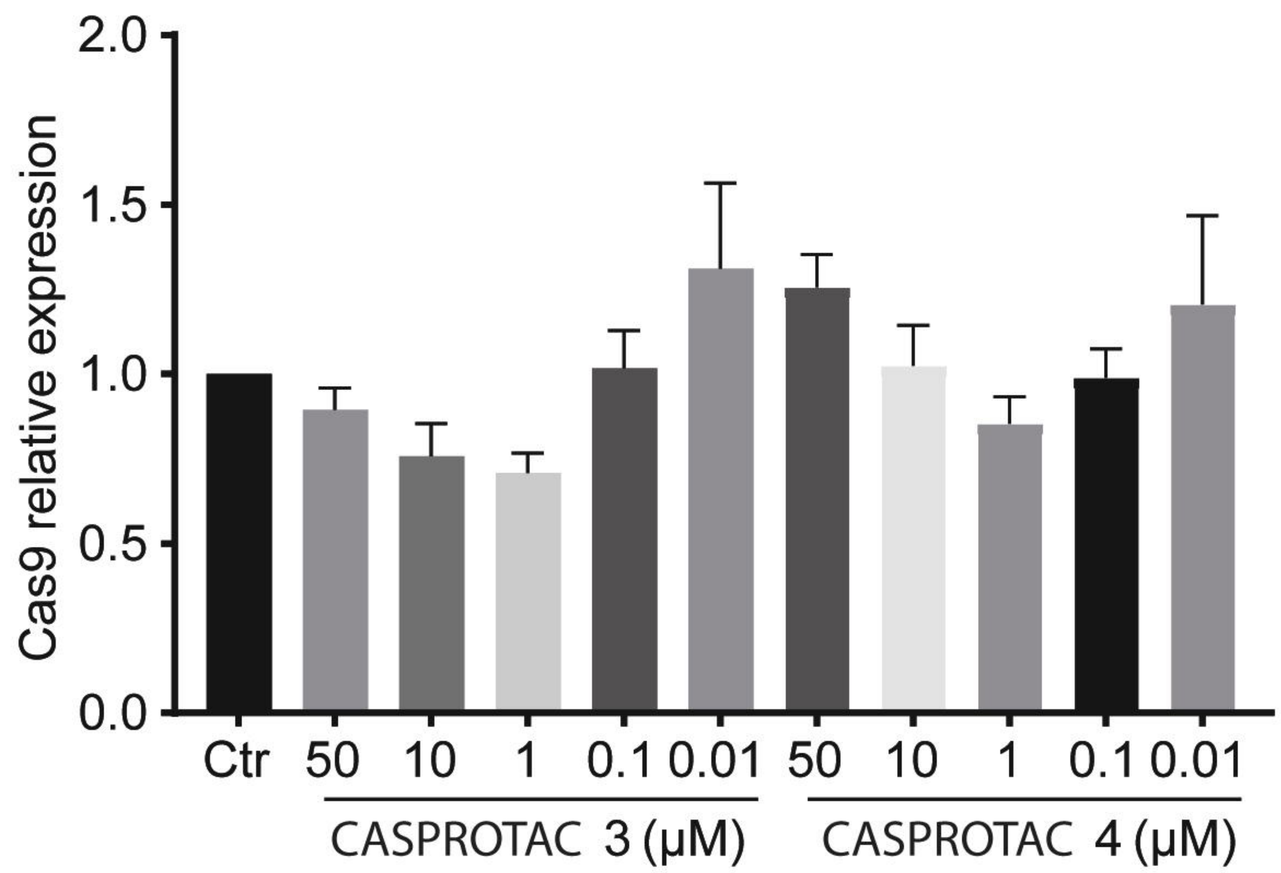

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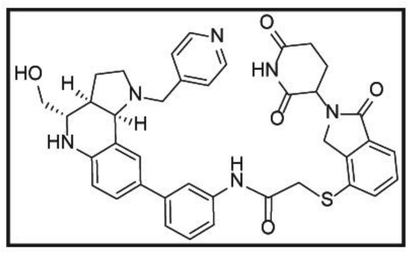

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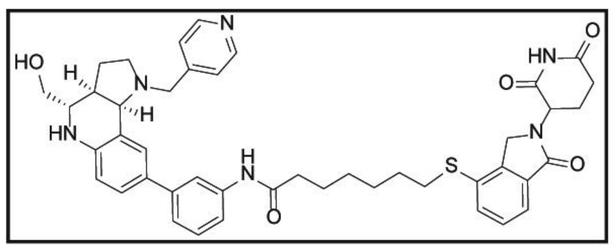

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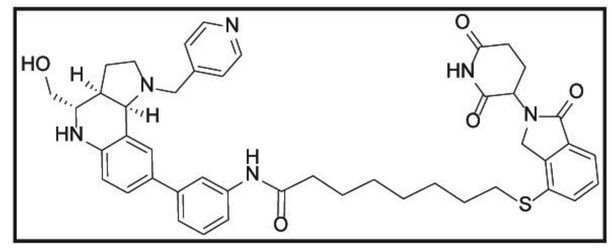

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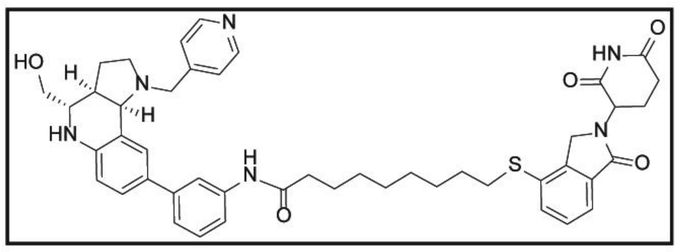

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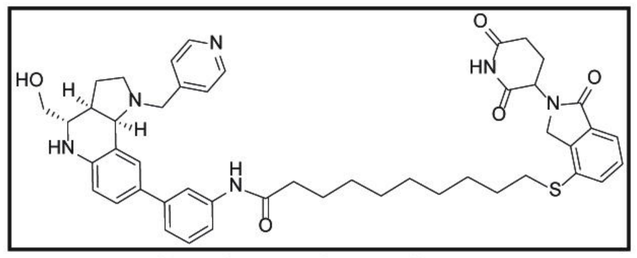

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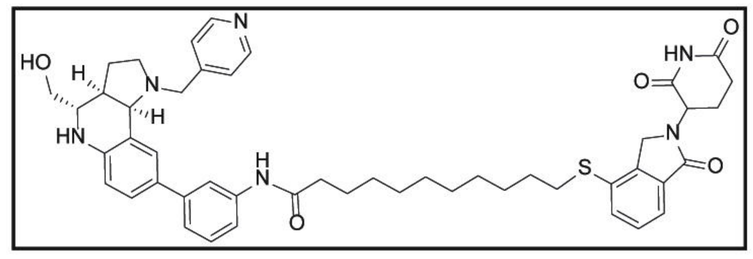

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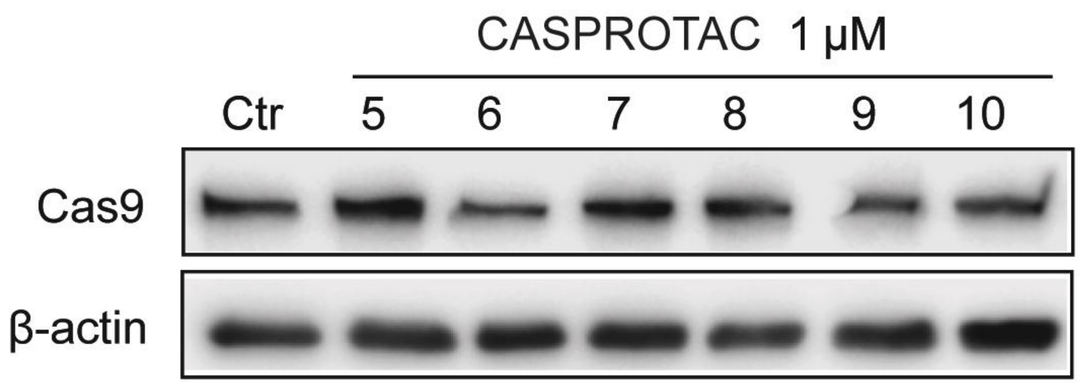

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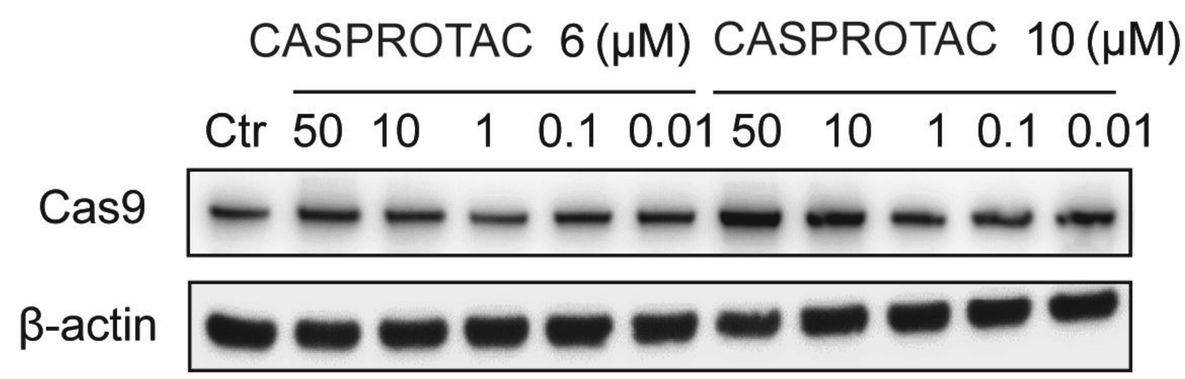

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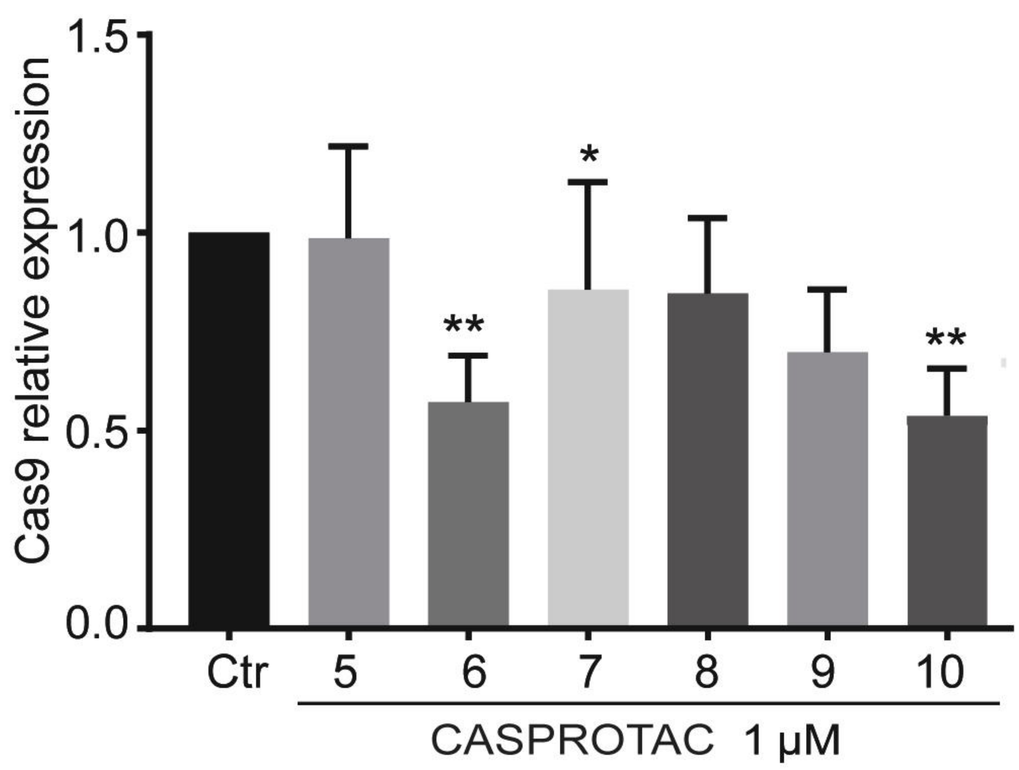

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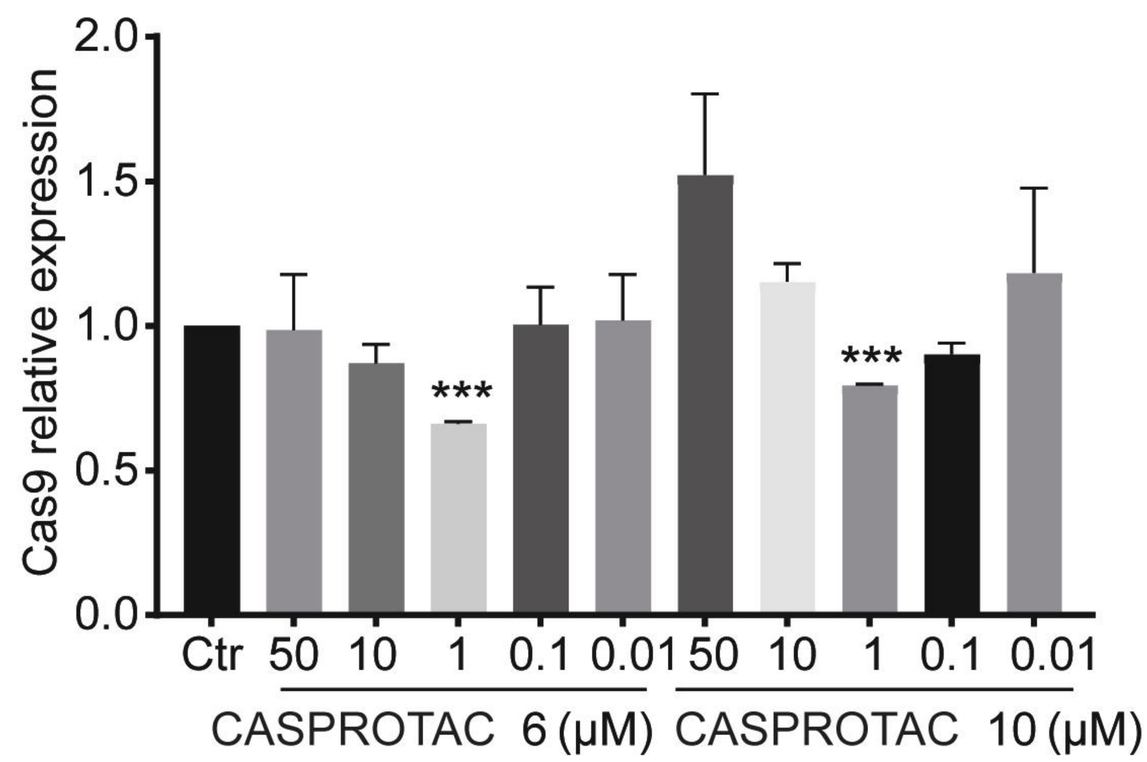

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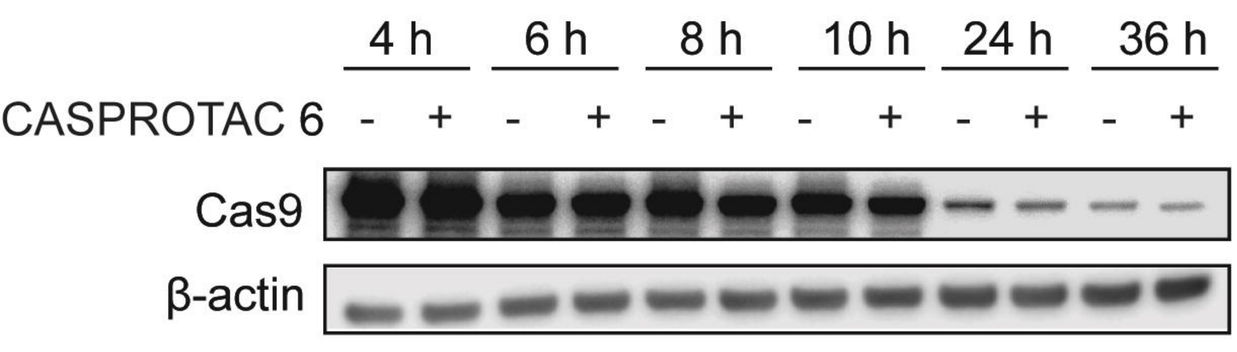

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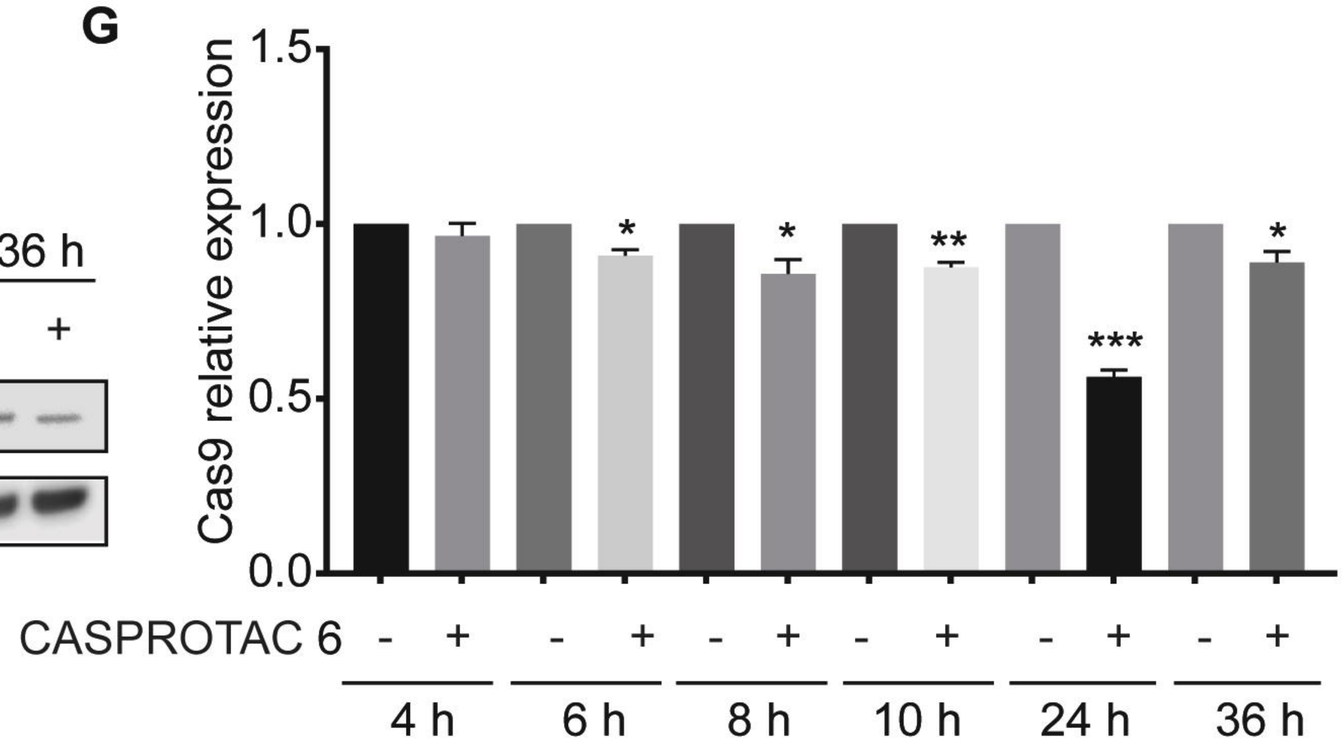

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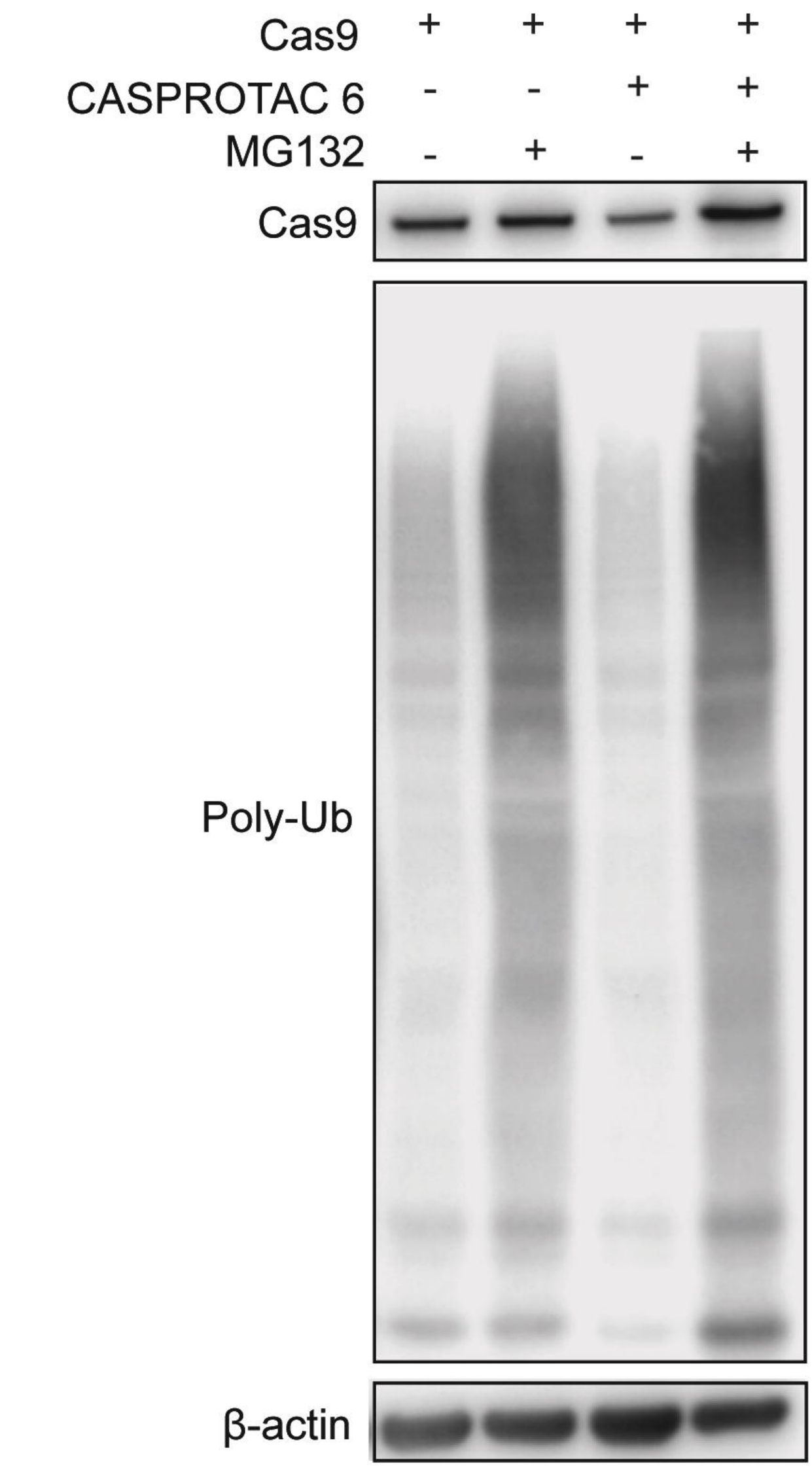
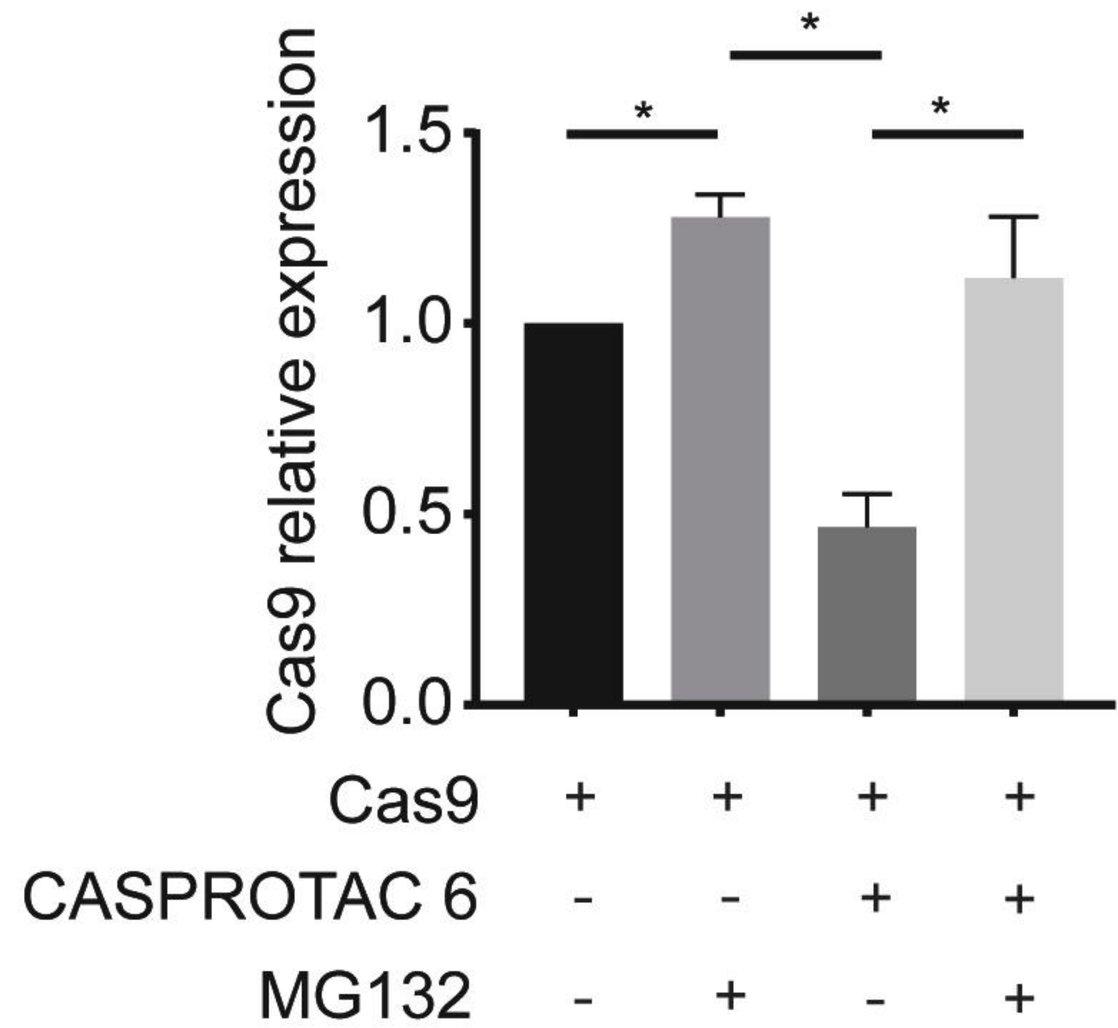

Cas9 + + + +
CASPROTAC 6 - - + +
MG132 - + - +
Cas9
Poly-Ub
β-actin

B


Cas9 relative expression
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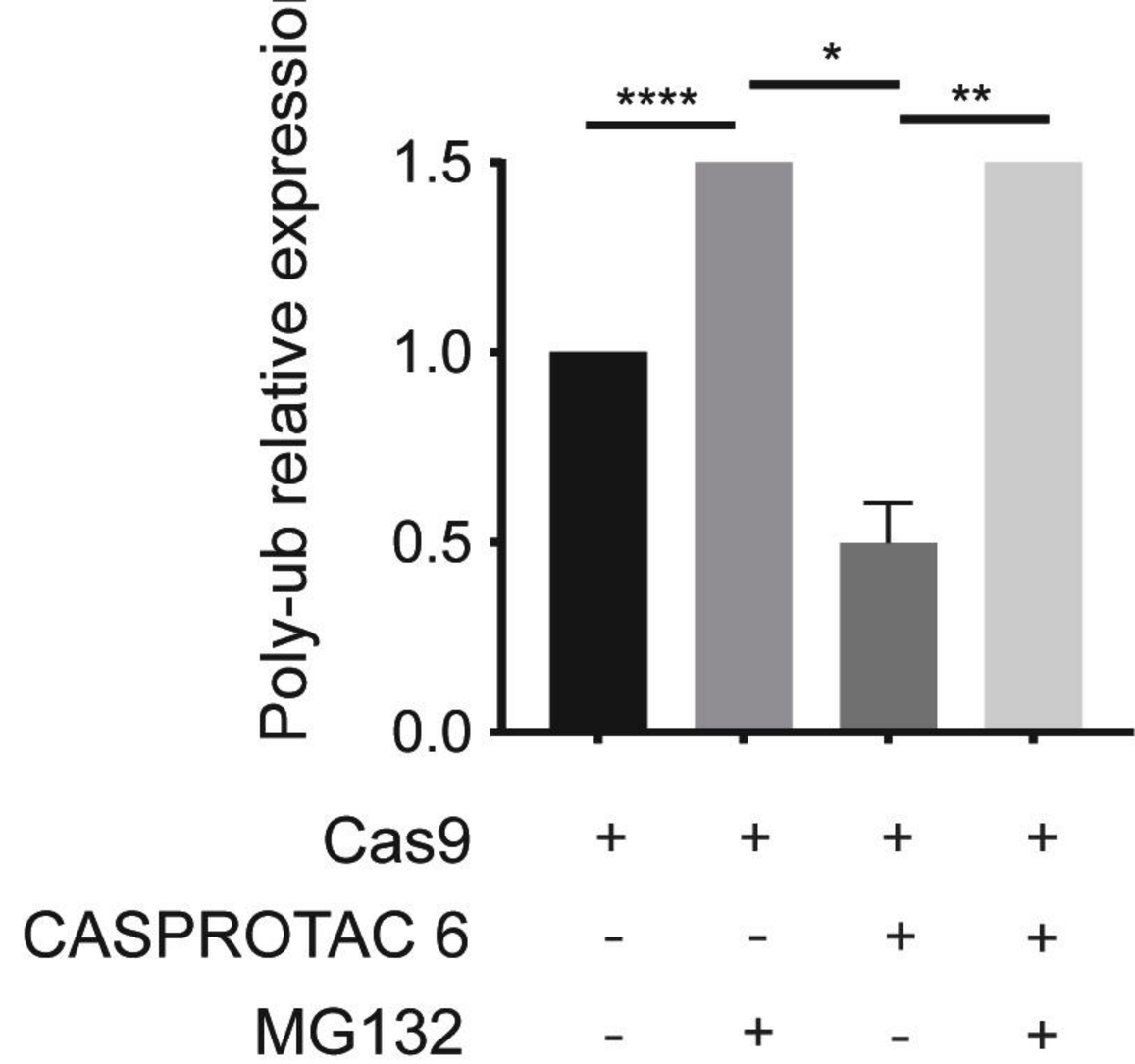
1.0
0.5
0.0
*
*
*
Cas9 + + + +
CASPROTAC 6 - - + +
MG132 - + - +

C


Poly-ub relative expression
1.5
1.0
0.5
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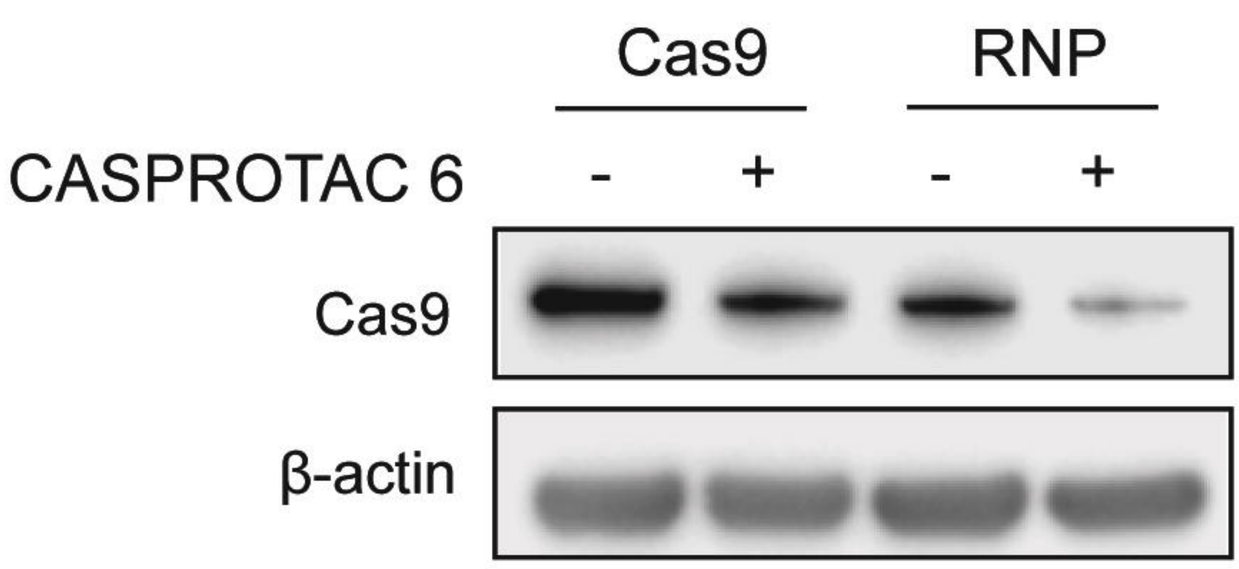
*
**
Cas9 + + + +
CASPROTAC 6 - - + +
MG132 - + - +

D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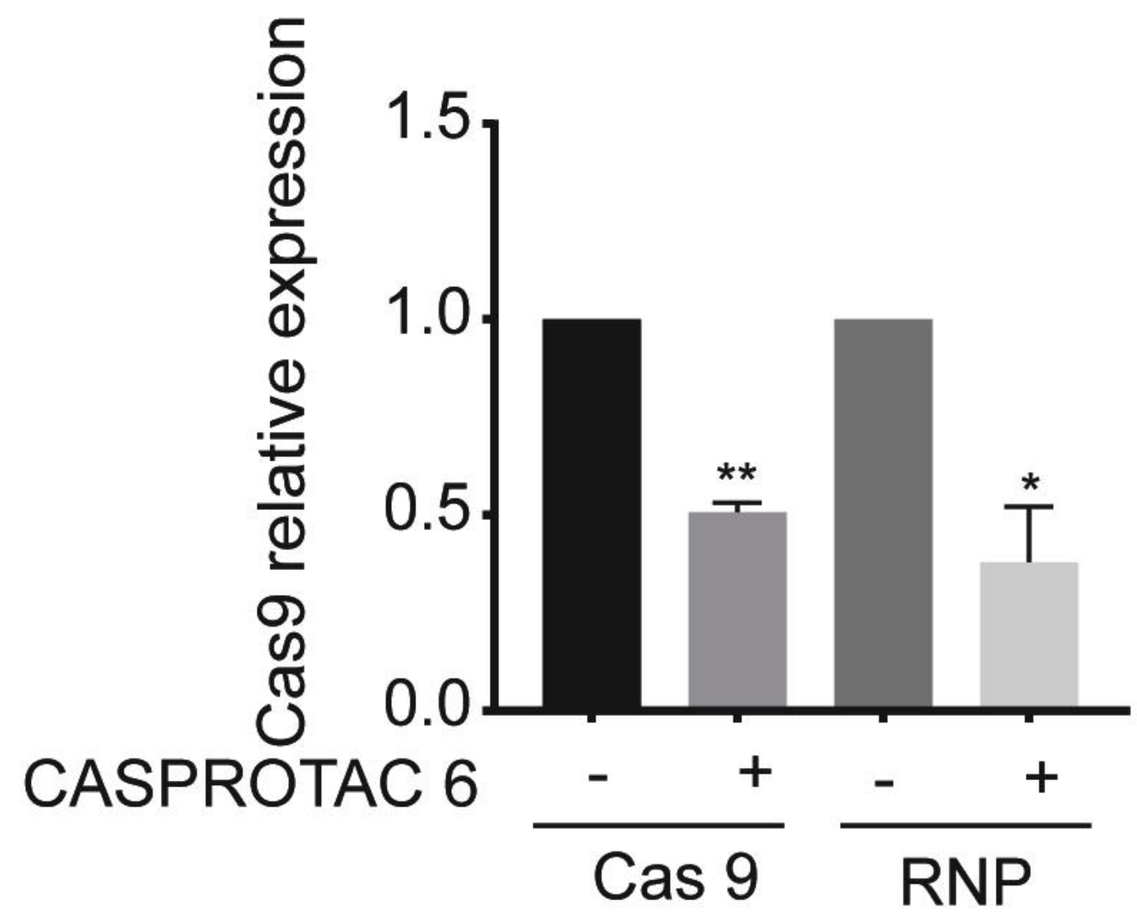
RNP
CASPROTAC 6 - + - +
Cas9
β-actin

F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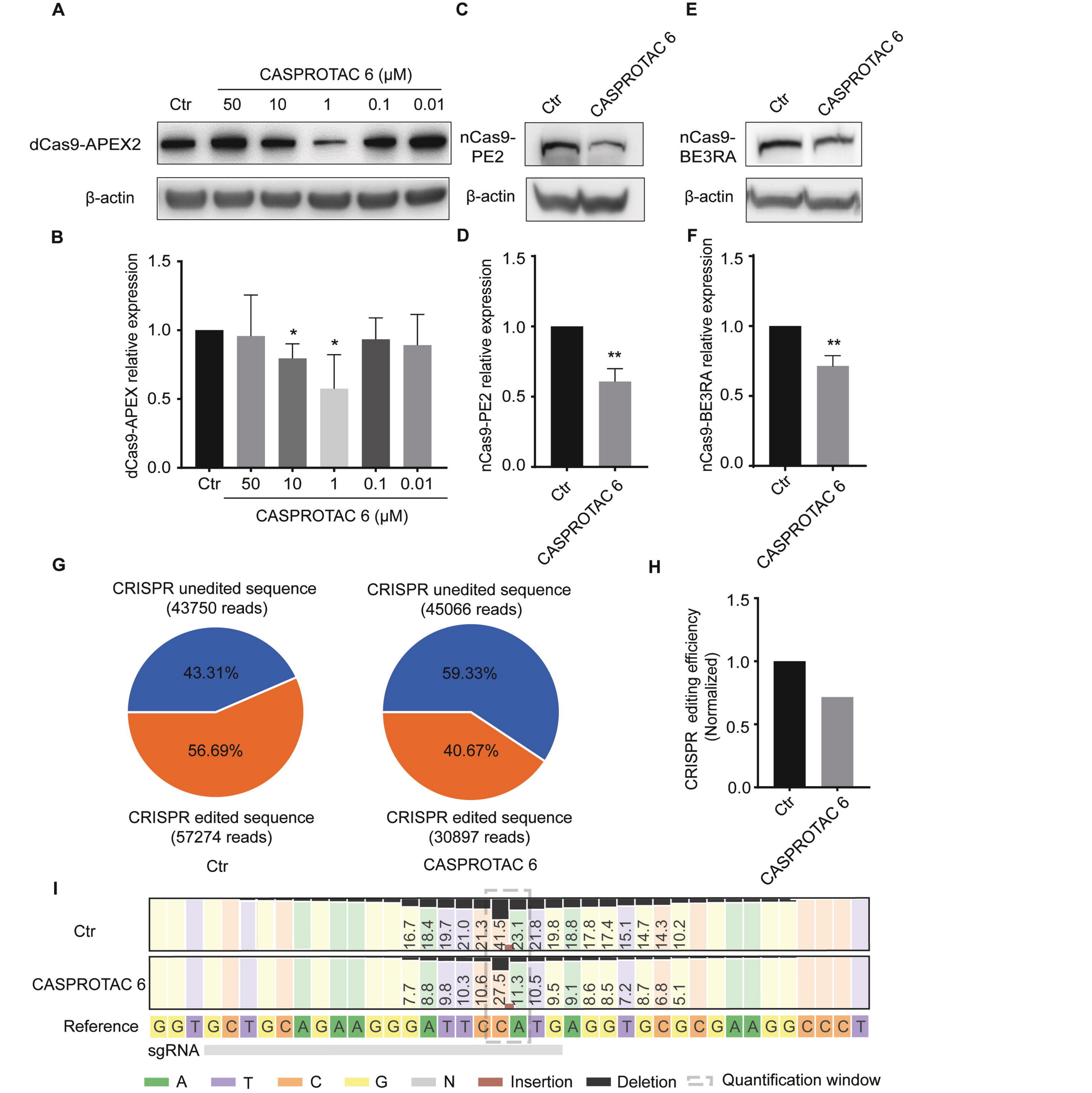
1.0
0.5
0.0
**
*
CASPROTAC 6 - + - +
Cas 9
RNP


A
CASPROTAC 6 (μM)
Ctr
50
10
1
0.1
0.01
dCas9-APEX2
β-actin
C
Ctr
CASPROTAC 6
nCas9-PE2
β-actin
E
Ctr
CASPROTAC 6
nCas9-BE3RA
β-actin
B
dCas9-APEX relative expression
1.5
1.0
0.5
0.0
*
*
Ctr
50
10
1
0.1
0.01
CASPROTAC 6 (μM)
D
nCas9-PE2 relative expression
1.5
1.0
0.5
0.0
**
Ctr
CASPROTAC 6
F
nCas9-BE3RA relative expression
1.5
1.0
0.5
0.0
**
Ctr
CASPROTAC 6
G
CRISPR unedited sequence
(43750 reads)
43.31%
56.69%
CRISPR edited sequence
(57274 reads)
Ctr
CRISPR unedited sequence
(45066 reads)
59.33%
40.67%
CRISPR edited sequence
(30897 reads)
CASPROTAC 6
H
CRISPR editing efficiency
(Normalized)
1.5
1.0
0.5
0.0
Ctr
CASPROTAC 6
I
Ctr
16.7 18.4 19.7 21.0 21.3 41.5 23.1 21.8 19.8 18.8 17.8 17.4 15.1 14.7 14.3 10.2
CASPROTAC 6
7.7 8.8 9.8 10.3 10.6 27.5 11.3 10.5 9.5 9.1 8.6 8.5 7.2 8.7 6.8 5.1
Reference
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sgRNA
A
T
C
G
N
Insertion
Deletion
Quantification window