A Chemical Toolbox for Labeling and Degrading Engineered Cas Proteins

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ABSTRACT: The discovery of clustered regularly interspaced short palindromic repeats and their associated proteins (Cas) has revolutionized the field of genome and epigenome editing. A number of new methods have been developed to precisely control the function and activity of Cas proteins, including fusion proteins and small-molecule modulators. Proteolysis-targeting chimeras (PROTACs) represent a new concept using the ubiquitin-proteasome system to degrade a protein of interest, highlighting the significance of chemically induced protein-E3 ligase interaction in drug discovery. Here, we engineered Cas proteins (Cas9, dCas9, Cas12, and Cas13) by inserting a Phe-Cys-Pro-Phe (FCPF) amino acid sequence (known as the π-clamp system) and demonstrate that the modified CasFCPF proteins can be (1) labeled in live cells by perfluoroaromatics carrying the fluorescein or (2) degraded by a perfluoroaromatics-functionalized PROTAC (PROTAC-FCPF). A proteome-wide analysis of PROTAC-FCPF-mediated Cas9FCPF protein degradation revealed a high target specificity, suggesting a wide range of applications of perfluoroaromatics-induced proximity in the regulation of stability, activity, and functionality of any FCPF-tagging protein.

KEYWORDS: CRISPR/Cas9, genome editing, π-clamp, Cas9 inhibitor, PROTAC, chemical-induced proximity

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems (Cas) constitute a family of nucleases responsible for adaptive immune responses in many bacteria and archaea against bacteriophages.1 In general, Cas enzymes use CRISPR RNA (crRNA), obtained from the incorporation of fragments of the invading genetic material, as a guide to target and cleave invading nucleic acids.1,2 These proteins have been classified into two classes (1 and 2) and six subtypes (I−VI) based on their molecular mechanisms. In comparison to class I proteins (types I, III, and IV), the class 2 Cas proteins (types II, V, and VI) utilize single nuclease effectors guided by crRNA to target nucleic acids. These nucleases have attracted attention among molecular biologists and biotechnologists.1,3 The most widely used type 2 protein is Cas9 from Streptococcus pyogenes Cas9 (SpyCas9, hereinafter referred to as Cas9); however, Cas9 proteins from other bacteria have also been shown to be effective.2 Cas9 proteins detect double-stranded DNA (dsDNA) and produce double-strand breaks (DSBs), resulting in genomic deletions. The dsDNA can then be repaired by either nonhomologous end-joining repair or homologous recombination, which can then mediate gene replacement or insertions.3 The most well-known example of type V proteins is Cas12 (isoform a and b), isolated from Prevotella buccae and Francisella novicida. The molecular mechanisms of Cas12 proteins display minimal differences to those of Cas9, although they both detect dsDNA and induce DSBs. Cas12 is also able to produce a collateral detection and trans-cleavage of single-stranded DNA (ssDNA), which makes it a powerful tool in several applications. In contrast, type VI proteins (Cas13a, Cas13b, and Cas13c) from Leptotrichia sp and Prevotella sp, among others, are RNA-guided ribonucleases, by means of their two higher eukaryote and prokaryote nucleotide-binding domains that identify and degrade single-stranded RNA (ssRNA), thus allowing a post-transcriptional regulation of gene expression, nucleic acid detection, RNA knockdown, and transcript tracking in mammalian cells and plants.4−7

Although the first in-human phase 1 clinical study has reported that CRISPR-Cas9-edited T-cells are well-tolerated by patients,8 off-target effects are still the major concern in its clinical applications. Studies have shown that the precise regulation of Cas9 activity significantly reduces the off-target effect, hinting that a hyperactivation of Cas9 is most likely the reason for the observed off-target DNA cleavages.9−11

Received: January 6, 2021
Published: May 12, 2021
Recently, we and other groups showed the impact of small-molecule inhibitors on the activity of Cas9. However, chemical inhibitors usually have multiple targets, which may be associated with severe side effects. Liu and co-workers inserted a 4-hydroxytamoxifen-dependent intein into Cas9 and achieved a specific Cas9 activation. Indeed, fusing an activator/repressor into nuclease-dead Cas9 (dCas9) has become a general strategy for CRISPR-dCas9-mediated epigenome editing. One of the technical challenges is the delivery of such large chimeric proteins into living cells and organisms. Moreover, highly expressed exogenous epigenetic regulators may impact the nature of cells and induce significant side effects. Thus, it is urgently required to develop a new CRISPR-(d)Cas9 system for a more specific and flexible modulation.

Proteolysis-targeting chimera (PROTAC) represents a recent approach that uses the intracellular ubiquitin-proteasome system in order to degrade a protein of interest (POI). A PROTAC is a heterobifunctional small molecule, consisting of two ligands connected by a linker: one targeting a proteasome system in order to degrade a protein of interest (POI). A PROTAC is a heterobifunctional small molecule, consisting of two ligands connected by a linker: one targeting a POI, and the other one recruiting an E3 ligase. The strategy of PROTAC to degrade a POI by inducing a POI-E3 ligase interaction offers a wide range of applications in the aspect of chemically induced proximity in drug development. Here, we inserted a sequence of four amino acids Phe(F)-Cys(C)-Pro(P)-Phe(F), also known as a π-clamp, into Cas proteins (herein referred to as CasFCPF). We demonstrate that this site-specific chemical modification made Cas9FCPF recognizable by perfluoraromatics as reported previously by Zhang et al., which could be utilized for labeling Cas9FCPF in living cells by a fusion of a fluorophore into perfluoraromatics, or to degrade FCPF tagging Cas proteins, including Cas9FCPF, dCas9FCPF, Cas12FCPF, and Cas13FCPF, by a new synthetic perfluoraromatics-containing PROTAC molecule (PROTAC-FCPF). This approach provides a new postexpression strategy to chemically regulate the stability, activity, and function of Cas and other proteins in genome editing and gene therapy.

### RESULTS

#### Labeling of Cas9FCPF with FITC-FCPF in Living Cells

The π-clamp system provides a useful approach to select FCPF-engineered proteins—for instance, the insertion of this short sequence has been used to form antibody-drug conjugates. To test if the π-clamp system can be applied for the recognition of exogenously expressed proteins, such as the Cas9 protein, we inserted FCPF at the C-terminus of Cas9, whose expression is controlled by a cytomegalovirus (CMV) promoter (Figure 1A). We first developed a simple labeling method to detect a Cas9FCPF expression by using a synthetic fluorescein (FITC)-conjugated perfluoraromatic moiety, which we obtained by coupling fluoro-1,1-biphenyl with NHS-activated 6-fluorescein (referred to as FITC-FCPF, Figure 1A in green). The synthesis is comprehensively described in the Materials and Methods section. We observed the anticipated green fluorescence signal in HeLa cells expressing Cas9FCPF after an incubation with FITC-FCPF (10 μM) for 2 h, which was similar to that of Cas9GFP (Figure 1B and Figure S1A). A fluorescence-activated cell sorting (FACS) analysis confirmed that the intensity of the green fluorescence signal in cells, expressing Cas9FCPF and treated with FITC-FCPF labeling, was similar to that in cells expressing Cas9GFP (Figure 1C and Figure S1B). That intensity was almost 20-fold higher compared to FITC-FCPF-treated cells expressing Cas9WT (Figure 1C and Figure S1B).
indicated by a strong signal at a molecular weight of ~150 kDa when observed under UV light (Figure 1D and Figure S1C), which might indicate the formation of a stable irreversible covalent bond between FCPF and perfluoroaromatics.22

PROTAC-FCPF Degradates Cas9\(^{\text{FCPF}}\)

We designed a perfluoroaromatics-based PROTAC molecule to investigate if a heterobifunctional small molecule can induce an artificial Cas9\(^{\text{FCPF}}\)-E3 ligase interaction for the regulation of Cas9\(^{\text{FCPF}}\) stability. Several compounds have been previously reported as ligands of E3 ligases for the development of PROTACs, including thalidomide, which binds to cereblon (CRBN).20,23 The replacement of FITC with the thalidomide derivative lenalidomide (Figure 1A in orange) yielded a novel PROTAC molecule that we called PROTAC-FCPF (a PROTAC-targeting FCPF amino acid sequence). In HeLa cells treated with 10 \(\mu\)M PROTAC-FCPF we detected the reduction of Cas9\(^{\text{FCPF}}\) as early as 6 h (Figure 2A and Figure S2A). The depletion lasted at least up to 72 h (Figure 2B and Figure S2B). Very recently, Sreekantan et al. developed a dTAG-system to degrade Cas9FKBP. They demonstrated that the small molecular degrader, dTAG-47, degraded Cas9 carrying two FK506-binding proteins (FKBPs, Cas9FKBP) at the N-terminal and loop region at concentrations of 0.1-1 \(\mu\)M in a 24 h treatment, which significantly increased the specificity of the CRISPR-Cas9 system.24 We evaluated the activity of PROTAC-FCPF for Cas9\(^{\text{FCPF}}\) degradation in both 8 and 24 h treatments. We found that 10 \(\mu\)M PROTAC-FCPF robustly degraded the Cas9\(^{\text{FCPF}}\) protein (Figure 2C and Figure S2C). A similar effect was observed at nanomolar concentrations in a 24 h treatment with an IC\(_{50}\) value of ~150 nM (Figure 2D and Figure S2D). Of note, higher concentrations (>500 \(\mu\)M) resulted in an attenuated degradation (Figure 2C and Figure S2C). Douglass et al. developed a comprehensive mathematical model for analyzing a three-component system, which at least partially elucidated the autoinhibition in the formation of ternary complex.25 This so-called hook effect has been also repeatedly described in both reversible and irreversible covalent PROTACs.26 Preincubation with free lenalidomide (50 \(\mu\)M) inhibited PROTAC-FCPF-mediated Cas9\(^{\text{FCPF}}\) degradation, which was probably caused by a competitive binding to CRBN E3 ligase (Figure 2E and Figure S2E). By contrast, the Cas9\(^{\text{WT}}\) protein was not affected by PROTAC-FCPF (Figure 2A and Figure S2F), suggesting that the PROTAC-mediated degradation of Cas9 protein was FCPF-specific. Moreover, we found a dose-dependent degradation of Cas9\(^{\text{FCPF}}\) induced by PROTAC-FCPF in HEK293T cells (IC\(_{50}\): 167.2 nM) similar to that in HeLa cells (IC\(_{50}\):143.4 nM, Figure 2D and Figure S2D). Because primary cells are more relevant to the application of CRISPR-Cas9-mediated genome editing, we tested PROTAC-FCPF-induced degradation in Jopaca-1 cells, a polymorphic primary cell line established from poorly differentiated ductal adenocarcinoma.27 The IC\(_{50}\) value was 72.0 nM in a 24 h treatment, which was slightly better than those in HeLa and HEK293T cells (Figure 2D and Figure S2D).

The T7E1 assay has been widely used to evaluate the efficiency of CRISPR-Cas9-mediated genome editing.28,29 and was recruited to evaluate if PROTAC-FCPF interrupted the function of the Cas9 protein on the AAVS1 gene, as recently established and reported by Niopak's lab.7 We detected similar efficiencies of delivery (Figure S2G) and genome editing for Cas9\(^{\text{FCPF}}\) and Cas9\(^{\text{WT}}\) (Figure 2F), implying that insertion of the FCPF sequence did not affect the biological activity of the Cas9 protein. As expected, we found that PROTAC-FCPF (1 \(\mu\)M) reduced the cleavage of target DNA in cells after a 24 h treatment, with similar activity as the anti-CRISPR/Cas9...
protein (AcrIIA4), which was coexpressed with Cas9 and AAVSI sgRNA.30 The result suggested that the PROTAC-FCPF-degraded Cas9FCPF efficiently repressed the biological function of the CRISPR-Cas9FCPF system in a time-controlled fashion. In agreement with the result reported by Srekanth et al.,24 the incubation of PROTAC-FCPF (1 μM) for 24 h reduced the off-target effect from 12.1% to 3.56% (Figure S2H), implying that Cas9 degraders can reduce the off-target effects of a CRISPR/Cas9 system.

**PROTAC-FCPF-Mediated Cas9FCPF Degradation Is Dependent on CRBN-Associated Ubiquitination**

PROTAC-mediated target protein degradation requires the ubiquitination machinery.31 As expected, coinubcation of PROTAC-FCPF (10 μM) with the proteasome inhibitor MG132 (5 μM), for 8 h, repressed the Cas9FCPF degradation (Figure 3A and Figure S3A). In comparison to Cas9WT cells, the level of polyubiquitination induced by PROTAC-FCPF in cells expressing Cas9FCPF was much higher compared to cells expressing Cas9WT (Figure 3A). Because lenalidomide recruits the CRBN E3 ligase for PROTAC-associated degradation,31,32 the PROTAC-FCPF-induced destabilization of the Cas9FCPF protein was considerably reduced in CRBN knockout HeLa cells (CRBN KD) using a specific small interfering RNA against CRBN (Figure 3B and Figure S3B), while Cas9WT was not affected (Figure S3B). Next, we generated HeLa CRBNKO cells using a CRISPR-Cas9 knockout system as previously reported (Figure S3C).32 In total six CRBNKO cell lines CRBNKO showed the best knockout efficiency and were used for an in vitro ubiquitination assay (Figure S3C). We overexpressed Cas9WT or Cas9FCPF in this CRBNKO cell line and collected cell extracts for an in vitro ubiquitination assay. The reaction was initiated with the addition of purified FLAG-CRBN. The degradation of Cas9 proteins was compared in the presence or absence of PROTAC-FCPF (100 μM for 90 min). As shown in Figure 3C (Figure S3D), only the Cas9 with the FCPF sequence was degraded in the presence of CRBNWT and PROTAC-FCPF, while no e

![Figure 3.](image-url)
was reproducible from three independent experiments. Eight of a total of 4436 protein are highlighted due to their significant alternations in PROTAC-FCPF-treated cells expressing Cas9WT as compared with those in Cas9WT cells. Cas9 was highlighted in red. Detailed information regarding the experiment protocol and an analysis of proteomics can be found in Materials and Methods.

MAPKAPK3.26 To determine the cellular specificity of PROTAC-FCPF-mediated Cas9CPFP degradation, we treated HeLa cells expressing either Cas9WT or Cas9CPFP with dimethyl sulfoxide (DMSO) (mock) or PROTAC-FCPF (10 μM) for 6 h and performed a proteome-wide analysis. The experimental and analytic details were exclusively described in Materials and Methods. We could quantify in total 4436 proteins in all samples in our high-resolution mass spectrometry-based proteomics and compared them for a specificity analysis. As shown in Figure 3G, none of the proteins were significantly differentially expressed in mock-treated cells transfected with Cas9WT or Cas9CPFP (Figure 3G left), while adding PROTAC-FCPF, eight proteins were significantly up- or down-regulated, namely, Cas9, CYR61, HADC2, HIST1H4A, INT51, ISG15, SRM2, and URB1 (Figure 3G right). Among them Cas9 showed nearly an eightfold lower expression in cells expressing Cas9CPFP compared to cells expressing Cas9WT. Moreover, in Cas9WT cells no proteins were significantly affected by PROTAC-FCPF (Figure S3G left). By contrast, PROTAC-FCPF repressed the expression of Cas9CPFP and 10 other proteins in Cas9CPFP cells (Figure S3G right). Of note, ISG15, INT51, and URB1 appeared synchronously with Cas9CPFP in both cases (Figure 3G and Figure S3G). It is well-known that ISG15 is a ubiquitin-like modifier and plays an important role in the antiviral immunity.39 Considering the original role of CRISPR-Cas9 in a host immune system, ISG15 might be involved in a PROTAC-FCFP-mediated Cas9CPFP degradation and related cellular responses. Lu et al. reported that lenalidomide recruited CRBN to degrade IKZF1,40 which was not detected in our experiment, because the expression of IKZF1 in HeLa cells is remarkably low according to our proteome data.

Degradation of dCas9CPFP, Cas12CPFP, and Cas13CPFP with PROTAC-FCPF in Living Cells

Catalytically inactive Cas9 (dCas9) is widely used for epigenetic repression and activation. As expected, PROTAC-FCPF degraded dCas9CPFP in a similar manner to Cas9CPFP, confirming that the PROTAC-FCPF-mediated degradation is independent of catalytic activity (Figure 4A and Figure S4A), showing the important nature of FCPF-mediated degradation in comparison to classic chemical inhibitors, which inhibit protein activity by occupation in the catalytic binding pocket. Generally, a classic inhibitor development program would require a substantial synthetic effort for each targeted protein. For instance, Maij et al. reported BRD0539 as a Cas9 inhibitor, which could not inhibit other members of Cas enzyme demonstrated in their work.41 We inserted an FCPF amino acid sequence into HA-Cas12 and HA-Cas13 vectors. Gratifyingly, as shown in Figure 4B, C, we observed a time- and concentration-dependent degradation of Cas12CPFP and Cas13CPFP, suggesting that this strategy presented here can be widely used to control the stability of various proteins tagging FCPF with the single PROTAC-FCPF molecule.

Figure 4. The PROTAC-FCPF degrades dCas9CPFP, Cas12CPFP, and Cas13CPFP. The PROTAC-FCPF induces dCas9CPFP, Cas12CPFP, and Cas13CPFP degradation. (A) PROTAC-FCPF degrades dCas9CPFP. HeLa cells expressing dCas9CPFP were treated with increasing concentrations of PROTAC-FCPF for 8 h. (B, C) PROTAC-FCPF degrades Cas12CPFP and Cas13CPFP in (B) concentration-dependent and (C) time-dependent manners. Cells expressing HA-Cas12aCPFP or HA-Cas13CPFP were treated with the PROTAC-FCPF as indicated. Immunoblotting was performed. We used HA antibodies to detect HA-tag proteins. We used ACTIN as a loading control. The densitometric analysis of proteins obtained from at least three independent experiments can be found in the Supporting Information (Figure S4), with one of the original pictures as an example.

Discussion

Despite the revolutionary role of CRISPR/Cas systems in biotechnology, in particular, in genetic manipulation and engineering, some limitations associated with this technology remain, including off-target mutations.37 To improve accuracy and reduce off-target activities, several research groups have been engaging in engineering Cas9 proteins and developing anti-CRISPR/Cas9 proteins.38–40 These anti-CRISPR proteins naturally occur in bacteria to block or diminish the activity of Cas9 proteins and, thus, regulate their function. Following this approach, anti-CRISPR/Cas12 inhibitors have also been identified.41 Moreover, BRD0539 and valproic acid have been identified as Cas9 small-molecular inhibitors from high-content screening, which opened a new window for the chemical regulation of Cas9 activity.2,13

Classic small-molecule inhibitors, such as various protein kinase inhibitors, inhibit the enzymatic activity of the POI, using a well-defined hydrophobic binding pocket.20 Numerous in vitro and in vivo results provide compelling evidence that an acquired drug resistance can develop during a treatment, such as mutations and activation of alternative compensatory downstream signaling pathways via the formation of homo/hetero-oligomers, which largely impacts the clinical outcome.42,43 PROTACs are a heterobifunctional molecule consisting of a chemical warhead, which brings small molecules to fast and short interaction with the POI and a ligand of E3 ligase, which leads to a ubiquitination and degradation of the POI.20 Thus, PROTACs can inhibit both catalytic-dependent and catalytic-independent activity of the target, which includes currently undruggable mechanisms.

In the past decade, a series of PROTACs has been synthesized. However, few PROTACs were reported to degrade undruggable proteins, probably due to the lack of proper chemical warheads. Site-selective chemistry provides a means to target the POI at predesigned sites through

https://doi.org/10.1021/jacsau.1c00007
JACS Au 2021, 1, 777–785

Figure 3. continued
postexpression modification.\textsuperscript{44} For instance, Virdee et al. incorporated a specific lysine into proteins to artificially generate an isopeptide bond with ubiquitin for degradation.\textsuperscript{45}

Recently, Pentelute’s group developed the $\pi$-clamp system, in which recombiant proteins tagged by the FCPF amino acid sequence are selectively recognized by perfluoroaromatic compounds in a nucleophilic aromatic reaction (S$_\text{Ar}$) at the thiol group of the cysteine residue (Cys) under physiological conditions.\textsuperscript{22} We showed that the $\pi$-clamp system can be applied to target exogenously expressed FCPF-engineered proteins, such as Cas9, dCas9, Cas12, and Cas13. After the perfluoroaromatic group is fused to lenalidomide, a ligand of the CRBN E3 ligase, the resulting PROTAC-FCPF selectively degraded FCPF-labeled proteins. In comparison, a substantial effort is required to develop a small-molecule inhibitor for each protein. Typically, chemical inhibitors require a well-defined hydrophobic binding pocket at an enzyme or receptor active site, which leads to unwanted off-target effects.\textsuperscript{20} Currently, two Cas9 small-molecule inhibitors have been reported.\textsuperscript{12,13} However, their specificity regarding Cas9 inhibition is not reported. PROTACs exhibit a relatively high target specificity.\textsuperscript{26,46} In line with this, our proteome-wide analysis showed that less than a dozen proteins of a total of 4436 were affected by PROTAC-FCPF.

Very recently, Choudhary and his co-workers developed a dTAG-based PROTAC system to degrade Cas9 tagging FKBP.\textsuperscript{24} They demonstrated significantly improved activity and specificity of Cas9FKBP-mediated genome editing under the control of a dTAG molecule. This new development highlights the potential application of Cas9 degraders in CRISPR-Cas9 genome editing. Using the $\pi$-clamp as an engineered sequence in Cas9 or other exogenously expressed proteins offers some advantages compared to other formats of PROTACs and approaches to target protein degradation. For instance, HALO- and dTAG-PROTACs require the insertion of a comparably large HALO tag or FKBP12 into the target protein. Such a large insertion could be problematic, as the large molecular weight increase (\approx 33 and 12 kDa, respectively) may interfere with protein interactions and other functions.\textsuperscript{26,47} In the case of Cas9FKBP, two insertions were required for a sufficient degradation.\textsuperscript{21} In contrast, an insertion of the FCPF sequence represents a minimal modification of the target protein. Therefore, multiple FCPF sequences could be inserted into a target protein, which makes this technology applicable for an endogenously expressed protein in combination with a CRISPR-Cas9 knockin system.

\section{CONCLUSION}
The CRISPR-Cas system is a powerful tool to manipulate the human genome for gene therapy. Although the first in-human phase I clinical trial confirmed more than 95\% on-target efficiency, rare off-target edits on various transcription factors, such as ZNF609 and ZNF808, were still observed,\textsuperscript{9} which may lead to unexpected side effects. Compelling evidence has shown that the timely regulation of Cas9 protein activity significantly reduces the off-target effects. Using a combination of the $\pi$-clamp system and PROTAC technology, we demonstrated a novel PROTAC-FCPF molecule able to sufficiently suppress the activity of Cas proteins through the ubiquitin-proteasome pathway. Because the size of insertion is relatively small, this approach may be applicable to label and functionalize not only exogenously but also endogenously expressed proteins in a combination of the CRISPR-Cas9 knockin system with proper heterobifunctional small molecules. One of the disadvantages of PROTAC is the lipophilicity of relatively large bifunctional molecules. According to the classic Lipinski’s rule of five, PROTAC-FCPF is still drug-like with a LogP of 4.42. However, further modifications are required on both protein-FCPF and PROTAC-FCPF to achieve the effective regulation of FCPF-tagging protein activity within a short incubation time.

\section{EXPERIMENTAL SECTION}
Plasmids, siRNAs, and Chemicals for Bioassays
dCas9 (Addgene, No. 61355), Cas12 (hPdCpf1, Addgene No. 69990), Cas13 (LwCas13a, Addgene No. 91902), and CRBN KO (plenti-px330-CRBN-T1-pGR-Pur and plenti-px330-CRBN-T2-pGR-Pur, 107382 and 107383) were purchased from Addgene. Insertion of the FCPF amino acid sequence was performed and analyzed by Genescript. DDB1, CRBN, CUL4A, MDM2, and VHL siRNAs were purchased from Thermofisher Scientific. Cas9 (No. 19526) and FLAG (No. 8146) antibody were purchased from Cell Signaling Technologies. CRBN antibody was from Thermofisher Scientific (PAS-38037). ACTIN antibody was from Santa Cruz Biotechnology (No. SC-4778). CMV-SpyCas9 (Addgene No. 113033), sgRNA targeting AAVS1 (Addgene No. 128119) plasmid, and Anti-CRISPR protein (ACRIIA4, Addgene No. 113037) are gifts from Dr. Niopek (IPMB, Heidelberg).\textsuperscript{54,55} CRISPR-Cas9 CRBN double nickase knockout plasmid was purchased from Santa Cruz Biotechnology (SC-412142). Lenalidomide, MG132, PYR41, and MLN4924 were purchased from Biomol.

Cell Culture and Transfection
HeLa and HEK293T cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) containing 10\% fetal bovine serum (FBS) (Gibco), as well as 100 Unit/mL penicillin and 100 $\mu$g/mL streptomycin (Gibco), at 37$^\circ$C in a humidified atmosphere under 5\% CO$_2$. Jopaca-1 cells were cultivated in RPMI 1640. Overexpression and knockdown were performed as previously described.\textsuperscript{48} Generally, lipofectamine 3000 was used for transfection. According to the manufacturer’s instructions, the mixture of 100 $\mu$L of lipofectamine 3000 and 100 $\mu$L of p3000 reagent with either 20 nM siRNA or 2500 ng of DNA was incubated at room temperature for 5 min. Cells with 60–70\% of confluency in a 6-well plate were transiently transfected with the mixture for 24 h in medium without antibiotics and incubated with refreshed medium for another 24 h. For a 77E1 assay, Cas9WT or Cas9$^{\text{FCPF}}$ were cotransfected with sgRNA in the presence or absence of anti-CRISPR protein (ACRIIA4). The sequences of siRNAs can be found in the Supporting Information.

Degradation in Cells
HeLa, HEK293, or Jopaca-1 cells were transfected with Cas9$^{\text{FCPF}}$ for 48 h as above-described. Generally, 10 $\mu$m of PROTAC-FCPF was added to induce an acute Cas9$^{\text{FCPF}}$ degradation (less than 8 h). For a 24 h treatment, 1000 ng of DNA was transfected with Lipofectamine 3000 and incubated for 48 h. PROTAC-FCPF (1 $\mu$m) was used for 24 h treatment.

FITC-FCPF Staining
HeLa cells were transfected with Cas9$^{\text{FCPF}}$ for 48 h. FITC-FCPF (10 $\mu$m) was added and incubated for 2 h. Cells were washed at least three times with phosphate-buffered solution (PBS), maintained in PBS with 1\% FBS, and monitored under a fluorescence microscope. Cas9$^{\text{WT}}$ and Cas9$^{\text{FCPF}}$ were used as controls. For an FACS analysis, cells were trypsinized, centrifuged (200 g, 5 min), washed at least three times with PBS, and resuspended in PBS containing 1\% BSA and 2 mM ethylenediaminetetraacetic acid (EDTA). Guava easyCyte was used as previously reported.\textsuperscript{48} For immunoblotting, cells were lysed in 6 M 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$_3$VO$_4$, 10 $\mu$g/mL pepstatin, 100 $\mu$m phenylmethylsulfonyl fluoride (PMSF), and 3 $\mu$g/mL aprotinin in PBS). Total protein was resolved
on 8% SDS-PAGE gels for Cas9. Images were taken under visible or UV light.

**In Vitro Ubiquitination Assay**

CRBN KO cells were transfected with Cas9WT or Cas9FCPF plasmid as above-described and lysed with immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM NαEDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin, 1 mM PMSE, pH 7.5) freshly prepared before use. FLAGCRBNWT or -CRBNWT was expressed in HeLa cells and freshly isolated using a FLAG immunoprecipitation kit (Sigma-Aldrich, Merck, No. FLAGIPT1-1KT) according to the manufacturer's instructions. Anti-FLAG M2 affinity gel (20 μL) was added into 500 μL of cell lysate and incubated at 4 °C overnight. The suspension was washed at least three times with a wash buffer (50 mM Tris HCl and 150 mM NaCl, pH 7.4) and eluted 100 μL of elution buffer (0.1 M glycine HCL, 1150 mM NaCl, pH 7.4). 10X Wash buffer was added to neutralize the pH value.

The assay was initiated with adding CRBN into cell lysis and incubated for 90 min at 37 °C in the presence or absence of PROTAC-FCPF (100 μM). The adenosine triphosphate (ATP) consumption was determined directly in ENLITEN ATP Assay (Promega, PE2000). Pretreatment of lenalidomide (100 μM) for 5 min sufficiently reduced the degradation of Cas9FCPF. The generation of CRBN KO cell lines can be found in the Supporting Information.

**T7 Endonuclease I Assay**

An EnGen mutation detection kit (NEB, No. E3321) was used to evaluate the efficiency of CRISPR-Cas9 in genome editing, which includes a positive control. HeLa cells were transfected with CasWT or Cas9FCPF or in a combination of Cas9FCPF and anti-CRISPR protein (ACR) in the presence of sgRNA AAVS1 for 48 h. CMV-SpyCas9 (Addgene No. 113033), sgRNA targeting AAVS1 (Addgene No. 128119) plasmid, and Anti-CRISPR protein (ACR1A4, Addgene No. 113037) were gifts from Dr. Niopek, University Heidelberg. HeLa cells expressing Cas9FCPF were treated with PROTAC-FCPF (1 μM) for 24 h. LuciferaseQuickExtract DNA Extraction Solution (Biozynyme, No. MA150E) was used to collect DNA. HeLa cells were washed with PBS and trypsinated. The pellet of 500,000 cells was lysed with 500 μL of LuciferaseQuickExtract DNA Extraction Solution and vortexed for 15 s. The lysis was first heated at 65 °C for 6 min and vortexed for 15 s and at 98 °C for 2 min. DNA can be stored at −70 °C for 6–8 weeks.

The target sequences of AAVS1 gene were amplified with Eppendorf Mastercycler. A mixture of Q5 Hot Start high-Fidelity 2x Master mix (12.5 μL), 10 μM forward and reverse primers (1.25 μL of each), and genomic DNA (100 ng) in nuclease-free water (25 μL in total) was prepared. The target sequence was amplified using the following setup: 1 cycle (98 °C for 30 min); 40 cycles (98 °C for 5 min, 70 °C for 10 min, and 72 °C for 20 min); and 1 cycle (72 °C for 2 min).

For heteroduplex formation between polymerase chain reaction (PCR) products with and without mutations 5 μL of PCR product was mixed with 2 μL of 10X NEBuffer2 in 12 μL of nuclease-free water. The mixture was denatured and annealed using the following program: 95 °C for 5 min; 95–85 °C (−2 °C/sec) and 85–25 °C (−0.1 °C/sec). Afterward, 1 μL of EnGen T7 Endonuclease I was added into the annealed PCR product and incubated at 37 °C for 15 min. Protease K (1 μL) was added and incubated at 37 °C for another 5 min to inhibit the activity of endonuclease. The sample was analyzed on a 2% agarose gel. The signal was quantified by Aida image analysis software. We quantified band intensities by ImageJ software and calculated genome editing efficiency using the eq 100(1-(1-fracton cleaved)1/2).10

The corresponding sequences can be found in the Supporting Information.

**Statistics and Reproducibility**

Data were analyzed with the GraphPad Prism software (v7.01). Two-way analysis of variance (ANOVA) was performed. All experiments except for proteomics were independently repeated at least three times, from which similar results were obtained. The standard deviation is noted as SD.
executed, and analyzed the proteomics analyses. X.C. designed the experiments, performed experiments, and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Funding
We thank DFG for the financial support (CH 1690/2-1 and 2-3). This work was supported in part by the LOEWE Center Frankfurt Cancer Institute funded by the Hessen State Ministry for Higher Education, Research and the Arts [III L S-519/03/03.001-0015].

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
We thank the Quantitative Proteomics Unit at IBC2, Dr. Woll for his support, and Dr. Niopek for sharing the materials.

■ ABBREVIATIONS
CRISPR, clustered regularly interspersed short palindromic repeats; crRNA, CRISPR RNA; dCas9, nuclease-dead Cas9; DSBs, double-strand breaks; dsDNA, double-stranded DNA; PROTAC, proteolysis-targeting chimera; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; TLC, thin layer chromatography

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