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CRISPR-Cas Φ from huge phages is a hypercompact genome editor

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Abstract

CRISPR-Cas systems are found widely in prokaryotes where they provide adaptive immunity against virus infection and plasmid transformation. We describe a minimal functional CRISPR-Cas system, comprising a single ~70 kilodalton protein, Cas Φ , and a CRISPR array, encoded exclusively in the genomes of huge bacteriophages. Cas Φ employs a single active site for both

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Author Contributions: BAS and JFB conceived initial study, further developed with PP and JAD. PP and BAS designed experiments and analyzed data with input from JAD and JFB. PP, EBR, and BAS cloned constructs with guidance from PP. BAS conducted metagenomic assemblies, genome curation, protein and crRNA sequence analysis, active residue identification and computational analyses for PAM depletion assays and RNA-seq. PP conducted PAM depletion assays, EOT assays, performed RNA-seq experiments, purified proteins and performed biochemical experiments. PP, BAS, CT and ZL designed genome editing experiments with input from SEJ. CT performed tissue culture and flow cytometry and ZL performed editing experiments in *A. thaliana*. BFC and GJK provided materials and experimental advice. BAS and PP wrote the manuscript with input from JAD and JFB. PP and BAS prepared figures. The manuscript was reviewed and approved by all co-authors.

Competing Interests: The Regents of the University of California, Berkeley and Los Angeles have patents pending for CRISPR technologies on which the authors are inventors. JAD is a co-founder of Caribou Biosciences, Editas Medicine, Intellia Therapeutics, Scribe Therapeutics, and Mammoth Biosciences. JAD is a scientific advisory board member of Caribou Biosciences, Intellia Therapeutics, eFFECTOR Therapeutics, Scribe Therapeutics, Synthego, and Mammoth Biosciences. JAD is a director at Johnson & Johnson and has sponsored research projects by Pfizer, Roche Biopharma, and Biogen. JFB is a founder of Metagenomi. SEJ is a scientific co-founder of Inari Agriculture and SEJ and JAD are members of its scientific strategy board.

Data and materials availability: All data are available in the manuscript or the supplementary material. Reagents are available through Addgene and upon request from JAD.

CRISPR RNA (crRNA) processing and crRNA-guided DNA cutting to target foreign nucleic acids. This hypercompact system is active *in vitro* and in human and plant cells with expanded target recognition capabilities relative to other CRISPR-Cas proteins. Useful for genome editing and DNA detection but with a molecular weight half that of Cas9 and Cas12a genome-editing enzymes, Cas Φ offers advantages for cellular delivery that expand the genome editing toolbox.

One Sentence Summary:

Phage-derived Cas Φ uses a single active site to process guide RNA and cut DNA for genome editing and nucleic acid detection.

Competition between viruses and their host microbes fostered the evolution of CRISPR-Cas systems that employ nucleases and non-coding CRISPR RNAs (crRNAs) to target foreign nucleic acids by complementary base pairing (1). Processing of CRISPR array transcripts, consisting of repeats and spacer sequences acquired from viruses or other mobile genetic elements (MGEs) (2), generates mature crRNAs that guide Cas proteins (3) to detect and destroy previously encountered viruses. Although found almost exclusively in microbial genomes, the recent discovery of ubiquitous huge bacteriophages (viruses of bacteria) revealed the surprising prevalence of CRISPR-Cas systems encoded in their genomes (4). These systems notably lack CRISPR spacer acquisition machinery (Cas1, Cas2 and Cas4 proteins) and generally harbor compact CRISPR arrays (median: 5 spacers per array), some of which target the genes of competing phages or phage hosts. Cas Φ (Cas12j) is a family of Cas proteins encoded in the Biggiephage clade (4). Cas Φ contains a C-terminal RuvC domain with remote homology to that of the TnpB nuclease superfamily from which type V CRISPR-Cas proteins are thought to have evolved (4, 5) (fig. S1). However, Cas Φ shares <7% amino acid identity with other type V CRISPR-Cas proteins and is most closely related to a TnpB group distinct from miniature type V (Cas14) proteins (Fig. 1A).

Cas Φ 's unusually small size of ~70–80 kDa, about half the size of the Cas9 and Cas12a (Fig. 1B), and its lack of co-occurring genes raised the question of whether Cas Φ functions as a *bona fide* CRISPR-Cas system. We investigated three divergent Cas Φ orthologs from metagenomic assemblies (fig. S2), hereafter referred to as Cas Φ -1, Cas Φ -2 and Cas Φ -3. To examine Cas Φ 's ability to recognize and target DNA in bacterial cells, we tested whether Cas Φ could protect *Escherichia coli* from plasmid transformation. CRISPR-Cas systems target DNA sequences following or preceding a 2–5 base pair (bp) Protospacer Adjacent Motif (PAM) for self-versus-non-self discrimination (6). To determine whether Cas Φ uses a PAM, we transformed a library of plasmids containing randomized regions adjacent to crRNA-complementary target sites, thereby depleting plasmids harboring functional PAMs. This revealed the crRNA-guided double-strand DNA (dsDNA) targeting capability of Cas Φ and minimal T-rich PAM sequences, including 5'-TBN-3' PAMs (where B is G, T, or C) depleted for Cas Φ -2 (Fig. 1C).

We next used the *E. coli* expression system and plasmid interference assay to determine the components required for CRISPR-Cas Φ system function. RNA-sequencing analysis revealed transcription of the *cas Φ* gene and the reduced CRISPR array but no evidence of other non-coding RNA such as a trans-activating CRISPR RNA (tracrRNA) within the locus (Fig. 1D).

In addition, Cas Φ activity could be readily reprogrammed to target other plasmid sequences by altering the guide RNA (fig. S3). These findings suggest that in its native environment, Cas Φ is a functional phage protein and *bona fide* CRISPR-Cas effector capable of cleaving crRNA-complementary DNA such as other phage (Fig. 1E). Furthermore, these results demonstrate that this single-RNA system is much more compact than other active CRISPR-Cas systems (Fig. 1F).

We next investigated the DNA recognition and cleavage requirements of Cas Φ *in vitro*. RNA-seq revealed that the crRNA spacer, which is complementary to DNA targets, is 14–20 nucleotides (nt) long (Fig. 1D). Incubation of purified Cas Φ (fig. S4) with crRNAs of different spacer sizes along with supercoiled plasmid or linear dsDNA revealed that DNA cleavage requires the presence of a cognate PAM and a spacer of 14 nt (Fig. 2A; fig. S5A). Analysis of the cleavage products showed that Cas Φ generated staggered 5' overhangs of 8–12 nt (Fig. 2B, C; fig. S5B, C), similar to the staggered DNA cuts observed for other type V CRISPR-Cas enzymes including Cas12a and CasX (7, 8). We also observed that Cas Φ -2 and Cas Φ -3 were more active *in vitro* than Cas Φ -1, and the non-target strand (NTS) was cleaved faster than the target-strand (TS) within the RuvC active site (Fig. 2D; figs. S6A, S7; Supplementary Text). Furthermore, Cas Φ was found to cleave ssDNA but not ssRNA *in cis* and *in trans* (fig. S6B, S8), suggesting that Cas Φ may also target ssDNA MGEs or ssDNA intermediates. The trans-cleavage activity of Cas Φ , observed only upon DNA recognition *in cis* (fig. S8), coupled with a minimal PAM requirement (Fig. 1C), may be useful for broader nucleic acid detection as previously demonstrated for type V and type VI Cas proteins (9–11).

CRISPR-Cas Φ systems must produce mature crRNA to guide foreign DNA cleavage. Other type V CRISPR-Cas proteins process pre-crRNAs using an internal active site distinct from the RuvC domain (12) or by recruiting Ribonuclease III to cleave a pre-crRNA:tracrRNA duplex (13–16). The absence of a detectable tracrRNA for Cas Φ hinted that Cas Φ may catalyze crRNA maturation on its own. To test this possibility, we incubated purified Cas Φ with substrates designed to mimic the pre-crRNA structure (Fig. 3A). Reaction products corresponding to a 26–29 nt-long repeat and 20 nt spacer sequence of the crRNA were observed only in the presence of wild type Cas Φ , corroborated by RNA-seq analysis of native loci (Figs. 1D; 3A, C; fig. S9). In control experiments, we found that pre-crRNA processing is strictly magnesium-dependent (Fig. 3B; fig. S9), which is different from other CRISPR-Cas RNA processing reactions and suggested a distinct cleavage mechanism. Notably, the RuvC domain requires magnesium to cleave DNA (17), and some RuvC domains have been reported to have endoribonucleolytic activity (15). Based on these observations, we tested Cas Φ containing a RuvC-inactivating mutation and found it to be incapable of processing pre-crRNAs (Fig. 3B; fig. S9A, B). Both wild-type and catalytically inactivated Cas Φ proteins bind crRNA, and their reconstituted complexes with pre-crRNA have similar elution profiles from a size exclusion column, suggesting no pre-crRNA binding or protein stability defect resulting from the RuvC mutation (fig. S10).

We hypothesized that if the RuvC domain is responsible for pre-crRNA processing, the products should contain 5' phosphate and 2' and 3' hydroxyl moieties as observed in RNAs generated by the RuvC-related RNase HI enzymes (17). In contrast, other type V

CRISPR-Cas enzymes process pre-crRNA by metal-independent acid-base catalysis in an active site distinct from the RuvC, generating 2'-3'-cyclic phosphate crRNA termini, as observed for Cas12a (18). Phosphatase treatment of Cas Φ -generated crRNA followed by denaturing acrylamide gel analysis showed no change in the crRNA migration, distinct from the change in mobility detected for crRNA generated by Cas12a (Fig. 3C; fig. S9C). This result implies that no 2'-3'-cyclic phosphate was formed during the reaction catalyzed by Cas Φ , in contrast to the acid-base catalyzed processing reaction by Cas12a (Fig. 3C, D). Together, these data demonstrate that Cas Φ uses a single RuvC active site for both pre-crRNA processing and DNA cleavage.

The versatility and programmability of CRISPR-Cas systems for genome editing in virtually any organism have sparked a revolution in biotechnology and fundamental research (19). To investigate whether Cas Φ can be harnessed for human genome editing, we performed a gene disruption assay (8) using Cas Φ co-expressed with a crRNA in HEK293 cells (Fig. 4A). We found that Cas Φ -2 and Cas Φ -3, can induce targeted disruption of a genomically integrated EGFP gene (Fig. 4A; fig. S11). In one case, Cas Φ -2 with an individual guide RNA was able to edit up to 33% of cells (Fig. 4A), comparable to levels initially reported for CRISPR-Cas9, CRISPR-Cas12a, and CRISPR-CasX (7, 8, 20). We next tested if Cas Φ -2 can be delivered as RNPs into plant protoplasts to edit the endogenous *Arabidopsis thaliana PDS3* gene (Fig. 4B; fig. S12). Next generation sequencing revealed that Cas Φ -2 introduces primarily 8–10 bp deletions (Fig. 4B), consistent with the cleavage pattern observed *in vitro* (Fig. 2C). The small size of Cas Φ in combination with its minimal PAM requirement will be particularly advantageous for both vector-based delivery into cells and a wider range of targetable genomic sequences, providing a powerful addition to the CRISPR-Cas toolbox.

Three other well-characterized Cas enzymes Cas9, Cas12a, and CasX, use one (Cas12a and CasX) or two active sites (Cas9) for DNA cutting and rely on a separate active site (Cas12a) or additional factors (CasX and Cas9) for crRNA processing (Fig. 4C). The finding that a single RuvC active site in Cas Φ is capable of crRNA processing and DNA cutting suggests that size limitations of phage genomes, possibly in combination with large population sizes and higher mutation rates in phages compared to prokaryotes (21–23), led to a consolidation of chemistries within one catalytic center. Such compact proteins may be particularly amenable to engineering and laboratory evolution to create new functionalities for genome manipulation, and highlight huge phages as an exciting forefront for discovery and biotechnological applications for human health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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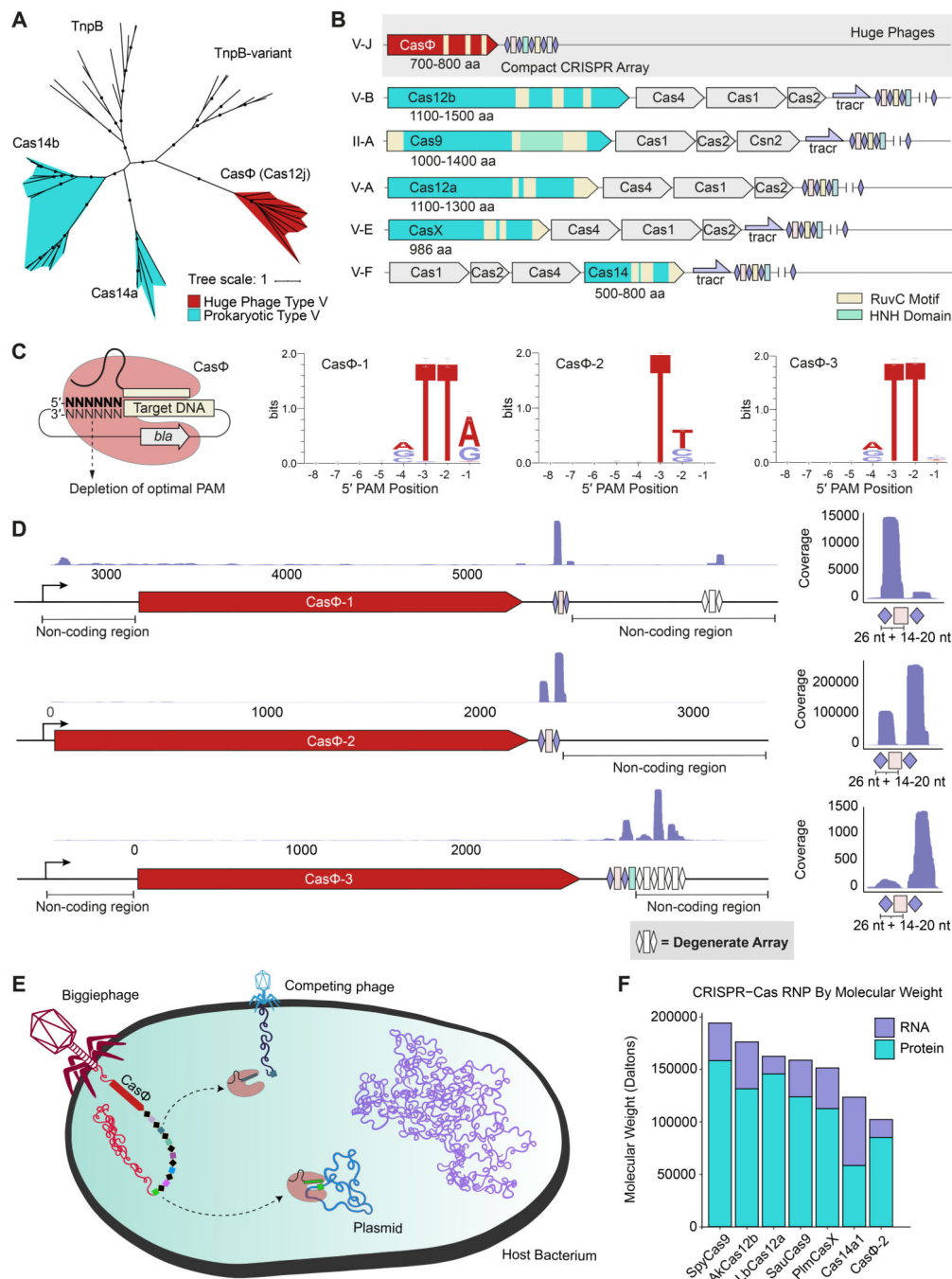


Fig. 1. CasΦ is a *bona fide* CRISPR-Cas system from huge phages. **(A)** Maximum Likelihood phylogenetic tree of type V effector proteins and respective predicted ancestral TnpB nucleases. Bootstrap and approximate likelihood-ratio test values ≥ 90 are denoted on the branches with black circles. **(B)** Illustrations of genomic CRISPR-Cas loci of CasΦ, Cas14, and systems previously employed in genome editing applications. **(C)** Graphical representation of the PAM depletion assay and the resulting PAMs for three CasΦ orthologs. **(D)** RNA-sequencing results (left) mapped onto the native genomic loci of CasΦ orthologs

and their upstream and downstream non-coding regions as cloned with reduced CRISPR-arrays into expression plasmids. Enlarged view of RNA mapped onto the first repeat-spacer pair (right). (E) Schematic of the hypothesized function of Biggiephage-encoded Cas Φ in an instance of superinfection of its host. Cas Φ may be used by the huge phage to eliminate competing mobile genetic elements. (F) Predicted molecular weights of the ribonucleoprotein (RNP) complexes of small CRISPR-Cas effectors and those functional in editing of mammalian cells.

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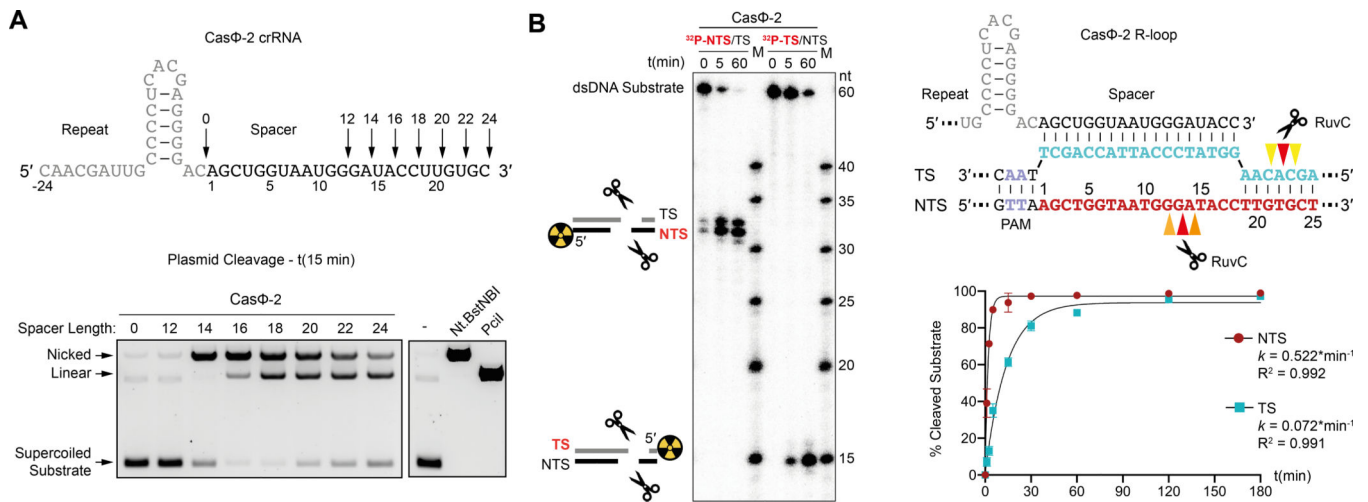


Fig. 2. CasΦ cleaves DNA. **(A)** Supercoiled plasmid cleavage assay testing CasΦ RNPs reconstituted with crRNAs of different spacer lengths. **(B)** Cleavage assay targeting dsDNA oligo-duplicates for mapping of the cleavage structure. **(C)** Scheme illustrating the cleavage pattern. **(D)** NTS and TS DNA cleavage efficiency ($n = 3$ each, mean \pm s.d.). Data is shown in fig. S7B.

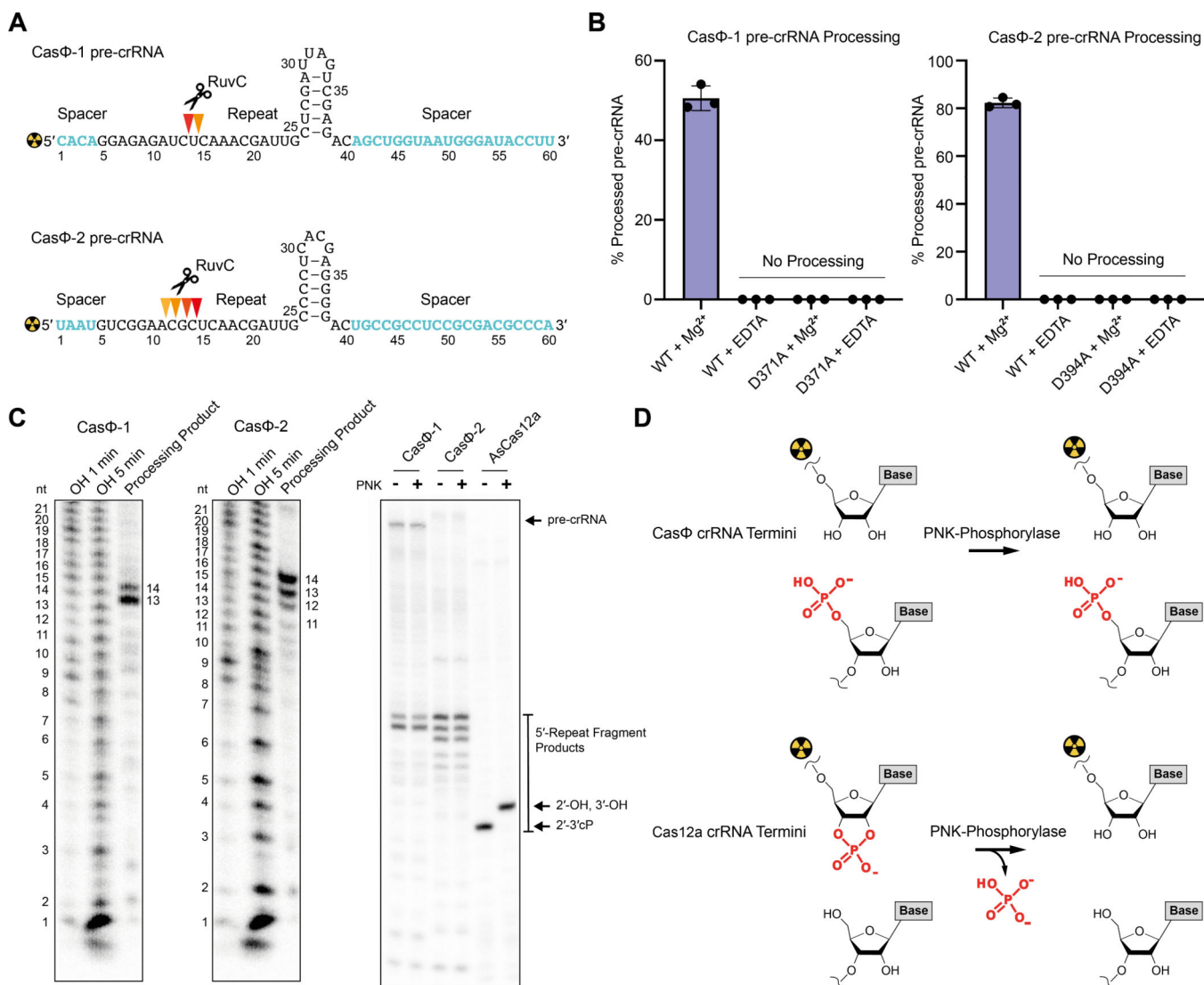


Fig. 3. CasΦ processes pre-crRNA within the RuvC active site. (A) pre-crRNA substrates and processing sites (red triangles) as derived from the OH-ladder in panel C. (B) Pre-crRNA processing assay for CasΦ-1 and CasΦ-2 in dependence of Mg²⁺ and RuvC active site residue variation (D371A and D394A) (n = 3 each, mean ± s.d.; t = 60 min). Data is shown in fig. S9B. (C) Left and middle: Alkaline hydrolysis ladder (OH) of the pre-crRNA substrate. Right: PNK-phosphatase treatment of the CasΦ and *Acidaminococcus sp.* Cas12a cleavage products. (D) Graphical representation of the mature crRNA termini chemistry of CasΦ and Cas12a and PNK-phosphorylase treatment outcomes.

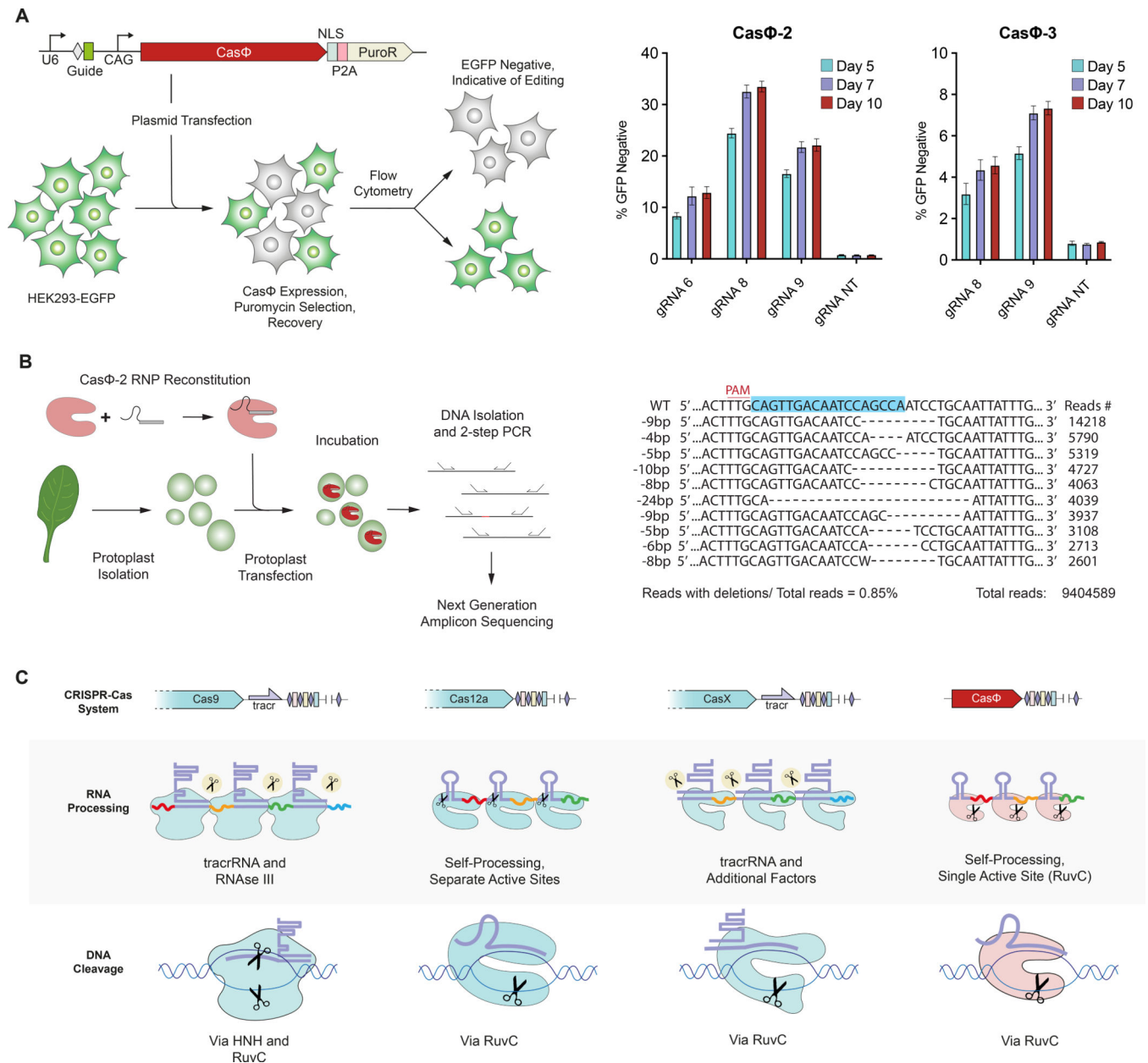


Fig. 4. CasΦ is functional for genome editing. **(A)** Experimental workflow of the GFP disruption assay (left) and GFP disruption using CasΦ-2 and CasΦ-3 and a non-targeting (NT) guide as a negative control (n = 3 each, mean ± s.d.). **(B)** Experimental workflow of CasΦ-2 RNP-mediated genome-editing in *A. thaliana* mesophyll protoplasts (left) and amplicon sequencing data (right) showing the most frequent deletions for gRNA33 in the targeted region (blue) within the *AtPDS3* gene. **(C)** Scheme illustrating the differences in RNA processing and DNA cutting for Cas9, Cas12a, CasX, and CasΦ.